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Aqueous and oil-adjuvant influenza vaccines and iso-immunization to group A substance

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INTRODUCTION

Because pregnant women tend to suffer severely from influenza they form one of the groups for whom influenza vaccine has been advised (Davenport, 1962). When Springer & Tritel (1962) reported that a concentrated extract of influenza vaccines stimulated a significant rise in anti-A iso-agglutinins in group O or B volunteers and Dr Springer was quoted (American Heart Association, cited by U.S. Public Health Service, 1962) as having warned that this might lead to ABO haemolytic disease in infants, much concern was caused.

Sussman & Pretshold (1963) were unable to demonstrate the presence of group A substance in commercial influenza vaccines but conflicting evidence as to whether anti-A antibodies were actually stimulated by similar vaccines was presented by Davenport (cited by Influenza Surveillance, 1962) and Mathieson, Banner & Harris (1963).

In view of this controversy it seemed appropriate to use the opportunity and specimens presented by two trials of influenza vaccines to test whether antibodies to group A red cells were elicited in volunteers of blood group O.

MATERIALS AND METHODS

Trials

In the first trial 339 volunteers, medical students at the Queen's University of Belfast in the first to fifth years of their course, received two inoculations and had four blood samples taken over a period November 1962 to February 1963.

All volunteers were randomly allocated to receive as first inoculation either 0·5 ml. of a saline placebo or 0·5 ml. of an aqueous polyvalent inactivated influenza virus vaccine (*Invirin*, Glaxo) subcutaneously. From this dose of vaccine each volunteer received 7500 haemagglutinating units (H.A.U.) of virus. The

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viruses in this, as in the other vaccines, had been propagated in embryonated eggs.

The second inoculation was given in January 1963, 2 months after the first inoculation. The volunteers were again distributed into two groups by an independent process. Each volunteer was given either 1·0 ml. of *Invirin*, containing 15,000 H.A.U. of virus, subcutaneously or 0·25 ml. of an oil-adjuvant inactivated vaccine intramuscularly. This adjuvant vaccine contained 3000 H.A.U. of virus of the same strains and in the same proportions as in *Invirin*. The effect of the adjuvant was to give this lesser amount of virus an antigenic effect approximately similar to that in 1·0 ml. of *Invirin*.

The blood samples were taken at the time of inoculation and 3 weeks after the first inoculation and 4 weeks after the second. The sera were separated and stored at −20° C. The blood group of each volunteer was determined at the time of the third bleeding.

In the second trial 97 volunteers, 68 first year medical students and 29 nurses from the Royal Victoria or the Royal Maternity Hospitals, Belfast, were given 0·25 ml. of an oil-adjuvant inactivated influenza virus vaccine (*Admune*, Evans Medical) intramuscularly. This dose contained 3500 H.A.U. of virus.

Specimens of blood were taken at the time of inoculation and one and three months after inoculation. The blood group of each volunteer was ascertained at the time of the first bleeding.

During the second trial a survey of the incidence of anti-A_1 haemolysins in the sera of group O donors to the Northern Ireland Blood Transfusion Service was made in an attempt to detect any seasonal variation which might affect the interpretation of the results from the trial.

Methods

Saline iso-agglutinin tests

A 5% (v/v) suspension of washed standard human A_1 red cells in saline was added to equal volumes of a range of serum dilutions from 1/1 to 1/512. The tubes were read after 1½ hr. at room temperature and the final end point was established microscopically. All sera of an individual were examined together.

Haemolysin tests

To drops of serum were added equal volumes of 5% (v/v) saline suspensions of standard human A_1 red cells. After incubation at 37° C. for 1½ hr. the degrees of haemolysis were recorded as:

No haemolysis, trace haemolysis (T), slight colouring of the supernatant fluid, partial haemolysis (P), or complete haemolysis (C).

Significance tests

The '*t*'-test was used and significance was read at the 5% level.

RESULTS

In the first trial 146 of the volunteers were of blood group O. The design of this trial was such that the effect of the first inoculation of vaccine could be compared with the effect of placebo after 3 weeks and after 2 months.

The mean pre-inoculation iso-agglutinin titres of the placebo and vaccine groups were different and the antibody titres of the placebo group also rose over the period of the test (see Fig. 1). However, it was possible to show that the group receiving vaccine experienced a greater rise in antibody by 3 weeks after inoculation ($0{\cdot}05 > P > 0{\cdot}0025$) and by 2 months after inoculation ($0{\cdot}005 > P > 0{\cdot}0025$).

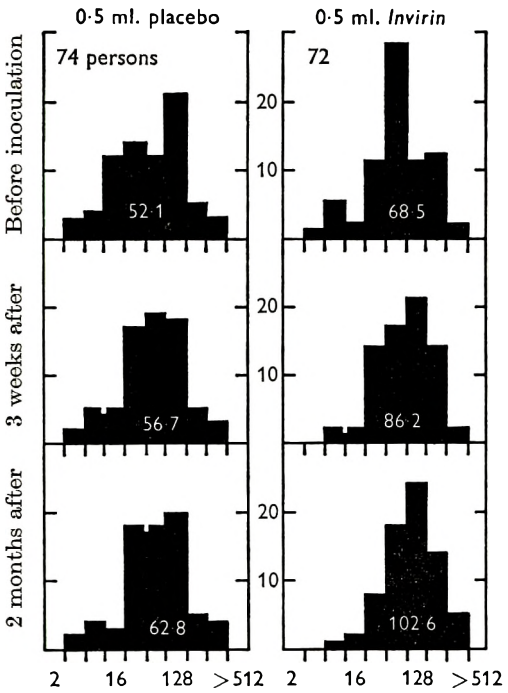

Fig. 1. Levels of anti-A iso-agglutinins in volunteers before and at intervals after the inoculation of saline placebo or 0·5 ml. *Invirin*. Geometric mean titres (G.M.T.) inserted.

When the effect of the two vaccines was compared after the second inoculation (see Fig. 2) no difference in the behaviour of antibody titres emerged ($0{\cdot}3 > P > 0{\cdot}2$). This lack of difference was not, apparently, affected by which inoculum the volunteers had received in the first part of the trial.

Of the 97 volunteers in the second trial, 44 were of group O. Levels of anti-A iso-agglutinins did not rise much after the inoculation of *Admune*. Geometric mean titres were:

Before inoculation	54·7
One month after	56·4
Three months after	68·2

The increase in the first month was within chance limits ($0{\cdot}7 > P > 0{\cdot}6$) but over 3 months was just significant ($0{\cdot}05 > P > 0{\cdot}025$). However, in the absence of a group given placebo no adjustment could be made for the upwards drift unassociated with vaccine which was noticed in the first trial.

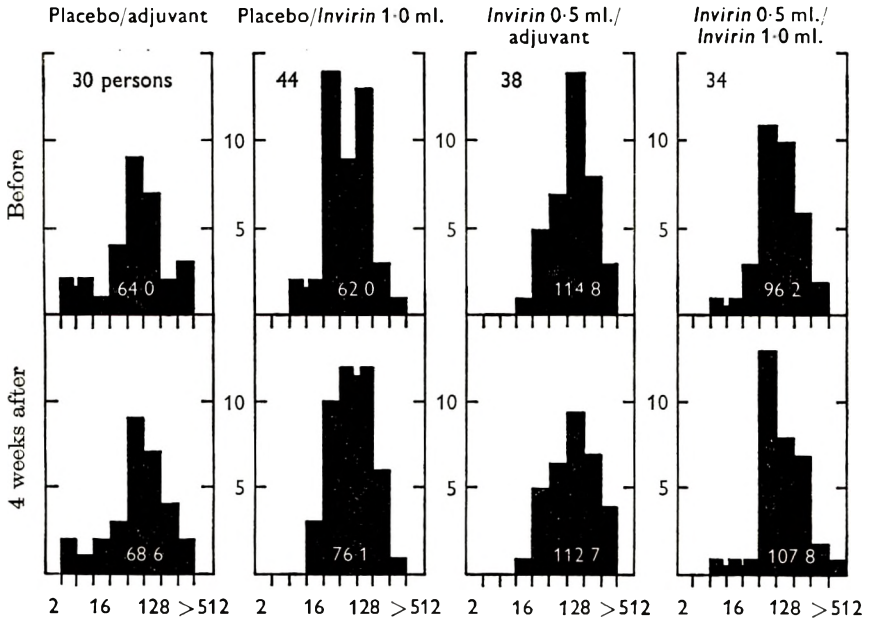

Fig. 2. Levels of anti-A iso-agglutinins in volunteers, who had been given placebo or *Invirin* 2 months previously, before and 4 weeks after either adjuvant influenza vaccine or *Invirin* (G.M.T. inserted).

Table 1. *The percentage incidence of anti-A_1 haemolysins active at 37° C. in group O blood donors and in group O volunteers receiving oil-adjuvant influenza vaccine* (Admune)

			Haemolysis			Total numbers
			Complete	Partial	Trace	
November	Before vaccine	Donors	0 %	4·7 %	11·1 %	342*
		Volunteers	0	4·4	0	44
December	1 month	Donors	0·01 %	3·4 %	11·1 %	1495
		Volunteers	0	2·2	0	44
January	2 months	Donors	0	2·0 %	7·7 %	1887
February	3 months	Donors	0 %	2·9 %	7·4 %	1506
		Volunteers	0	2·2	8·9	44

* Examination of donor sera started only in the latter part of November.

During the second trial sera were tested to find the effect of the vaccine used on the production of haemolysins active against A_1 red cells at 37° C.

Only weak haemolysins were found in the pre-inoculation sera of few of the volunteers and the sera taken after vaccination showed no clear tendency for haemolysins to be increased (see Table 1). Over the period of the trial the variations

in haemolysin incidence of the volunteers' sera were not greater than those seen in the sera of unselected group O blood donors (see Table 1).

Over this same period, the effect of the standard dose of 1500 units of equine anti-tetanic serum (A.T.S.), administered for the usual indications, on the production of haemolysins was investigated in a number of casualty patients attending the Royal Victoria Hospital. Blood samples were taken before the prophylactic A.T.S. and again about 10 days later in some cases and a month later in others. While only a few pairs of suitable specimens from group O patients were available there did seem to be some tendency for haemolysin to be stimulated (see Table 2).

Table 2. *The effect of* 1500 *units of equine anti-tetanic serum* (A.T.S.) *on anti-*A_1 *haemolysins in group O patients*

		Before A.T.S.	After A.T.S.
(i) 9–15 days between sera	Trace haemolysis	—	1
Four patients	Partial haemolysis	—	1
	Complete haemolysis	1	2
(ii) 1 month between sera	Trace haemolysis	3	1
Six patients	Partial haemolysis	1	3
	Complete haemolysis	0	1

In all these tests for haemolysin the sera were tested fresh, within hours of being taken. This excluded the error introduced by adding extraneous serum as a source of complement, but increased the day-to-day test variations. While many of the donor sera were taken in Belfast, the mobile unit collected blood from many towns in Northern Ireland and this could be expected to increase the spread of results.

DISCUSSION

From the evidence described there does appear to be an association between the administration of influenza vaccine and a rising titre of saline reactive iso-antibodies to group A substance. However, from the slight increase in antibody in the volunteers given the placebo inoculation of saline, it is clear that more than one factor was operating.

If the vaccines had stimulated the formation of haemolysins, as an index of immune-type antibodies, it would have appeared more significant in relation to haemolytic disease of the new-born than the rise of saline-reactive agglutinins actually observed, as these latter types of antibodies are less prone to cross the placenta (Tovey, 1945). However, the relation of any pre-existing antibody to ABO type haemolytic disease is obscure, as shown by the frequency with which such cases occur in first-born children (Levine, Vogel & Rosenfield, 1953) and may not occur in succeeding susceptible infants (Crawford, Cutbush & Mollison, 1953).

The adjuvant vaccine did not appear to stimulate the formation of haemolysins and the fact that the administration of A.T.S. did elicit haemolysins weakens the force of the suggestion that the Northern Ireland population, with its low incidence of haemolysins, might be inherently insensitive to the relevant antigenic stimuli.

However, it is possible that the aqueous vaccine, *Invirin*, could have been more active in this respect than the adjuvant vaccine as might be suggested by the iso-agglutinin studies.

SUMMARY

Influenza vaccines used in trials appeared to stimulate a small increase of saline-reactive anti-A_1 antibodies in group O volunteers. There was no evidence that haemolysins were elicited by adjuvant vaccine. It is unlikely that influenza vaccines would cause ABO haemolytic disease in infants.

We would like to thank Dr M. C. Huth for permitting these investigations to be carried out in the laboratories of the Northern Ireland Blood Transfusion Service, Dr G. H. Tovey and Mr J. W. Lockyer, B.Sc., of the South-west Regional Transfusion Centre, Bristol, for their advice and assistance and Mr F. Corr, A.I.M.L.T., for technical help. Mr A. O. H. Roberts, B.Sc., guided the statistical analysis of the material.

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Sociological factors in the spread of variola minor in a semi-rural school district

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Several studies have emphasized the role of the nursery or the primary school as a source of cases introducing communicable diseases into the household, as well as the subsequent occurrence of real intra-household outbreaks (Lidwell & Sommerville, 1951; Ipsen, Donovan & James, 1952; Knight *et al.* 1954; McCarroll, Melnick & Horstmann, 1955; Meyer, 1962). The present paper records an examination of the roles of the school class and the household in the spread of an epidemic of variola minor (alastrim) through Vila Guarani, a semi-rural school district of the City of São Paulo, Brazil. A detailed study on the spread of the disease in the district school has been published already (Angulo, Rodrigues-da-Silva & Rabello, 1964), as well as an examination of the spread in the district households (Angulo, Rodrigues-da-Silva & Rabello, 1967).

MATERIALS AND METHODS

Twenty-two households, in which fifty-two cases of variola minor occurred, were surveyed. In two of these households it was not possible to obtain data referring to all members of the household, and these households are excluded from the analysis of data in the paper. A description of the school and its population, as well as the procedures employed there during the field study, have been reported (Angulo *et al.* 1964). The characteristics of the households with cases, the methods used in surveying these households and the definitions used in that and in the present study have also been reported (Angulo *et al.* 1967). Twenty-two households without cases but including one or more members who had known contacts with patients were also surveyed and visited until three or more weeks after last contact. When referring here to a 'social unit' we mean a discrete group of persons regularly associated for a defined social function, like family life or school-class attendance.

An environmental survey of the district was conducted to determine its socio-economic characteristics and to establish space relations between infectives and contacts. Vila Guarani was connected with the remainder of the City of São Paulo through a single street. The district had no public sewerage or water-supply

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system and consisted of poorly built small houses without proper sanitation. A primary day-school was located in the district but there was no other similar place for customary gathering of persons. Although minor differences in the standard of living could be detected among the study households, these differences were not great. This group of households may be taken as representative of the district population because the socio-economic structure of the district was extremely simple and homogeneous. Heads of families were low-income, unskilled workers with little education.

RESULTS

Spread in the district

Chain of contagion

The chain of disease transmission depicted in Fig. 1 includes the fifty-four cases infected in the district, two travellers with variola who stayed for a short time in Vila Guarani and six related cases appearing in a home from the City of Rio de Janeiro (Rodrigues-da-Silva, Rabello & Angulo, 1963). The accuracy of the diagram in Fig. 1 is supported by the following facts. (*a*) Systematic tracing of sources of infection and of their contacts clearly disclosed how the disease was introduced in the district. (*b*) There was no report of any previous occurrence of variola in epidemic or endemic form in Vila Guarani. None of the school teachers knew of any previous occurrence in the district, nor did the school orderly who had lived in Vila Guarani for the previous twenty-two years. In the households surveyed, persons with a history of previous variola unanimously reported that they had had the disease in other places, most of them in north-eastern Brazil. A great many of the inhabitants of Vila Guarani had moved there in the past few years. (*c*) The chain of contagion was compiled from dates of onset obtained through personal interviews. In addition, the dates of onset and information about personal contacts were usually confirmed by members of the household and neighbours. Contacts with other patients were also investigated and, in these few instances, dates of onset and the type and frequency of contacts were utilized to determine the most probable infecting contact. (*d*) The social structure of the district was extremely simple and personal associations were similarly simple and easy to trace. (*e*) In the few instances where the date of onset could not be accurately established, it is believed that the date recorded did not differ from the actual date of onset by more than three days. (*f*) The evidence supporting person-to-person transmission as the mode of transmission was extremely strong and no indication or suggestion of other modes of spread was obtained (Angulo *et al.* 1964, 1967).

The role of social units

The chain of person to person transmission of the disease appears to be composed, not of individuals, but of discrete groups of cases related in time and space (Fig. 1). These relationships correspond to a well-defined social unit, either a household or a school class.

The flow of the epidemic through the district obviously included two distinct types of transmission, inter-social unit and intra-social unit. Since the district

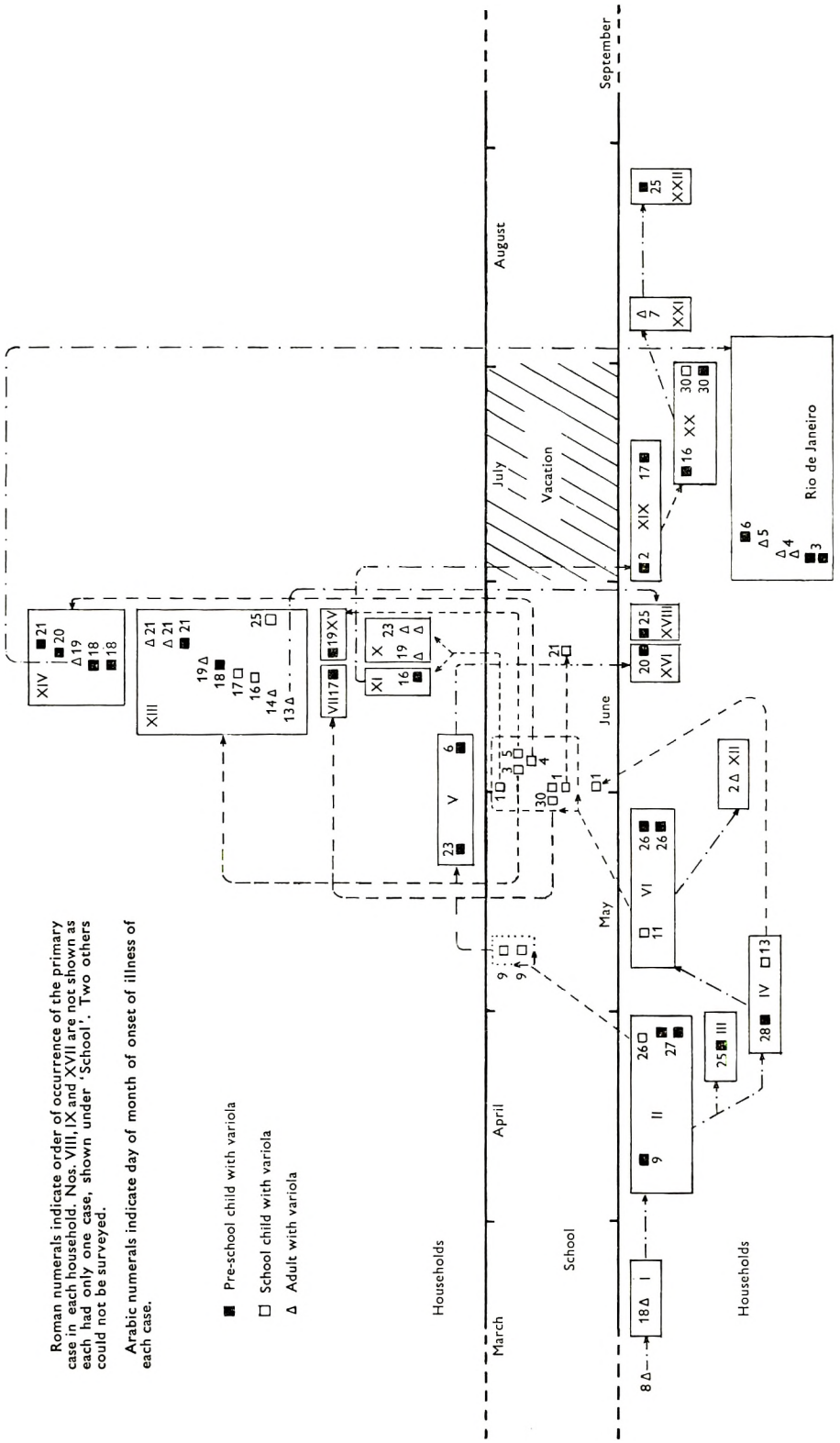

Fig. 1. Epidemic spread by date of onset, house and school, Vila Guarani.

consisted of only two types of social units, the household and the school class, the following possibilites of inter-social unit transmission existed: household to household, household to school class, school class to school class, and school class to household. The chain of disease transmission in the district shows that all four types of inter-social unit transmission actually occurred during the epidemic (Fig. 1). Moreover, each type predominated during a certain interval of the epidemic period, and thus four stages of the epidemic might be recognized. These stages compose a cycle whose completion coincided with the ending of the epidemic.

Most inter-social unit transmission of disease occurred, not between members of well-separated houses, but between persons from houses sharing a latrine, a clothes-washing basin and a yard. According to the information consistently obtained from household members, it was in this yard that personal contacts between members of different households most frequently occurred. Small crowded houses impelled people to gather in this yard and there was general reluctance to quarantine patients, since the eruption was benign. Nine classes briefly met daily in the central yard of the school, before and after leaving classrooms. It was at this time that class to class transmission probably occurred (Angulo *et al.* 1964).

Factors influencing the spread in the district

Social-unit composition

As shown in Table 1, a greater proportion of the households with cases had at least one school child. Forty per cent of these households included two or more school children, while less than 15% of the households without cases had two or

Table 1. *Study households by specified number of school children and pre-school children and vaccination level*

Type of household	Total number of households	Number of households with specified number of: School children 0	1	2+	Pre-school children 0	1	2+	Number of households with vaccination level of: < 50%	⩾ 50%
With cases	20	6	6	8	4	4	12	13	7
Without cases	22	11	8	3	10	6	6	5	17

more school children. In addition, a greater proportion of the households with cases had at least one pre-school child, compared with the households without cases. Households with a vaccination level of 50% or higher are significantly more frequent among households without cases (Yates corrected $\chi^2 = 6{\cdot}02$, $P = 0{\cdot}014$). The proportion of persons with a previous (successful) vaccination was also higher in the total population of the households without cases (62·7% compared with 50·5%). Although the characteristics of school classes without cases were not determined, the evidence obtained in classes where cases occurred indicate that the vaccination level was influential (Angulo *et al.* 1964).

Type of inter-social unit transmission

Table 2 shows the number of resulting cases by type of inter-social unit transmission. Because the actual length of the infectivity period for all cases was unknown, the total period of transmission is calculated as the interval between the first and last onset of illness for any given type of inter-social unit transmission. This criterion cannot be applied to occurrences in the school, because here the students responsible for the introductions did not attend the school during the whole eruptive stage nor during the pre-eruptive stage but on certain known days of their eruption (Angulo *et al.* 1964). Thus the total period for household to school class transmission is calculated from the date (29 April 1956) of the first class attendance by a student in the eruptive stage to the date (19 May) of the last

Table 2. *Number of cases of variola minor by type of inter-social unit transmission*

Type of inter-social unit transmission	Total period of transmission (days)*	Social units affected†		Number of resulting cases
		Number	Rate of transmission (per day)	
School class to household	43	12	0·28	23
Household to household	160	11	0·07	20
Household to school class	20	3	0·15	9‡
School class to school class	18	2	0·11	2

* See text for definition of this period.

† The households include twenty-one households actually surveyed plus two households which were not surveyed but whose primary cases were identified in the school. Household I is not included, since the source of infection for the traveller introducing variola minor in this household (the first household infected in the district) is unknown.

‡ Includes only those cases infected in the same class as the student introducing the disease.

class attendance by a student in the eruptive stage. The total period of class to class transmission is calculated from the date (29 April) of the earliest introduction resulting in transmission to another class to the date (17 May) of the last introduction which resulted in transmission to another class.

Household to household transmission was responsible for spread of variola minor from household I, through which it was introduced into the district, only to households II, III and IV, all living in the same group of houses sharing living facilities (Figs. 1 and 2). A restricted household to household transmission also occurred in the neighbourhood comprising the households XIX, XX, XXI and XXII where the disease was spreading after closure of the school for vacation in July. In contrast, the topographical distribution of homes affected by school class to household transmission was widespread. This is observed in spite of school class to household transmission being in operation for only one fourth of the period of household to household (Table 2). School class to school class transmission seems to have been the least efficient in the spread of variola minor. Class to class spread

occurred in only two instances, although twenty-seven classes used nine classrooms and shared two latrines (Angulo *et al.* 1964).

Family status of persons involved in inter-social unit transmission

All inter-social unit transmissions involved only one person from each school class or household, whatever the social units involved. When the person infected in an inter-social unit transmission was a school child, the secondary attack rate (SAR) in his household was twice the rate found in the households where the disease was introduced by pre-school children (Table 3). In the households where adults introduced the disease very small numbers were involved.

Table 3. *Inter-social unit transmission of variola minor. Secondary attack rate by family status of primary cases*

Family status (school attendance)*	Total number of households	Total number of susceptible household members	Secondary cases	Secondary attack rate (%)
Pre-school children	6	26	5	19·2
School children	11	66	25	37·9
Adults	3	2	0	0·0
Total	20	94	30	31·9

* Of patient introducing variola minor into the household.

Table 4. *Intra-social unit transmission of variola minor. Secondary attack rate by family status of primary case*

	Transmission to persons with same family status			Transmission to persons with other family status		
Family status* (school attendance)	No. of persons	No. of cases	SAR (%)	No. of persons	No. of cases	SAR (%)
Pre-school children	8	3	37·5	18	2	11·1
School children	17	3	17·6	49	22	44·9
Adults	1	0	0·0	1	0	0·0
Total	26	6	23·1	68	24	35·3

* Of patient introducing variola minor into the household.

Family status of persons involved in intra-social unit transmission

Pre-school children introducing variola minor into their households showed the greatest ability to transmit the disease to housemates with the same family status compared with school children transmitting the disease to housemate school children (Table 4). When transmission to persons with different family status is examined, school children had the greatest ability to transmit variola minor. It is difficult to determine the efficiency of adults to transmit variola minor to housemates with the same or different family status, since very small numbers are involved.

Place of occurrence of disease-transmitting contacts

Disease-transmitting contacts between members of different households could occur at the school or at places (houses, playgrounds, streets, bars, etc.) other than the school. The information about the possible source of infection for the twenty-one persons introducing variola minor in the twenty-two households with cases which were surveyed, and for the three students introducing the disease into school classes, strongly suggested that transmission almost constantly occurred at home or at the school. Only one disease-transmitting contact seemingly occurred in a playground and no contact with an infective from another household was recorded as occurring in the streets, bars or groceries for the remaining patients who had a primary infection. Moreover, all persons reporting frequent personal contacts with patients referred to neighbours exclusively.

Eleven school children seemingly infected in the school introduced variola minor into twelve households (Fig. 1). Of the eighteen school children developing the disease, eleven were infected in the school and seven were infected at home (Fig. 1). Furthermore, although there were eight school children in the population of the eleven households affected through household to household spread, only one school child was infected in these eleven inter-household transmissions (the first household affected in the district is not included, as the corresponding inter-household spread did not occur in Vila Guarani). The evidence on class to class spread suggested that this spread occurred outside the classroom and, probably, in the school central yard, where children met before and after attending classes (Angulo *et al.* 1964).

Degree of exposure

For unvaccinated susceptible persons in school classes the SAR was 19·6% (Angulo *et al.* 1964). This is significantly lower (Yates corrected $\chi^2 = 11{\cdot}26$, $P < 0{\cdot}001$) than the SAR of 55·3% for unvaccinated susceptible persons in households (Angulo *et al.* 1967). There were at least 55 exposed susceptible persons previously vaccinated in the three school classes where the students introducing disease attended classes during the eruptive stage of illness (Angulo *et al.* 1964). None of these fifty-five persons developed variola. In contrast, nine of the fifty-six susceptible persons, previously vaccinated, exposed in their household showed variolous manifestations. This difference is significant (Yates corrected $\chi^2 = 9{\cdot}62$, $P = 0{\cdot}002$).

In two of the three school classes in which secondary cases did not occur it was firmly established that the children with variola did not attend classes during illness (Angulo *et al.* 1964). In the two classes with secondary cases, the patients introducing the disease attended classes during the eruption. The child attending classes for the longest period of time during the eruptive stage gave rise to the largest number of cases.

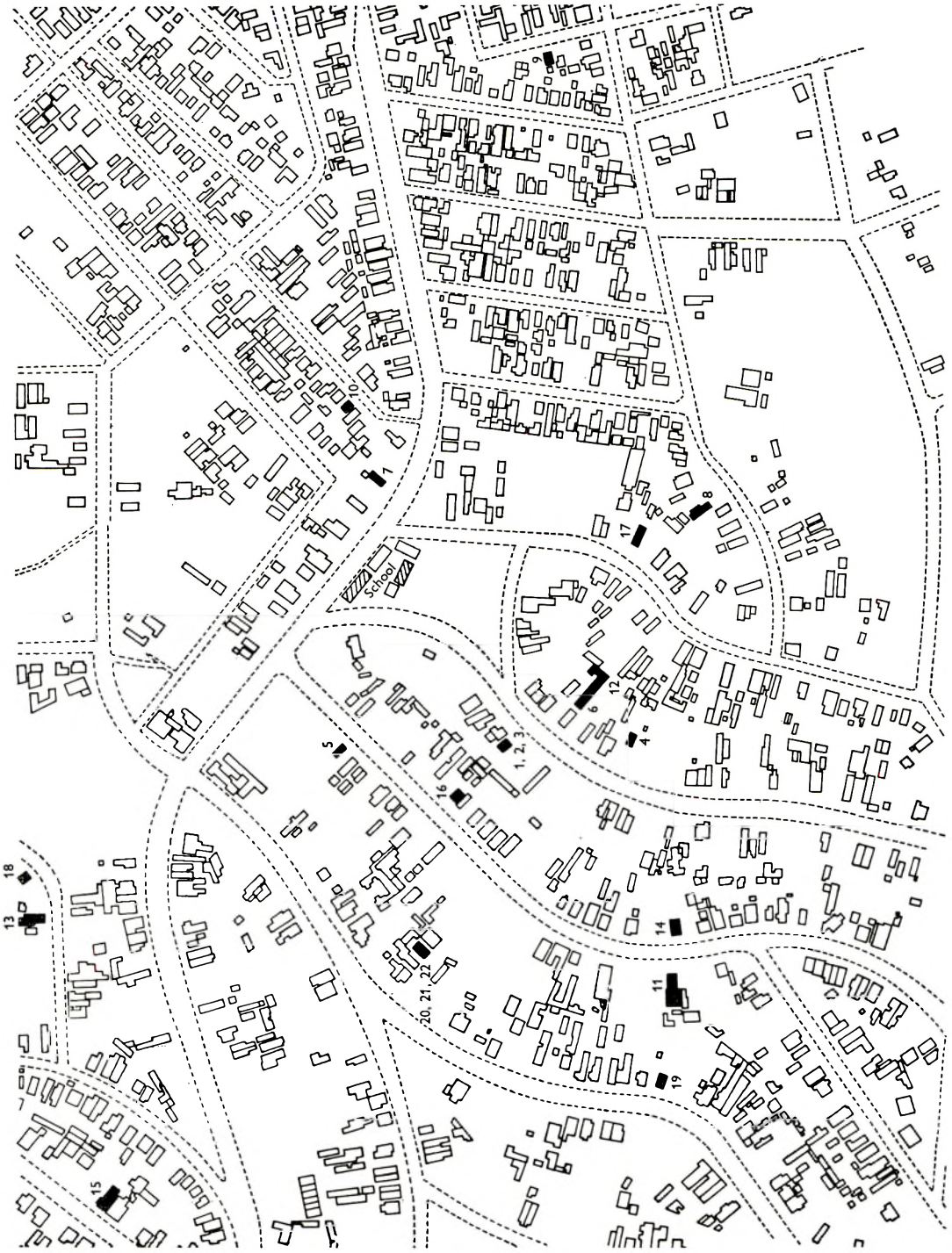

Fig. 2. Geographic distribution of households with cases of variola minor, Vila Guarani.

Preventive measures

With the exception of two patients admitted on the first day of eruption, eight other patients were isolated on the fourth to the tenth day of eruption in a hospital located 10km from the district. Two additional patients were isolated at unknown intervals after onset of eruption. The SAR is higher, though not significantly, in the eleven households where the patients remained at home than in the six where the patients were isolated in hospital, even though the former households had a higher vaccination rate (Table 5).

Table 5. *Effect of hospitalization on secondary attack rate*

Transmission of disease in households*	Number of households	Vaccination level† (%)	Exposed susceptible persons	Secondary cases	SAR (%)
With hospitalization of one or more patients	6	33·3	25	6	24·0
Without hospitalization of patients	11	56·3	69	24	34·8

* Includes the seventeen households with exposed susceptible household members.
† Includes the primary case in each household.

The City of São Paulo Department of Health conducted a compulsory mass vaccination of the school population and offered vaccination to the district inhabitants. Vaccinations were initiated on 6 June, when all but one of the cases infected in the school had already occurred, and continued until 30 June (Angulo *et al.* 1964). No remarkable difference was found when the SAR was compared between households with at least one primary vaccination after onset of the first case and households with no primary vaccination after onset of the first case.

DISCUSSION

The study households without cases differed from the households with cases in having a smaller proportion of school children and pre-school children and having a higher vaccination level. Variables such as sex, ethnic group or number of adults showed no remarkable difference when compared between the two groups of households. Although the characteristics of school classes without cases were not determined, it seems that the vaccination level influenced the number of cases appearing (Angulo *et al.* 1964).

Once the disease was introduced into a social unit (a household or a school class), intra-social unit spread progressed independently, since it was not associated with spread in neighbouring social units (Angulo *et al.* 1964, 1967). The chance for transmission of the disease outside homes or the school was very small, as suggested by the constant occurrence of a single introduction into households and school classes. The degree of exposure to infective persons was clearly higher in households than in school classes. The contrast between the relatively high transmissibility of variola minor in households and the very narrow flow of the disease through the

district, a very large part of which was strikingly spared, has its counterpart in an epidemic of poliomyelitis (Nolan, Wilmer & Melnick, 1955).

Household to household transmission occurred almost exclusively in groups of dwellings sharing a yard, a latrine and a basin for washing clothes. In this respect certain places of social activities, like backyards and stairways common to several houses, have been found to influence the spread of several diseases, as they increase the rate of personal contacts (Peters, 1910; Halliday, 1928). Introduction of the disease into the school was difficult because it implied class attendance by school children during the period of illness (eruption) where clinical manifestations could easily be recognized or suspected by teachers and classmates. Also, some school children were kept at home, in spite of the mildness of the eruptive stage. The subsequent intra-class spread was seemingly influenced by the length of school attendance during illness (Angulo *et al.* 1964). These factors, together with a relatively high vaccination level (compared with that of households), class discipline and the short stay in the school (approximately four hours daily) were seemingly responsible for the very limited dissemination of disease in the school.

It is believed that closure of the school for the month of July (the customary winter vacation in the Southern Hemisphere) noticeably influenced the extent of spread during the epidemic because (*a*) after the school was closed, the only mechanism of spread in the district was household to household transmission, this mechanism being self-limiting and affecting restricted areas; (*b*) the school played a predominant role in the spread of disease through the district; (*c*) there was a pronounced decrease in the number of cases in July; (*d*) the mass vaccination campaign and isolation of patients were both incomplete and started too late for their results to be effective.

Transmission of variola minor between social units included all four types possible according to the simple social structure of the district. The rate of spread from school classes to households was at least twice the rate of any other type of inter-social unit spread. This type of transmission was directly and indirectly reponsible for the majority of cases during the epidemic, for the peak of the epidemic curve and for the extent of spread. It may thus be inferred that, if introduction into the school with subsequent spread among school children had occurred for a longer time, the epidemic would have lasted longer and would have included a greater number of cases. Also, if the disease had not been introduced into the school the epidemic would have been limited to the cluster of houses where introduction into the district occurred.

The outstanding influence of the school on the spread of variola minor in the district may be due to any of the following factors. (*a*) The school was the only place in the district where persons living in various neighbourhoods had regular repeated contacts. The school thus increased the rate of contact between persons from different families and acted as a nodal centre. The daily coming and going of school children represented lines of communication converging on the school, when variola minor was introduced into the school, and diverging from the school, when children infected in the school conveyed the disease to neighbourhoods other than those where variola minor was spreading before introduction into the school;

(*b*) The concentration of school children in a single school clearly contributed to spread of the epidemic through the district. (*c*) The great majority of school children were susceptible (had no previous variola). (*d*) The social activities of school children gave them the highest potential as transmitters of variola minor, not only in extra-household contacts but in intra-household spread as well.

Closure of primary schools may be an important measure to take in an epidemic of variola minor, at least when cases are occurring among school children. In contrast, some epidemics of variola (usually variola major) in Europe have involved mostly adults (de Jongh, 1956; Dixon, 1962), resulting in a different epidemic pattern. It seems that, in those countries where endemicity of variola has disappeared, small, sporadic epidemics develop following importation of the disease by adults, with subsequent spread in the hospitals to which these patients are admitted. On the other hand, variola minor is endemic in Brazil and patterns essentially identical with that found in the present study have been observed in larger epidemics occurring in two small cities, in spite of the more elaborate urban environment (Angulo, unpublished observations).

SUMMARY

The mechanism of spread of an epidemic of variola minor (alastrim) occurring in 1956, in Vila Guarani, a semi-rural school district of the City of São Paulo, Brazil, is examined. Cases were grouped according to time and space relationships corresponding to either a household or a school class, as once variola minor was introduced into a household or a school class spread was independent of occurrences in neighbouring social units. The constant occurrence of a single introduction in school classes and households implied that the chance for transmission of the disease outside the homes or the school was very small. The secondary attack rate was higher in households than in school classes. Twenty households with cases had more pre-school children and school children and a lower vaccination level than twenty-two neighbouring households without cases but also including one or more persons who were known to have had personal contacts with patients.

Examination of the chain of contagion clearly shows that spread in the school was directly and indirectly responsible for the majority of cases in the epidemic, for the peak of the epidemic curve and for the extent of topographical spread of the epidemic. This predominant role of the school was accentuated by incomplete preventive measures (vaccination campaign and isolation of patients) applied late in the epidemic and it is attributed to the following facts. (*a*) There was a single school in the district, and school children were thus concentrated. (*b*) The school was the only place of the district where persons living in different neighbourhoods had regular, repeated contacts. (*c*) The great majority of school children had had no previous variola and many had no vaccination scar. (*d*) The social activities of school children gave them the highest potential as transmitters of variola minor.

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A whey complement fixation test. Its relation to whey agglutination and isolation of *Brucella abortus* from the milk of individual cows

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The Milk Ring Test (MRT) (Fleischhauer, 1937) has been widely used for 30 years as a screening test to detect brucella-infected herds. At the present time the identification of the infected individual animals within these herds is of considerable interest because of the eradication policy announced by the British Government (Hansard, 1966), the voluntary attempts to eradicate brucellosis from dairy herds particularly by the producer-retailers, and the Ministry of Health's indication that the existing legislation against the sale of raw untreated brucella-infected milk should be more vigorously applied (Min. of Health Circular 17/66).

In the early stages of the British eradication scheme it is proposed that a herd must show three negative milk ring tests at not less than three-monthly intervals before it may be registered as a 'Supervised Herd'. Thereafter full registration will follow blood testing of every animal in the herd.

In the meantime, to safeguard the health of the public, the local health authorities have neither the facilities nor the authority to take blood samples from cows and are limited solely to sampling milk. There is a need, therefore, for an adequate test for the routine examination of individual cow's milk, the prerequisite of any such test being that it should leave the least area of doubt whether the animal is infected or not. In our opinion published work suggests that the MRT is not an adequate test for the examination of individual milk samples. However, there are four quantitative serological techniques for the examination of milk which have proved to be of value in detecting brucella infection of the udder. These are:

(i) The whey agglutination (AG) test (Smith, Orcutt & Little, 1923; Traum & Maderious, 1947).

(ii) The milk plate agglutination test (Blake, Manthei & Goode, 1952).

(iii) The whey plate agglutination test (Cameron, Kendrick & Merriman, 1956).

(iv) The quantitative MRT (Ferguson & Robertson, 1960).

Kerr, Pearson & Rankin (1959) made a major contribution by showing that the udder is capable of producing antibody in response to the introduction of brucella antigens into one or more quarters, thus demonstrating the local production of antibodies. They showed that, at the beginning and at the end of lactation, serum globulins are present in milk. Therefore, depending on the stage of lactation, whey AG antibodies in milk could have arisen either from local production in an infected udder or they could be serum globulins from the blood.

In an unvaccinated animal or in an adult animal vaccinated in calfhood (5–8 months old) the presence of agglutinins in the whey at any stage of lactation is indicative of infection, either local or general. After vaccination of an adult cow, irrespective of the stage of lactation, agglutinating antibodies are present in whey for 3 months but persist in the blood for at least 2 years. In the animal vaccinated as an adult—and very often the vaccination history of an animal is not available—the presence of agglutinating antibodies is not necessarily indicative of infection.

The four tests listed above detect agglutinating antibodies and consequently cannot distinguish between adult vaccination and natural infection. In acute human brucella infections we have found that complement-fixing antibodies in the blood reflect the activity of the disease. We have been unable to find a published reference to a whey complement fixation (CF) test for brucella antibodies and it was decided to investigate this test with milk from individual cows, and to compare the results with those depending on whey agglutination and isolation of the organism.

METHODS AND MATERIALS

Milk ring test

All samples were screened by the MRT and the results were graded + + +, + +, + and − according to the standards set down by a Working Party of the Public Health Laboratory Service (PHLS) (Report, 1956).

Whey tests

Whey was prepared from whole milk using the method recommended by the PHLS Working Party (Report 1956).

Agglutination (AG)

The AG test was performed on whey dilutions (using 0·4% phenol saline as diluent) ranging from 1/10 to 1/640 with both *Brucella abortus* and *B. melitensis* antigens (obtained from the Standards Laboratory, PHLS). The dilution of whey showing 50% agglutination was taken as the endpoint of the titration.

Complement fixation (CF)

The CF test was performed as described by Bradstreet & Taylor (1962), using the method of 'long' (overnight) fixation. The antigens used were the Standards Laboratory agglutinable suspensions. They were washed free of phenol and resuspended in veronal buffer. The optimum antigen dilution was obtained from a chessboard titration against a whey of known antibody content. The complement is titrated in the presence of antigen and a 1/10 dilution of pooled negative whey, using 1·2 units of complement in the test. Usually this combination has an anticomplementary effect, so the unit of complement determined by this method does not have any relation to the 1 MHD (minimal haemolytic dose) determined by the traditional method of titration of complement in the presence of saline. In practice the 1·2 units are approximately equal to 2 MHD but the relationship between the two varies with the batches of reagents used (Farrell & Robertson, 1967).

Culture

Culture of MRT-positive individual cow samples of milk was made from overnight gravity cream (Huddleson, Hasley & Torrey, 1927) on two different selective agars—Mair's medium (Mair, 1955) and a modification of Morris's medium (Morris, 1956). The identification of organisms isolated was confirmed using the criteria laid down by FAO/WHO Expert Committee on Brucellosis (Report. 1964*a*).

RESULTS

Of the 293 MRT-positive individual milk samples which were examined 142 were MRT + + +, 60 were MRT + + and 91 were MRT +. Three hundred MRT-negative individual milk samples were also examined as controls by the whey AG and CF tests, and 4/300 (1 %) contained CF antibodies.

Table 1. *The relation between MRT, AG and CF antibodies in whey from* 293 *MRT-positive cows*

	MRT		
	+ + +	+ +	+
Total	142	60	91
No. both AG and CF positive	120 (84)	29 (50)	19 (20)
No. AG positive only	12 (8)	7 (11)	3 (3)
No. CF positive only	4 (3)	3 (5)	2 (2)
No. both AG and CF negative	6 (4)	21 (34)	67 (73)

Figures in parentheses are percentages.
Three hundred MRT-negative individual samples yielded 4 (1 %) positive CF tests (see text).

Table 2. *A comparison of the isolation rates for* Brucella abortus *obtained on culture from MRT-positive, whey AG-positive, and whey CF-positive milks*

	MRT					
	+ + +	No. of isolations	+ +	No. of isolations	+	No. of isolations
MRT-positive	142	95 (67)	60	20 (33)	91	9 (10)
AG-positive	132	91* (71)	35	17* (57)	22	7* (41)
CF-positive	124	95 (76)	32	20 (63)	21	9 (43)

* There were nine isolations when the whey AG was negative (Table 4). Figures in parentheses are percentage isolation rates.

In Table 1 the results of MRT, AG and CF tests on the 293 MRT-positive milk samples are shown. From these results it is evident that the number of MRT-positive milk samples in which whey AG and CF antibodies could not be demonstrated increases as the intensity of the MRT reaction decreases, i.e. MRT + + + 4 %; MRT + + 34 %; and MRT + 73 %. This would appear to suggest that many MRT + + and MRT + reactions on individual milk samples are in fact false positive results.

The isolation rates of *B. abortus* are shown in Table 2 for MRT-positive; MRT and whey AG-positive; MRT and whey CF-positive milk samples. These are subdivided according to the intensity of the MRT reaction.

Table 3. *The relationship between whey CF titre and isolation of* Brucella abortus *in* 293 *MRT-positive individual milk samples*

Whey CF titre	MRT			Total
	+ + +	+ +	+	
⩾ 640	5/5	2/3	0/0	7/8 (88)
320	11/14	2/3	0/1	13/18 (72)
160	24/25	0/1	1/2	25/28 (89)
80	14/21	3/6	1/2	18/29 (62)
40	17/22	1/3	0/0	18/25 (72)
20	12/18	5/5	4/7	21/30 (70)
10	12/19	7/11	3/9	22/39 (56)
< 10	0/18	0/28	0/70	0/116

All titres expressed as reciprocals of the whey dilutions.
Figures in parentheses are percentages.
The figures represent number of positive cultures/total number cultured.

Table 4. *The relationship between whey AG titre and isolation of* Brucella abortus *in* 293 *MRT-positive individual milk samples*

Whey AG titre	MRT			Total
	+ + +	+ +	+	
⩾ 640	29/33	2/3	0/0	31/36 (86)
320	21/26	2/3	0/1	23/30 (77)
160	24/32	3/7	3/5	30/44 (68)
80	9/17	4/8	2/6	15/31 (47)
40	8/19	2/5	1/5	11/29 (38)
20	0/5	4/9	1/5	5/19 (26)
< 20	4/10	3/25	2/69	9/104 (9)

All titres expressed as reciprocals of the whey dilutions.
Figures in parentheses are percentages.
The figures represent number of positive cultures/total number cultured.

It is evident that the correlation between the MRT and isolation rate is increased if the presence of either whey AG or CF antibodies is taken into account. The correlation between the whey AG test and isolation rate for the three MRT categories (+ + +, + + and +) was 71, 57 and 41 % respectively compared with the correlation of 76, 63 and 43 % respectively between whey CF and isolation rate.

The serological test giving the highest correlation with isolation rate was Complement Fixation and it is important to note that *B. abortus* was isolated only from MRT-positive milk when whey CF antibodies were detected, as opposed to nine isolations from milk which was whey AG-negative. These nine milk samples were MRT and CF positive.

The relationship between the titre of the CF antibody and the isolation rate is shown in Table 3 for the three grades of the MRT reaction.

B. abortus was not isolated from any of the 116 MRT-positive milk samples without detectable brucella complement-fixing antibody.

The relation between whey AG titre and isolation is shown in Table 4. It is evident from Tables 3 and 4 that the relation between the isolation rate and CF titre remains constant from 1/20 upwards, and even at the lowest level (1/10) at which CF antibody was detected a 54% isolation rate was obtained. This contrasts with the whey AG test where there is a progressive decrease in the isolation rate from 86% at a titre of 1/640 to 26% at a titre of 1/20.

DISCUSSION

This investigation shows that the presence of whey CF antibodies strongly suggests a brucella infection within the bovine udder, for *B. abortus* was isolated from 124 of the 293 MRT-positive milks and in each culture-positive milk whey CF antibody was present. Although the whey CF showed a slightly higher correlation with isolation as compared with the whey AG test the difference between the two is not appreciable (Table 2). But the CF test is a more specific index of infection than the AG test because *B. abortus* was not isolated from whey CF-negative samples, whereas it was isolated from nine milk samples which were whey AG-negative.

The whey AG test has been widely used by veterinary bacteriologists, who have reported isolation of *B. abortus* from whey AG-negative milk samples. The working density of antigen used by the veterinary bacteriologists (Weybridge Abortus antigen) is optically 6·75 times as dense as that used for the examination of human serum (Standards Laboratory, PHLS). Because of its greater density the Weybridge antigen is less sensitive for the detection of the brucella antibodies than the PHLS antigen (Farrell & Robertson, 1967). We have isolated *B. abortus* from some milk samples which were whey AG-negative even when the more sensitive PHLS antigen was used in the test.

The biotypes of *B. abortus* have the antigenic structure of either abortus or melitensis. The abortus antigen is predominant in biotypes 1 and 2, whereas the melitensis antigen is predominant in biotypes 5 and 9. These four are the most common biotypes in Great Britain, and biotypes 5 and 9 are present in 12% of the infected herds in the north of Lancashire (Farrell & Robertson, 1967). For this reason both abortus and melitensis antigen are used in the whey tests. This is of particular importance in animals which are at an early stage of infection when, although *B. abortus* is excreted in the milk, the level of CF antibody is often low. Moreover, in infections with biotypes 5 and 9 melitensis CF antibodies may be present before the abortus CF antibodies. The converse is true in infections with biotypes 1 and 2. Because of the low levels of CF antibody in the initial stages of infection it is necessary to make the CF test as sensitive as possible while retaining specificity.

In 94 (32%) of the 293 MRT-positive milk samples examined brucella antibodies

could not be demonstrated by either the whey AG or CF tests, but 67 of these were only MRT +, and a further 21 were MRT ++. A further 22 were whey AG-positive but whey CF-negative.

This investigation indicates that brucella organisms are not excreted in the milk of whey CF-negative animals, irrespective of the MRT or whey AG results. When the whey CF test is negative but the MRT and/or whey AG test is positive the animal may be in the early stages of an udder infection. In a further investigation (Robertson & Farrell, to be published), the lactating animals from a large calfhood-vaccinated herd were examined on seven separate occasions over a 10-month period and 43 cows were found to be excreting *B. abortus*, biotype 5 in their milk. In this study a number of cows were seen whose milk was MRT-positive and whey AG and CF negative at first, but became positive to all three tests 2 months later. In such cows the disease showed a steady progression thereafter, as indicated by an increase in whey AG and whey CF titres.

Our experience in this and in other instances has been that any animal which was in the early stages of infection has given a whey CF-positive test on a further sample taken 2 months later. If these two successive whey CF tests are negative, irrespective of the MRT or whey AG result, we suggest that the animal can be regarded as free from udder infection. But this does not exclude the possibility of another focus of infection which can only be determined by blood testing.

It was estimated that 36,500 cows had udder infections due to brucellosis in the British Isles in the year 1960/61 (Report 1964*b*). In north-west Lancashire there are approximately 900 producer-retailers, 20% of whose herds are known to be infected (Robertson, 1967). In attempts to safeguard the health of the public, milk from these producer-retailers is sampled frequently by local authorities and examined for the presence of tubercle bacilli and *B. abortus*. The isolation of *B. abortus* from a sample of herd milk is usually followed by a Pasteurization Order in accordance with the Ministry of Health Circular (17/66). The detection of the infected animals within these herds is difficult, as local authorities have neither the authority nor the facilities to take blood samples from the suspect animals. This limits testing to milk samples; the MRT followed by either direct culture or biological examination of all MRT-positive milk samples is impracticable when large numbers of individual samples have to be examined. For the success and smooth working of an eradication programme, the examination of individual samples by the MRT only is not advisable because it may be concluded from the present study that up to 32% of MRT-positive milk samples are not due to brucella infection of the udder.

The limitations of the serological tests which detect agglutinating antibodies are twofold. First, in their failure to detect antibodies in the 9/104 milk samples which were MRT, CF and culture positive, and secondly, in their failure to distinguish between a natural brucella infection and adult vaccination. In an animal vaccinated more than 6 months before testing, a positive whey CF test is indicative of a brucella infection, irrespective of whether it is lactoglobulin or a serum globulin that is detected. CF antibody disappears from the blood within 6 months of adult vaccination (W. R. Kerr, pers. comm.).

We have found the whey CF test to be useful for the detection of infected animals within the herds of producer-retailers in the north of Lancashire and believe it could be a valuable additional test in an eradication programme.

SUMMARY

Two hundred and ninety-three milk ring test-positive individual milk samples were examined by whey complement fixation and agglutination tests and the results were compared with the isolation of *B. abortus* by cultures made from the milk.

There was a closer correlation between whey CF tests and isolation of the organism than between whey AG tests and isolation. Brucella organisms were not isolated from 116 MRT-positive milk samples when the whey CF titre was < 1/10; whereas 9/104 (9 %) isolations were made from milk when the whey AG titre was < 1/20.

Because of its closer correlation with culture results it is suggested that the whey CF test would be valuable in an eradication programme for the detection of infected udders.

The limitations of the milk ring test for the examination of individual milk samples are emphasized and it should be used only as a preliminary screening test. In the individual milk samples examined, 32 % of the MRT-positives showed no evidence of infection by either the whey AG, whey CF, or culture tests.

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The isolation and characterization of foot-and-mouth disease virus from clinically normal herds of cattle in Botswana

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INTRODUCTION

Since van Bekkum, Frenkel, Fredericks & Frenkel (1959) demonstrated the virus of foot-and-mouth disease (FMD) in the saliva of some cattle over a period of months after recovery from clinical disease, other workers have used similar techniques for further studies on the carrier state in cattle. Sütmoller & Gaggero (1965) were able to recover FMD virus from the oesophageal fluid of cattle on a farm some months after natural infection, and Burrows (1966), working with experimental cattle, was able to show that the main sites of carrier virus multiplication were the mucosae of the pharynx and the dorsal surface of the soft palate.

Little is known, however, of the incidence of the carrier state under natural conditions in areas where FMD is enzootic, and where, owing to the emphasis on livestock and meat exports, knowledge of the ecology of FMD virus assumes considerable importance. This paper gives the results of a survey of the FMD carrier state in cattle in such an area in Botswana, formerly known as the Bechuanaland Protectorate.

The methods of collection and transport of the samples are particularly important in work carried out at such a distance from the testing laboratory. The isolation of virus on tissue cultures of bovine thyroid cells is described. Sera were assayed for FMD antibodies and an attempt is made to correlate the antibody levels demonstrated with the carrier state.

Antigenic studies were made on some of the carrier viruses in which they were compared with each other, with a strain of virus isolated during the most recent FMD outbreak and with an attenuated strain of virus of the same type used as a vaccine at the time of the outbreak.

MATERIALS AND METHODS

Area and cattle

The survey was carried out in three localities of the Botletle river area of Northern Botswana. This area, its importance, its ecology and system of animal husbandry is described in more detail elsewhere (Hedger, Bachmann & Herniman, to be published). For many years foot and-mouth disease has occurred periodically in this region where the disease must be considered enzootic. In the first locality chosen (Rakops) there had been an outbreak of FMD earlier in the year with the

last remaining clinical cases being observed 7 months before the survey. In the second locality (Lake Dow) there had been a severe infection 12 months before the survey. These outbreaks were both caused by an SAT 3 virus, subtype 4, strain Bec. 1/65 (referred to in the remainder of this paper as outbreak virus). Vaccination with an attenuated SAT 3 vaccine strain, SA 57/59 (referred to as vaccine virus), subsequently shown to be a different subtype, did not completely prevent or control the outbreaks. In the third locality in the eastern part of Ngamiland, a considerable distance from the more recently infected areas, there was no known history of FMD or vaccination in the cattle under test.

The cattle (*Bos indicus*), mainly long horned with pre-thoracic humps, were all native owned indigenous Tswana stock ranched under harsh conditions with a low standard of husbandry. They were chosen at random from different herds at each cattle post but were mainly adult cattle.

Collection and handling of samples

The oesophageal/pharyngeal samples were collected in cups similar to that described by Sütmoller & Gaggero (1965). To ensure adequate samples the following technique was used. The cup was passed through the mouth into the pharynx and by movements directed dorsally and laterally attempts were made to scrape quickly the surface epithelium of the pharynx and soft palate. The cup was then passed down into the upper portion of the oesophagus and withdrawn, preferably after the animal had swallowed, depositing the pharyngeal scrapings together with mucus and saliva into the cup. Particular care was taken to ensure that each specimen contained some visible cellular material.

Samples collected in the morning from cattle which have passed the night in dry and dusty kraals often contain quantities of fine soil and faecal matter. Many may contain variable amounts of ruminal contents. When it is not practical to water these animals immediately before collection, clean usable samples are easily obtained by drenching each animal with normal saline solution immediately before collection. In Europe and in other well watered areas adequate quantities (5–10 ml.) of oesophageal/pharyngeal fluid are not difficult to obtain. In dry countries, however, where available grazing may be a long distance from water, animals frequently are so dehydrated that little or no fluid may be collected in the cup. Satisfactory samples were obtained from such animals by rinsing their months with a few millilitres of normal saline solution immediately before collection.

The cups were disinfected with sodium hypochlorite and rinsed several times in water between animals. All equipment was autoclaved at the conclusion of sampling.

Immediately after collection each sample was mixed with an equal quantity of buffered diluent containing antibiotics. Oesophageal/pharyngeal samples are alkaline and in previous work it had been found that their addition to phosphate buffered saline (PBS) or M/25 phosphate buffer at pH 7·6 resulted in high pH values (mean of 14 samples pH $8{\cdot}16 \pm 0{\cdot}16$). On this survey therefore M/12·5 phosphate buffer containing 0·001 % phenol red indicator and adjusted to pH 7·2 was used, resulting in a final mean pH of $7{\cdot}55 \pm 0{\cdot}05$ (9 samples). Each millilitre

of diluent contained 1000 units of penicillin, 100 units of Neomycin, 50 units of Polymyxin B and 100 units of Mycostatin.

Transport and storage of samples

As other workers (Burrows, 1966; van Bekkum, Straver, Bool & Frenkel 1966) have noted that oesophageal/pharyngeal samples quickly lose their infectivity when stored at 4° C. and −20° C., comparative parallel tests of oesophageal/pharyngeal samples containing minimal virus stored at 4° C. and in dry ice at −70° C. were carried out at Pirbright before the field survey. The virus recovery rate from samples stored at −70° C. over periods of up to one month was significantly higher than after storage at 4° C. The greatest loss of titre in both sets of samples occurred during the first 48 hr. after collection. Thereafter the amount of recoverable virus in those samples stored at −70° C. remained fairly constant over the 30-day period of the tests, while a steady loss of recoverable virus occurred during storage at 4° C.

Dry ice was therefore used for the transportation of samples and arrangements were made for them to reach the laboratory at Pirbright within 48 hr. of their collection. Sealed boxes of 2 in. polystyrene proved to be adequate for transporting the specimens in dry ice, and were relatively light.

To maintain adequate stocks of dry ice over the many weeks of this survey in a remote area with ambient shade temperatures reaching 43° C., a sheet metal cavity walled box large enough to hold four 56 lb. blocks of dry ice was constructed. The walls of this box were 4 in. thick and the cavities insulated with polystyrene. Stocks of dry ice when covered with sawdust lasted for up to 2 weeks in the box provided that the unoccupied space was taken up with packing material.

Sera

All cattle under test were bled, clinically examined and identified with numbered ear tags the day before the oesophageal/pharyngeal samples were taken to avoid undue delay at the time of or after sampling. The sera were separated on the day of collection using a small variable speed portable centrifuge* developed to run off either a 6 V. or a 12 V. car battery. Weighing only $3\frac{1}{2}$ lb. this centrifuge was fitted with a nylon head designed to hold four 9 ml. disposable tubes with caps and was capable of speeds up to 5500 r.p.m. Antibiotics were added to the sera to give a final concentration of penicillin 100 i.u./ml., polymyxin B 50 units/ml., Neomycin 100 units/ml. and Mycostatin 100 units/ml.

Isolation of virus

Isolation of virus was made on monolayer cultures of calf thyroid cells in 6 in. × $\frac{5}{8}$ in. tubes on roller drums as described by Snowdon (1966) who showed calf thyroid cells to be more sensitive to unmodified FMDV than either BHK 21 cultures, unweaned mice, secondary pig kidney cultures or cattle by intradermolingual inoculation. Since this observation the World Reference Laboratory at Pirbright has routinely used calf thyroid tissue cultures not only for the isolation

* Luckham Ltd., Labro Works, Burgess Hill, Sussex.

of virus from oesophageal/pharyngeal samples, but also in addition to other systems, for the isolation of virus present often in only minimal quantities from samples of epithelium submitted from the field for typing.

Although, as mentioned by Snowdon (1966) the infected thyroid tissue culture maintenance fluid containing 3% ox serum is generally a satisfactory antigen in complement fixation tests, it has on occasion been found to be anticomplementary. For this reason, and to avoid any possible non-specific inhibition of the minimal virus in oesophageal/pharyngeal samples, ox serum was omitted from the maintenance medium after inoculation of the tubes. Once the monolayers were established the omission of serum did not affect their susceptibility to FMDV, nor was there observable inhibition of cell metabolism over the 48 hr. period of the test. Cytopathogenic effect (CPE) was normally apparent by 27 hr. after inoculation of the tubes but a final check reading was made at 48 hr. Only rarely were tubes negative at 27 hr. found to be positive at 48 hr.

Virus specificity

The specificity of all the viruses isolated was checked by complement-fixation tests on infected cultures using the microtitre technique described by Casey (1965).

Two of the viruses isolated were adapted to guinea-pigs for specific antiserum production and antigenic differences were studied between these viruses and previously known subtype strains by cross-complement-fixation tests, using the method described by Davie (1964) in addition to the microtitre method.

Serum neutralization tests

The cattle sera were assayed by the cell metabolic inhibition test or colour test (Martin & Chapman, 1961) using primary monolayers of pig kidney cells and a type SAT 3 (strain Bec. 1/65) virus isolated from the most recent field outbreak of FMD in the locality of the survey. Before its use in the test this virus was adapted to grow in pig kidney cell cultures by serial passage. Neutralization titres are expressed as the reciprocal of the final dilution of serum present in the serum virus mixture at the 50% end-point estimated according to the method of Kärber (1931).

Mouse neutralization tests with homologous virus strains were also used to confirm the absence of antibodies in some of the sera from virus-positive animals.

RESULTS

General survey

The areas from which the cattle were drawn, the numbers sampled and the percentages of carrier animals in which virus was demonstrated, together with the virus type and details of previous FMD history and vaccination, are summarized in Table 1. The percentage of positive carriers identified at the three cattle posts in the Rakops locality remained fairly constant at approximately 20%. The cattle sampled at these cattle posts were drawn from nine individual herds, but under the

prevailing conditions of husbandry each cattle post may, from an epizootiological aspect, be considered as one herd. The total number of cattle at each cattle post during the survey varied from 500 to 1700 head, thus the sample represented approximately between 3·5 and 12% of each cattle population. Although the cattle were selected at random for sampling, emphasis was placed on adult animals in the first instance which were more likely than the calves to have been among the infected animals during recent outbreaks.

Table 1. *Summary of the results of a carrier virus survey in three localities in Botswana*

Locality	Cattle post	Number of cattle sampled	Positive carriers	%	Virus type	Period since last infection	Vaccination history: type and period before sampling
Rakops	Sokwani I	60	12	20	SAT 3	7 months	Bivalent attenuated SAT 3 (SA 57/59) and SAT 1 (RV 11) —14 months previously
	Sokwani II	60	13	23	SAT 3	7 months	
	Tsienyani	60	11	18	SAT 3	7 months	Monovalent attenuated SAT 3 (SA 57/59) or SAT 1 (RV 11) —7 months previously
Lake Dow	Goi	60	2	3	SAT 3	12 months	Bivalent as above—14 months previously Monovalent SAT 3—11 months previously
	Machana	60	12	20	SAT 3	12 months	Monovalent SAT 1 or SAT 3 as above—7 months previously
Ngamiland	Manwelo's	69	0	0	—	No history of infection	Nil

In the Lake Dow locality where sampling was approximately 12 months after infection the expected lower carrier incidence was realized at Goi, but the 20% incidence at Machana was surprising. While it is possible that this high rate of virus recovery may have been in part due to some intermingling and movement of cattle from area to area after quarantine restrictions were withdrawn, the lower degree of virus excretion (Table 2) and lower group mean antibody titre (Table 3) do suggest that the result may be a true assessment of the herd at this longer period after infection.

There were no indications of correlation between the sex and age of the animals in this survey and their carrier status.

Virus was isolated from one calf estimated to be 5–6 months old and born since the last outbreak of FMD in the locality. The serum antibody titres in this calf and its dam were high (1/178) but no virus was isolated from the single oesophageal/pharyngeal sample taken from the dam.

Serology

The association of high serum antibody titres with the carrier state has been previously reported (Sütmoller & Gaggero, 1965; Burrows, 1966 and van Bekkum

et al. 1966). In this survey 46 of the 50 carrier animals identified also had high neutralizing antibody titres in their serum, but in four of the positive animals, all adults, no serum antibody could be detected in the colour test against the outbreak strain of virus. These sera were therefore also assayed for antibodies against their homologous carrier viruses in mouse neutralization tests and no significant titres were found.

Table 2. *Virus isolations and serum antibody titres against the outbreak virus (Bec. 1/65) in individual animals at two representative cattle posts*

Sokwani II (7 months after infection)			Machana (12 months after infection)		
Animal no.	No. of positive T.C. tubes/ten	Reciprocal serum antibody titre	Animal no.	No. of positive T.C. tubes/ten	Reciprocal serum antibody titre
208	10	128	514	3	—
220	10	128	516	3	⩽ 6
195	8	45	517	3	64
186	7	90	536	3	128
217	7	256	555	2	64
212	4	64	522	1	⩽ 6
180	4	128	526	1	178
218	3	16	534	1	⩽ 6
174	2	178	537	1	90
209	2	128	540	1	22
224	2	128	558	1	90
197	1	⩽ 6	566	1	178
205	1	22			

Table 3. *The geometric mean reciprocal antibody titres of groups of carrier virus-positive cattle compared with similar groups of virus-negative cattle*

Cattle post	Virus-positive	Virus-negative
Sokwani I	275	88
Sokwani II	64	112
Tsienyani	128	95
Machana	42	54

Table 2 compares the individual antibody titres of a group of positive carrier animals from one of the cattle posts 7 months after infection with those of one of the cattle posts 12 months after infection. An indication of the amount of virus being excreted by each animal is given by the number of tissue culture tubes positive out of the ten inoculated with each oesophageal/pharyngeal sample. There is no apparent correlation between the amount of virus being excreted and the serum antibody titre in the individual animals, although, as expected, considerably more virus was isolated from the group 7 months after infection than from the group 12 months after infection. Table 3 compares the geometric means of antibody titres against the outbreak virus of groups of virus-positive animals with similar groups of virus-negative animals at different cattle posts. There is no apparent significant difference between the mean antibody titres of those animals from which virus was successfully isolated and those from which virus was not

isolated at each cattle post. Not unexpectedly, however, the mean titres in the cattle 12 months after infection are of a lower order than those 7 months after infection.

Antigenic studies

Antigenic studies included the screening of a number of carrier viruses by micro-technique complement-fixation test against a range of type SAT 3 subtype specific antisera. The resultant histograms indicated not only an antigenic variation of some strains from the outbreak strain of virus, but also at least one antigenic

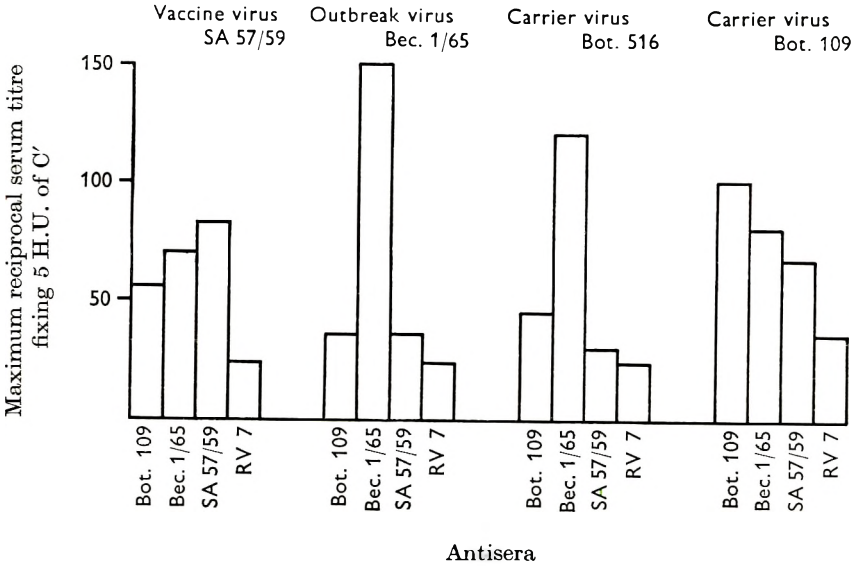

Fig. 1. The results of complement-fixation tests comparing two carrier virus strains with the outbreak and vaccine strains of virus.

difference between strains of carrier virus isolated from different animals at the same time in the same herd. Figure 1 compares the histograms of two of the carrier virus strains (Bot. 109 and Bot. 516) with the outbreak virus strain (Bec. 1/65) and the vaccine strain (SA 57/59) administered to the cattle earlier in the year.

Specific guinea-pig antisera were prepared to both those carrier strains, and cross-complement-fixation tests (Davie, 1964) were carried out in tubes with these strains, the outbreak strain and the vaccine strain. Table 4 illustrates the relationship of these virus strains to each other expressed as cross-fixation products. Carrier virus Bot. 516 is shown to be identical with the outbreak strain both in its relationship to the latter and in its similar relationship to the other two virus strains. Carrier virus Bot. 109 on the other hand, although related to the outbreak strain, shows a definite degree of antigenic variation from it. Neither carrier virus showed a close relationship with the vaccine virus, the wider variation being with Bot. 516.

It has not been possible in this present study to show a relationship between the degree of antigenic change of the carrier virus and the time lapse following clinical

disease. Carrier virus Bot. 516 which proved to be identical with the outbreak strain was isolated approximately twelve months after clinical infection in the herd, while Bot. 109 which did show antigenic variation, was isolated only 7 months after clinical disease in the herd.

Table 4. *The relationship of two carrier strains, the field outbreak strain and the vaccine strain of virus expressed as cross fixation products*

Bot. 109	1·0			
Bot. 516	0·49	1·0		
Bec. 1/65	0·51	1·25	1·0	
SA 57/59	0·165	0·09	0·05	1·0
	Bot. 109 (carrier)	Bot. 516 (carrier)	Bec. 1/65 (outbreak)	SA 57/59 (vaccine)

DISCUSSION

Both Sütmoller & Gaggero (1965) and Burrows (1966) observed that FMD virus may be demonstrable only intermittently in some carrier animals, and mention has already been made of the rapid drop in titre of carrier virus in samples stored after collection.

In view of the conditions under which this survey was carried out, the distances over which the samples had to be transported and the resultant unavoidable time lapse of nearly 48 hr. before the specimens reached the laboratory, the isolation of FMD virus in 20% of the animals tested at least 7 months after natural infection or vaccination is significant and probably represents a much higher rate of virus excretion in the herd than was recorded.

It is not possible to state unequivocally that the origin of all the carrier virus strains was directly from the virulent virus strain causing the recent infection. In the thirty virus strains studied to date no antigenic similarity to the vaccine strain of virus has been noted, nor were any of them typed SAT 1, an attenuated strain of which was used concurrently with SAT 3 vaccine earlier in the year. It seems unlikely therefore that vaccine virus was persisting in the carrier state. The strains studied all show some relationship to the field outbreak strain, indeed one (Bot. 516) obtained 12 months after infection, was shown to be identical with the field strain. There seems little doubt therefore that the origin of the carrier virus was in fact the field outbreak of disease.

It has long been suspected that antigenic changes leading to the appearance of new subtype strains of foot-and-mouth disease virus may result from the spread of virus through a partially immune population. Hyslop & Fagg (1965) have recently been able to confirm experimentally that this phenomenon may occur when virulent virus is passaged serially in vaccinated cattle. The results presented here show the possibility of such antigenic variation during the spread of virus in a partially immune cattle population under natural conditions.

The isolation of virus from a calf born after the recent clinical outbreak of foot-and-mouth disease is evidence of the transmission of the carrier state from animal to animal within the herd in the absence of visible infection. It cannot be stated

that the source of this virus was necessarily the calf's dam, but the dam's high antibody titre does suggest that she had fairly recent experience of the virus. Whether the high serum antibody level of the calf was due to the persistence of maternal transferred antibody or the multiplication of persisting virus in the calf's own tissues or was the result of a previous subclinical infection is a matter for conjecture.

It is tempting to assume that the absence of detectable antibody in 8·0 % of the virus-positive animals is further evidence of the transference of carrier virus from animal to animal within the herd. This assumption is supported by the findings of de Mello, Honigman & Fernandez (1966) who reported the transference of modified FMD vaccine virus from vaccinated to susceptible in-contact cattle. They were unable to detect antibody in these virus-positive contact cattle and suggested that the amounts of virus in these cattle were insufficient to stimulate the formation of antibodies. It is, of course, well known that some animals fail to show a detectable antibody response to primary stimulation by virus and that others show a minor response which wanes quickly. Therefore the absence of antibodies in some of the virus-positive animals in this survey does not rule out the possibility that some or all of them were infected by virulent virus at the time of the outbreak. The absence of detectable antibody in this percentage of virus-positive animals urges caution in the acceptance of negative results to antibody assay as criteria for the movement of animals. It is evident, however, that the waning of antibodies to a negligible level in animals in which unmodified virus is persisting may not be the only condition necessary for the recrudescence of disease.

There may of course be transference of carrier virus to other fully susceptible animals which, either then or at a later date, manifest the disease clinically. Epizootiological evidence on this point is conflicting. R. Burrows (personal communication) working with another SAT 3 strain of virus (Bec. 3/64) from Botswana has produced clinical FMD with generalization in susceptible cattle by intradermolingual inoculation of oesophageal/pharyngeal fluid taken from carrier animals 16 weeks after infection. He had to use large quantities, up to 4 ml., of fluid to produce disease and it may be that in the natural state the amounts of carrier virus transferred from animal to animal are too small to initiate clinical disease.

It is possible, however, that some 'trigger' factor or factors may enhance the virulence of the carrier virus or provide a more favourable environment for its growth with the consequent appearance of clinical disease in its host. Such 'trigger' factors might include antigenic change in the virus or stress in the animal or animals with which it is in contact.

SUMMARY

The results of a survey of the foot-and-mouth disease carrier state in three localities of an FMD enzootic area of Botswana are presented. The collection, storage and handling of the samples is mentioned and the isolation of virus on calf thyroid monolayer tissue cultures is described. Type SAT 3 virus was isolated from up to 20 % of the animals sampled at periods between 7 and 12 months after

natural infection. Sera were tested for all the animals from which virus was recovered and compared with similar numbers of sera from virus-negative animals. No correlation was found between the carrier state and serum antibody titres.

Antigenic studies are described which indicated not only an antigenic variation of some carrier viruses isolated from the outbreak strain but also antigenic differences between strains of carrier virus from different animals in the same herd. There was no evidence at the time of sampling of the persistence as carrier virus of the attenuated vaccine strains of FMDV used previously in the cattle sampled.

The results suggest that transference of carrier virus from animal to animal may occur in the absence of clinical infection.

The author wishes to express his gratitude to the Director of Veterinary Services, Botswana, for providing the facilities and help which made this survey possible. He is also indebted to Mr R. Burrows for advice in the preparation of this work, Mr I. T. R. Barnett for carrying out the serum assay and to Mr Franco Guecol and the staff of the World Reference Laboratory for technical assistance.

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The serum opacity reaction of *Streptococcus pyogenes*: general properties of the streptococcal factor and of the reaction in aged serum*

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Although the serum opacity factor of *Streptococcus pyogenes* was first described by Ward & Rudd (1938) as an aid to differentiating non-capsulated group A streptococci from those of other groups, it was soon evident that a large proportion of non-capsulated strains of *Str. pyogenes* failed to produce an opalescence in serum. Gooder (1961) and Köhler (1963) found an apparent inverse relationship between production of serum opacity and of the M antigen. Subsequent studies (Top & Wannamaker, 1968) have confirmed the frequency of production of serum-opacity among strains for which it has been difficult to prepare satisfactory anti-M sera, but a consistent inverse relationship was found only among type 12 strains.

Krumwiede (1954) made some preliminary investigations of the serum opacity reaction and concluded that it was due to a lipoproteinase. Rowen (1961), and Rowen & Martin (1963) have made intensive studies of the reaction in fresh human serum and concluded that the opacity was related to cholesterol esterification. He also noted considerable opacity formation in aged serum that was not due to cholesterol esterification and which is still unexplained. In this paper we will present some observations on the reaction in aged serum and on the factor responsible for it. For convenience this factor will be referred to as an enzyme, although this is perhaps premature, since the exact nature of the substrate and of the reaction is unknown.

MATERIALS AND METHODS

Strains

Strains of group A streptococci were classified by conventional methods, by T-typing (Griffith, 1934) and by M-typing (Swift, Wilson & Lancefield, 1943). Strain AN 124, a T-type 11 M-negative strain, was used in most experiments. In addition, 3497, an M-type 4 strain, AN 369, a T-pattern 3/13/B 3264 M-negative strain, together with strains of M-types 4, 11, 13, 22, 25, 48 and 49 and M-negative

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strains of T-types 2, 3, 4, 5, 11, 12, 28, 8/25/imp 19 and 3/13/B 3264 were used to detect strain variations (e.g. in heat sensitivity) of the enzyme. All cultures were grown in Difco Todd–Hewitt broth at 37° C. for 18 hr. unless otherwise stated.

Measurement of serum opacity

A mixture of 0·5 ml. of enzyme sample (cell suspension or extracted enzyme solution) and 3 ml. of horse serum (Grand Island Biological Co.) was incubated at 37° C. Opacity formation was followed by measuring the increase in optical density at 475 mμ in a Beckman DU spectrophotometer. When cell suspensions were used as enzyme sample, the suspensions were diluted to give an optical density at 600 mμ of 1·00.

Cell fractionation

Suspensions of washed cells were shaken with glass beads in a Mickle disintegrator at 0° C. (Michel & Gooder, 1962) and the resultant suspension centrifuged at 10,000 *g*. for 30 min. at 4° C. The supernatant fluid represented the cytoplasmic material whilst the deposit contained cell wall and cell membranes, which were separated by the differential centrifugation method of Freimer (1963).

Isolation of α-lipoprotein

α-Lipoprotein was isolated by flotation (Korn, 1959). Horse serum was dialysed against running water for 3 days at 4° C. to precipitate the euglobulin fraction. The density of the supernatant (pseudoglobulin) fraction was adjusted to 1·07 by the addition of potassium bromide and the resultant solution was centrifuged at 90,000*g*. for 16 hr. at 4° C. in the SW 39 head of a Spinco model L ultracentrifuge. The clear colourless fraction floating on top was discarded and the density of the remainder was brought to 1·21 by the addition of potassium bromide. Centrifugation of this solution at 90,000*g*. for 16 hr. at 4° C. caused the α-lipoprotein to float to the top from where it could be removed in a much purified state. Potassium bromide was removed from the α-lipoprotein solution by dialysis.

Analytical methods

Glycerol was determined by the method of Korn (1959). This method is based on the conversion of glycerol to formaldehyde, the latter being estimated by colour formation with chromotropic acid. The method cannot distinguish between glycerol and glycerol-1-phosphate. Free fatty acid was determined by the method of Duncombe (1963), cholesterol and cholesterol esters by the method of Kingsley & Schaffert (1949) and protein by the modified Folin method (Lowry, Rosebrough, Farr & Randall, 1951).

Determination of the effect of cations

Horse serum was dialysed against running water at 4° C. for 3 days. This resulted in the precipitation of euglobulins which were removed by centrifugation. The remaining pseudoglobulin fraction contained the substrate for the serum opacity reaction. Solutions of metal chlorides (analytical reagent grade) were then added

to the dialysed serum. The best mixture contained 2 ml. of dialysed horse serum, 1 ml. of the appropriate salt in the appropriate concentration and 0·5 ml. of enzyme solution (or suspension in the case of the cell-bound enzyme) in distilled water.

Heat sensitivity determination

A number of tubes containing 0·5 ml. amounts of enzyme solution (or suspension) were placed in a water bath at the required temperature. Tubes were removed at 5 min. intervals and immediately chilled in an ice-water bath. The remaining enzyme activity was determined by incubating the heated enzyme samples with 3 ml. of horse serum at 37° C. for 16 hr. and comparing the opacity produced with that produced by an unheated sample.

RESULTS

Variation of enzyme activity with age

Five ml. volumes were removed periodically from a growing culture of AN 124, merthiolate was added, and the cells were sedimented by centrifugation. The pellets were washed in distilled water, then suspended in distilled water to give an

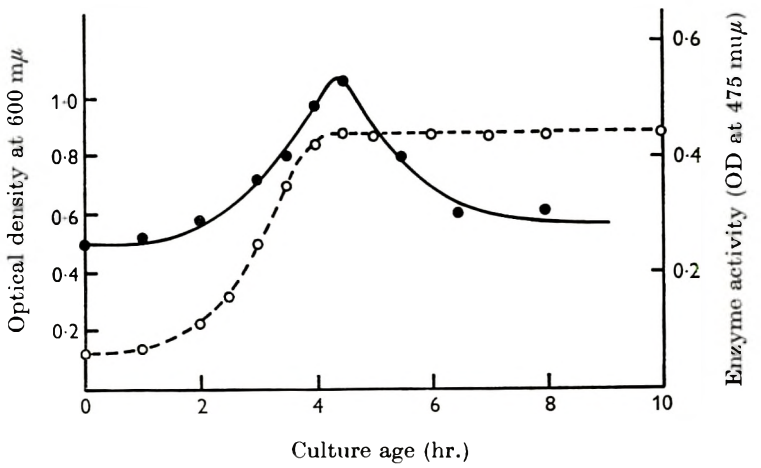

Fig. 1. Variation in the activity of the serum opacity factor of strain 3497 with age. ●—● enzyme activity; ○----○ growth curve (using the optical density at 600 mμ as a measure of the growth).

OD 600 = 1·00, and 0·5 ml. of this suspension was incubated with 3 ml. of horse serum at 37° C. for 16 hr. It was then centrifuged to remove the cellular material, and the OD of the supernatant fluid read at 475 mμ. The growth cycle was followed by measurement of the optical density of samples from the growing culture at 600 mμ.

Cells are able to produce an opacity in serum at all stages of the growth cycle (Fig. 1). The activity of the enzyme increased during the early exponential phase of growth, reaching a maximum towards the end of that phase, and decreased to a

variable extent during the stationary growth phase. This behaviour has been exhibited by all four strains tested (AN 124, AN 369, 3497, and the CDC type 22 strain).

Cellular location of the enzyme

Ward & Rudd (1938) found the enzyme in the Seitz filtrate of serum-broth cultures. Krumwiede (1954) and Rowen (1961) stated that in serum-free media the enzyme was cell bound with no extracellular activity. In initial studies with AN 124 we could find no extracellular enzyme activity, but subsequent studies have indicated that with certain strains some activity can be found in the supernatant, even when serum-free broth is used (L. W. Wannamaker, S. Skjold & F. H. Top, unpublished observations).

Table 1. *Activity of the serum opacity enzyme in cell wall and cell membrane fractions of AN* 124

	Enzyme activity (OD 475/hr./mg. protein)	
Sample	Cell-wall fraction	Cell-membrane fraction
1	0·06	0·19
2	0·02	0·14

When cells were fractionated into cytoplasmic membrane and cell wall components by mechanical breakage and differential centrifugation, no enzyme activity was detected in the cytoplasmic material but the membrane and cell-wall fraction both contained the enzyme. The enzyme activity was greatest in the membrane fraction (Table 1). Enzyme was determined in terms of increase in OD 475 per hr. per mg. protein. Protein content was estimated after extraction with 0·3 N-NaOH at 37° C. for 16 hr.

Extraction of the enzyme in a cell-free state

Krumwiede (1954), Rowen (1961) and Rowen & Martin (1963) obtained the enzyme in a soluble form by extraction of whole cells with urea. In their hands this gives a product of high activity and considerable stability. We have had much less success with this method and have, therefore, investigated other methods of extraction. Krumwiede also reported that the enzyme could be extracted with serum from whole cells; we have confirmed this.

Dissolution of the cell wall from wall-membrane fractions with phage-associated lysin (Maxted, 1957) releases some enzyme into solution, but the enzyme released is often of rather low activity and is unstable, 50% of its activity being lost at −10° C. in 7 days. The addition of albumin or β-mercapto-ethanol failed to stabilize the enzyme. In addition to being unstable, the enzyme was also very impure, since it contained all of the protein material of phage-lysin itself and that released by phage lysin. Attempts at purification using ammonium sulphate precipitation were unsuccessful, since the enzyme activity was always lost. This method of extraction was highly unsatisfactory.

Extraction with 1 % sodium deoxycholate yielded a solution of enzyme of very high activity and also of great stability. Cells from 1 litre of overnight growth in Todd-Hewitt broth were Mickle disintegrated (Michel & Gooder, 1962) and the cell wall-membrane material sedimented by centrifugation at 10,000*g.* for 30 min. Using this extraction method, an easily detectable opalescence (increase in OD 475 greater than 0·10 in 16 hr.) could be obtained using a 1/4000 dilution of the extract. By passing the extract through a column of G-50 Sephadex the enzyme could be freed from deoxycholate but was then less stable. Extracts freed from deoxycholate lost 50 % of their activity in 20 days at 4° C.; in the presence of deoxycholate no activity was lost in 3 months at 4° C.

None of the methods completely extracts the enzyme; some residual activity can always be found in the extracted cells. However, we find that the deoxycholate method of extraction is both easy to use and also very effective. All of our soluble enzyme preparations are made routinely by this method.

Enzyme substrate

Rowen & Martin (1963) have shown that, in fresh serum, extracts containing the opacity factor facilitate the transfer of fatty acids from the lecithin component of α-lipoprotein to cholesterol. A serum enzyme is responsible for the actual esterification, but the streptococcal factor makes the lecithin more available for reaction. They also showed that in aged serum there is no enhanced cholesterol esterification although there is still a serum opacity reaction. We have confirmed that in our system there is no enhanced cholesterol esterification. We have also found that pre-heating serum at 56° C. for 30 min. (which would destroy any remaining esterifying enzyme in the serum) does not reduce the amount of opalescence formed. Further, the addition of iodoacetate does not inhibit the reaction even at a concentration of 10^{-2} M. These three facts indicate that there must be some as yet unelucidated explanation for the opacity formation in our system.

In agreement with the results of Krumwiede and Rowen we found that, during the course of the reaction, the mobility of the α-lipoprotein, as determined by paper electrophoresis, is reduced. α-Lipoprotein was isolated by the flotation method and served as a substrate (or as an indicator) for the reaction in the presence of 1 % crystalline bovine serum albumin (Armour). During the course of the reaction there was a small increase in the amount of glycerol or glycerol-1-phosphate but no concomitant release of free fatty acid. We could obtain no opalescence in the absence of added albumin (in agreement with the results of Krumwiede, even though our method of lipoprotein isolation was much gentler).

It is of interest that extraction of whole serum with an equal volume of chloroform does not markedly reduce the ability to yield an opalescence when incubated with the enzyme. Obviously free chloroform-soluble lipids are not involved in the reaction.

An attempt was made to find a non-serum substrate for this streptococcal enzyme. The enzyme was unable to reduce the optical density of a suspension of streptococcal protoplast membranes (either from the homologous strain or heterologous strains). Strains which gave the serum opacity reaction were egg-yolk

negative when tested according to the method of Gillespie & Alder (1952). Further, the enzyme was unable to lyse sheep red blood cells.

Heat sensitivity of the enzyme

The sensitivity of cell-bound enzyme to heat at 60, 80 and 100° C. revealed considerable strain variation in this respect. The rate of loss of activity of a typical heat-sensitive strain (AN 369) is shown in Fig. 2. It is seen that 50 % of the activity

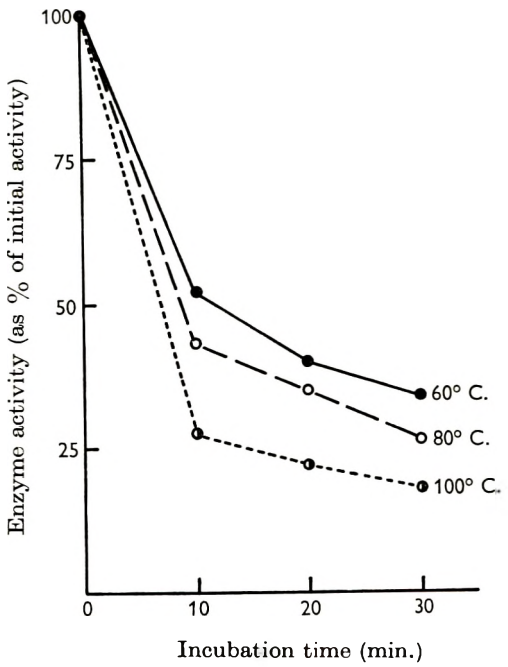

Fig. 2. The effect of heat at various temperatures on the activity of the serum opacity factor of strain AN 369.

is lost after only 10 min. of heating at 60° C., whilst 75 % is lost in 10 min. at 100° C. Approximately half of the strains tested behave in this way. The cell-bound enzyme in many strains, however, was resistant to heat at 100° C. for 60 min. and showed no loss of activity after such treatment. The heat denaturation curve for such a strain, 3497, is shown in Fig. 3. Extraction of the enzyme with deoxycholate reduces the heat resistance and removal of the deoxycholate renders the enzyme still more heat sensitive (Fig. 3). Even the deoxycholate-free enzyme of strain 3497 has a half-life of 40 min. at 100° C. The variation in heat sensitivity of the enzyme at various stages in the growth cycle was examined for strain 3497 (a heat-resistant strain) and strain AN 369 (a heat-sensitive strain). No variation in sensitivity was observed for either strain.

The pH of the suspension fluid used for incubating the enzyme is less critical than might be expected. For strain 3497 there was no reduction in activity after heating cell-bound enzyme at 100° C. for 60 min. at pH values between 4·0 and 8·6. Outside this range of pH the enzyme was much more heat sensitive (Table 2).

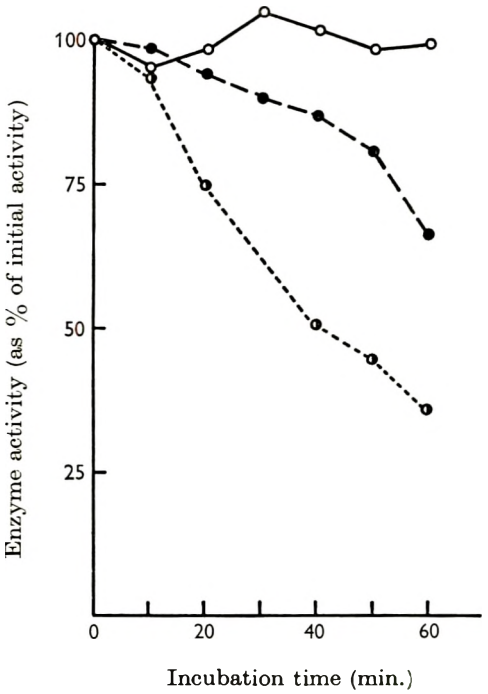

Fig. 3. The effect of heat at 100° C. on the activity of various samples of enzyme of strain 3497. ○—○ cell-bound enzyme; ●- - -● deoxycholate extract of enzyme; ◑·····◑ enzyme extract after removal of the deoxycholate.

Table 2. *Variation in heat sensitivity with the pH of the suspension fluid for enzyme from strain* 3497

pH of the suspension fluid	Time at 100° C. for 50 % reduction of enzyme activity (min.)
3·0	20
3·9	60
5·8	60
6·9	60
8·6	60
9·3	7

Other characteristics of the enzyme

The pH optimum of cell-bound and cell-free enzyme was determined using as substrate whole serum and α-lipoprotein. The pH optimum using the crudest enzyme substrate system (whole cells with whole serum) was 5·8 (Fig. 4); this was also the optimum pH for the purest system—cell-free enzyme with α-lipoprotein.

The enzyme was sensitive to the proteolytic enzymes trypsin and pepsin (both enzymes were crystalline products from Armour and Co. and were used at a concentration of 0·005 % for 2 hr. at 37° C.), but was resistant to streptococcal

proteinase. A crystalline product (which was kindly given to us by Dr S. D. Elliott) was used at a concentration of 0·01 %; the reaction was performed at 37° C. for 2 hr. at pH 7 in the presence of 0·05 M sodium thioglycollate. This latter observation is consistent with the fact that some of the best producers of α-lipoproteinase are also good producers of proteinase. The enzyme was destroyed

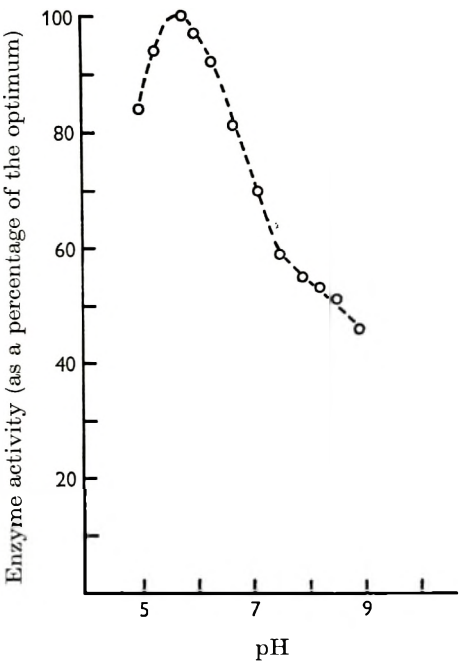

Fig. 4. Variation in the activity of the serum opacity enzyme with pH.

Table 3. *Effect of cations at various concentrations on the activity of cell-bound enzyme of strain AN* 124

	OD 475 produced at various cation concentrations							
Cation	3×10^{-1} M	3×10^{-2} M	1×10^{-2} M	3×10^{-3} M	1×10^{-3} M	3×10^{-4} M	1×10^{-4} M	0
K^+	1·05	0·70	0·57	0·47	0·49	0·49	0·48	0·50
Na^+	1·02	0·71	0·59	0·50	0·49	0·51	0·50	0·50
Mg^{++}	0·31	0·39	0·52	0·74	0·72	0·47	0·45	0·50
Ca^{++}	0·19	0·31	0·57	0·96	0·79	0·47	0·45	0·50

by incubation with 0·1 % formaldehyde or with 0·05 M sodium periodate for 30 min. at room temperature. The cell-bound enzyme, after such treatment followed by extensive washing with saline, was unable to produce any opacity in serum in 16 hr at 37° C. The inactivation with periodate was not reversible by treatment with reducing agents such as sodium arsenite or sodium thioglycollate.

The effects of cations on the reaction are complex. High concentrations of the divalent cations Ca^{2+} and Mg^{2+} (0·1 M or above) inhibited the reaction of both cell-bound and cell-free enzyme whilst lower concentrations had an activating effect

(Table 3). High concentrations of the univalent cations K^+, Na^+, Li^+, and NH_4^+ (10^{-2} M or above) activated cell-bound enzyme but were without effect on cell-free enzyme. The effects of the univalent cations were additive, as were the effects of the divalent cations. The action of univalent and divalent cations on cell-bound enzyme were not independent, since 0·3 M-NaCl failed to enhance the serum opacity reaction in the presence of divalent cations at a concentration of as little as 10^{-3} M. Further, the activating effect of 10^{-2} M-Ca^{2+} or Mg^{2+} was abolished by the presence of univalent cations. Univalent cations did not interfere, however, with the repression of the opacity reaction by higher concentrations of divalent cations.

DISCUSSION

The work reported here was done with aged serum so the results differ somewhat from those obtained by Rowen using fresh serum. For example, preheating the serum or adding iodoacetate, both of which were reported by Rowen to inhibit the reaction in fresh serum by repressing or destroying the cholesterol esterifying enzyme in the serum, had no effect on the serum opacity reaction in the system used here. This was presumably because the serum enzyme was no longer present, having been lost during the aging process. This conclusion is supported by the fact that we could detect no enhancement of cholesterol esterification during the serum opacity reaction.

These studies suggest that the cell-bound enzyme responsible for the serum opacity reaction may be primarily associated with the protoplast membrane. The proportionately smaller amount of enzyme activity found in the cell wall may be due to contamination of the walls with membrane material or to the leakage of the enzyme from the membrane through the cell wall. The latter explanation is not incompatible with the failure to detect extracellular enzyme in some strains, since cell-free enzyme can be very unstable. Enzyme leakage with subsequent stabilization provides a possible explanation for the apparent extraction of the enzyme with serum, since it is hard to envisage a membrane-bound enzyme being extracted so readily from whole cells. Another cell-bound activity of group A streptococci, the cell-bound streptolysin (Ginsburg, Harris & Grossowicz, 1963) is also extractable with serum. This appears to be able to diffuse into the medium and bind to lipoprotein, albumin and other constituents.

The difference in effect of univalent cations on cell-bound and cell-free enzyme is of interest. Since they have no effect on cell-free enzyme, the activation must be indirect. Presumably they assist in exposing the enzyme to the substrate via some action on the cell membrane. Since the action of univalent cations is inhibited by the presence of divalent cations, it could be postulated that this is competition for sites at the membrane, with the divalent cations being more tightly bound. The effect of divalent cations does not depend on the enzyme being cell bound; consequently it can be postulated that they are acting directly on the enzyme-substrate system and that this is a true case of cation inhibition.

All preparations of soluble enzyme leave much to be desired with respect to purity. Preliminary investigations using sucrose gradients, DEAE-cellulose columns

and ammonium sulphate fractionation proved fruitless. However, the enzyme appears to be precipitable with alcohol and this, together with its resistance to streptococcal proteinase, may provide a means of obtaining more satisfactory preparations. The best method of obtaining a stable enzyme preparation with high activity is to extract the cell wall membrane fraction with 1 % sodium deoxycholate at 0° C. for 16 hr. Deoxycholate has detergent properties and will dissolve protoplast membranes; it has also been shown to be capable of fractionating bacterial cell walls (Hill, 1967) and may prove to be a valuable tool in the extraction of enzymes or antigens from cells.

SUMMARY

The capacity of certain strains of *Streptococcus pyogenes* to produce opacity in aged horse serum has been studied. Cells from all stages of the growth cycle are able to produce opacity. Maximal activity is reached towards the end of the exponential phase of growth.

Examination of cell fractions obtained by mechanical breakage and differential centrifugation suggested that the cell-bound activity is predominantly associated with the membrane fraction. Extraction with sodium deoxycholate yields a soluble fraction of high activity.

There is considerable strain variation in heat stability of the serum opacity factor. Cell-bound activity is often quite resistant to heat, whereas extracted activity is less stable.

Low concentrations of divalent cations have an activating effect, whereas high concentrations inhibit the serum opacity reaction. High concentrations of univalent cations are without effect on the cell-free enzyme but have an activating effect on the cell-bound enzyme.

For both the cell-bound and the cell-free enzyme the pH optimum was 5·8.

Although sensitive to trypsin and pepsin, the serum opacity factor appears to be resistant to streptococcal proteinase. Its activity is destroyed by formaldehyde and by periodate but is unaffected by a number of reducing agents.

Pre-heating of the serum or the addition of iodoacetate did not affect the serum opacity reaction. The enhanced cholesterol esterification previously described with fresh serum appears to be a secondary reaction. Even when isolated by relatively gentle methods, α-lipoprotein serves as a substrate only in the presence of crystalline serum albumin.

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The serum opacity reaction of *Streptococcus pyogenes:* frequency of production of streptococcal lipoproteinase by strains of different serological types and the relationship to M protein production*

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The serum opacity reaction of *Streptococcus pyogenes* (Ward & Rudd, 1938) has been found to be associated with a lipoproteinase which acts upon the α_1 lipoprotein of the serum of various species to produce opalescence (Krumwiede, 1954; Rowen & Martin, 1963). Some observations on the general properties of the streptococcal factor and on the nature of the reaction in aged serum are recorded in an accompanying paper (Hill & Wannamaker, 1968). Although the nature of the reaction is not fully defined and other factors may possibly produce opalescence in serum, the terms serum opacity reaction (SOR) and lipoproteinase will be used interchangeably in this communication.

Data presented by Ward & Rudd (1938), Gooder (1961), and Köhler (1963) suggested to us that production of the serum opacity reaction was rather closely associated with serotype as determined by M and T antigens. Furthermore, both Gooder and Köhler concluded that strains which were difficult to type by the M-precipitin method generally produced a SOR but that M-typable strains rarely produced this reaction. If an inverse relationship between M-antigen and SOR could be substantiated, the serum opacity reaction might be useful as a preliminary test to characterize group A strains as M-positive or M-negative. Further investigation of the production of this enzyme in individual strains in relationship both to serotype and to production of an M-antigen and an investigation of the consistency of production of lipoproteinase by individual strains were therefore undertaken.

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METHODS

Source of strains

The strains examined were obtained from a wide variety of sources. The majority were obtained from an investigation of pyoderma at the Red Lake and Cass Lake Indian Reservations in Minnesota (Anthony, Perlman & Wannamaker, 1967), from a study of pharyngitis conducted at the St Paul-Ramsey Hospital (Top, Kaplan & Wannamaker, unpublished observations), and from the diagnostic bacteriology laboratory of the University of Minnesota Hospitals. Strains from our laboratory stock collection were examined, as were the prototype strains obtained from the Communicable Disease Center in Atlanta, Georgia. Other strains were obtained through the courtesy of Dr Rebecca Lancefield of the Rockefeller University, Mr W. R. Maxted of the Central Public Health Laboratory at Colindale, England, and Dr Hugh Dillon of the University of Alabama, Birmingham, Alabama.

All strains were examined concurrently for the serum opacity reaction, group specific carbohydrate, M-antigen, T-antigens, and, when indicated, the 28R-antigen.

Determination of streptococcal serotypes

Grouping antisera were obtained from the Communicable Disease Center in Atlanta, Georgia, as were M-typing antisera of types 1–6, 8, 11–15, 17–19, 22–26, 28–33, 36–44, 46, and 47. M-typing antisera for types 27, 34, 48, 49, and 51 were generously supplied by Dr Rebecca Lancefield who also supplied 28R antiserum. Type 9 antiserum was obtained from the Central Public Health Laboratory, Colindale, England. T-antisera were most generously supplied through the courtesy of Dr M. T. Parker and Mr W. R. Maxted of the Central Public Health Laboratory. The presence of M and 28R antigens on group A strains was determined by the capillary precipitin technique of Swift, Wilson & Lancefield (1943). Group A strains were examined for T-agglutination pattern by the method described by Williams (1958).

Screening of streptococcal strains for the serum opacity reaction

Production of the serum opacity reaction was determined as follows: strains were grown in 5 ml. of Todd–Hewitt broth* overnight at 37° C. and one drop of 1 % merthiolate solution was added. After collection by centrifugation, the cells were thoroughly resuspended in 2 ml. of horse serum† plus one drop of 1 % merthiolate solution and the suspension was incubated overnight at 37° C. After centrifugation, the presence of opalescence in the supernatant serum was determined visually by comparison with the opalescence produced by two control strains—one producing lipoproteinase, the other not producing lipoproteinase. Measurement of the opalescence produced was not attempted; the results were scored only as positive or negative.

* Obtained from Difco Laboratories, Detroit, Michigan.

† Obtained from Grand Island Biological Company, Grand Island, New York.

RESULTS

Production of lipoproteinase by strains of different serological types

Table 1 shows the production of the serum opacity reaction by streptococci of various groups. Only strains of group A streptococci produced a SOR, a finding previously noted by Ward & Rudd (1938) and by Köhler (1963). Streptococci of groups B, C, D, G and F did not produce a SOR.

The production of lipoproteinase by types of group A streptococci as determined by M-antigen is seen in Table 2. A distinct association between M-type and SOR was found; all strains examined of a given type invariably produced lipoproteinase or invariably failed to produce it. Types consistently producing lipoproteinase were types 2, 4, 8, 9, 11, 13, 22, 25, 27, 28, 35, 44, 48 and 49. Strains of types 1, 3, 5, 6, 12, 14, 15, 17, 18, 19, 23, 24, 26, 29, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 46, 47, 50, 51, and provisional types Schoenborn, Hanson and Kingbird (Top, Wannamaker, Maxted & Anthony, 1967) were never found to produce a SOR.

Table 1. *Serum opacity reaction (SOR) of streptococcal groups*

Group	Serum opacity negative (SOR−) (no. of strains)	Serum opacity positive (SOR+) (no. of strains)
A	733	602
B	14	0
C	20	0
D	3	0
G	36	0
F	5	0

Our results of lipoproteinase production by M-typable strains are in general agreement with the results reported by Gooder (1961). Among types which we invariably found to produce a SOR, Gooder encountered a few strains which were apparently SOR negative. Whereas we found that all types 2, 11, 27, and 35 strains examined produced lipoproteinase, Gooder found no SOR in 1 of 30 type 2 strains tested, 2 of 13 type 11 strains tested, 7 of 9 type 27 strains tested, and 2 of 3 type 35 strains tested. These differences may be due to differences in strain examined. In addition, both type 27 and 35 strains in our experience often produce weak serum opacity reactions, and it seems possible that the growth of certain weak SOR-producing strains such as these by Gooder may have been insufficient to demonstrate the reaction with these strains. Among strains of types which we have found never to produce lipoproteinase, Gooder encountered a few strains which were SOR positive. Aside from types 5 and 12 strains, the exceptions were limited to but one strain of each type tested. The unique production of lipoproteinase by types 5 and 12 strains will be examined more fully subsequently. With these infrequent exceptions, the earlier data of Gooder support the clear indication from our findings that there is a close relationship between lipoproteinase production and type among M-typable strains.

The results of lipoproteinase production by group A strains not typable by the precipitin method are shown in Table 3. Among these non-M-typable strains, a close association between SOR and serotype as determined by T-agglutination was also apparent. Non-M-typable strains of T-patterns 2, 4, 5/27/44, 9, 11, 12 and 28, produced a SOR with but two exceptions. All non-M-typable strains of T-patterns 1, 6, 15/17/19/23/47 and 18 failed to produce the reaction. Variable

Table 2. *The serum opacity reaction (SOR) of strains typable by the precipitin method*

M type	SOR− (no. of strains)	SOR+ (no. of strains)	M type	SOR− (no. of strains)	SOR+ (no. of strains)
1	31	0	31	38	0
2	0	24	32	2	0
3	31	0	33	2	0
4	0	33	34	1	0
5	12	0	35*	0	2
6	46	0	36	2	0
8	0	4	37	2	0
9	0	1	38	1	0
11	0	5	39	2	0
12	59	0	40	2	0
13	0	2	41	142	0
14	6	0	42	2	0
15	3	0	43	2	0
17	3	0	44	0	2
18	10	0	46	2	0
19	7	0	47	2	0
22	0	3	48	0	55
23	5	0	49†	0	15
24	6	0	50‡	3	0
25	0	2	51	5	0
26	2	0	Type Schoenborn§	65	0
27	0	5	Type Hanson§	29	0
28	0	4	Type Kingbird§	26	0
29	2	0			
30	2	0	Total	556	157

* Typing antiserum not available in our laboratory; strains originally typed by Dr Rebecca Lancefield. Type 35 is now believed to be identical with type 49 (Subcommittee on Streptococci and Pneumococci, in press).

† Typed as type 49 strains by Dr Rebecca Lancefield.

‡ Typing antiserum not available; strains examined originally typed as type 50 in other laboratories.

§ Provisional types 52, 53, 54 respectively (Top *et al.* 1967).

production of lipoproteinase was, however, evident among strains in the T-agglutination patterns 3/13/B3264, 8/25/imp.19, and 14. Since these latter T-patterns include two or more established M-types within each pattern, it seems reasonable to consider that the variation in lipoproteinase production may be due to the inclusion within them of currently undefined but distinct strains, only some of which produce lipoproteinase.

The majority of established types of group A streptococci were thus found not to

produce lipoproteinase. The types which failed to produce a SOR are types for which the production of M-antisera has been achieved without undue difficulty. However, the M-types found to produce a SOR—types 2, 4, 8, 9, 11, 13, 22, 25, 27, 28, 35, 44, 48 and 49—are types for which the production of M-antisera has generally been difficult (Williams & Maxted, 1953). The majority of strains which could not be typed by the M-precipitin method were also found to produce lipoproteinase.

Table 3. *The serum opacity reactions (SOR) of non-M-typable strains*

T-agglutination pattern	SOR− (no. of strains)	SOR+ (no. of strains)
1	7	0
2	0	5
3/13/B 3264	16	43
4	0	16
5/27/44	0	85
6	3	0
8/25/imp. 19	112	52
9	0	5
11	0	117
12	0	21
14	2	16
15/17/19/23/47	4	0
18	1	0
22	0	3
28	2	80
NT	30	2
Total	177	445

Table 4. *Serum opacity reaction of matt and glossy variants of strains*

	Matt variant			Glossy variant		
Strain	M	T	SOR	M	T	SOR
S 43	6	6	−	NT*	6	−
T 12/126/3	12	12	−	NT	12	+
Colindale 1130	12	12	−	NT	12	+
6184	15	15/17/19/23/47	−	NT	15/17/19/23/47	−
6186	14	14	−	NT	14	−
6188	44	5/27/44	+	NT	5/27/44	+

* NT = Not typable.

Lipoproteinase production in relationship to M-antigen production

Gooder (1961) reported that glossy strains of types 5 and 12 produced a SOR, whereas matt colonies of those strains failed to produce the reaction. Köhler (1963) reported a type 26 strain originally SOR negative which on subculture failed to produce M-antigen but produced lipoproteinase. These observations of an inverse relationship between M-antigen and lipoproteinase production suggest that many strains which fail to produce this enzyme might become lipoproteinase producers when they revert to the glossy state. In order to investigate this possibility, M-

positive and M-negative variants of individual strains were examined for M-antigen, T-antigens, and SOR. The results are shown in Table 4. Variants of a type 6, a type 14 and a type 15 strain were found not to produce a SOR in either the matt or glossy phase. The type 44 strain gave a SOR in both the matt and glossy phase. The two type 12 strains examined produced a weak SOR in the glossy phase but did not give this reaction in the matt phase, and so did exhibit the inverse relationship described by Gooder. We were unable to produce a glossy variant of a type 5 strain to confirm Gooder's observations of SOR variation in type 5 strains. Since all type 5 strains that we examined were found to be SOR negative, while all non-M-typable strains of T-pattern 5/27/44 were SOR positive, an inverse relationship between SOR and M-antigen production seems possible. With the exception of these two types, we found no difference in the SOR between M-positive and M-negative variants of individual strains.

Table 5. *SOR of types whose T-antigens are as specific as their M antigens*

	M-typable		Non-M-typable	
T pattern	SOR− (no. of strains)	SOR+ (no. of strains)	SOR− (no. of strains)	SOR+ (no. of strains)
1	31	0	7	0
2	0	24	0	5
6	46	0	3	0
12	59	0	0	21
22	0	3	0	3

Further evidence bearing on the suggested inverse relationship between M-antigen and SOR production was sought from examination of M-typable and non-M-typable strains with specific T-antigens. In some strains—types 1, 2, 6, 12 and 22, the T-antigen appears to be as strain specific as the M-antigen in that only a single M-antigen has been identified among strains with the particular T-antigen. Within these T-patterns, strains not typable by homologous M-antiserum can be considered with some confidence to be M-negative variants of that type. The results of serum opacity determinations of such strains is shown in Table 5. Both typable and non-typable strains with T-antigens 1 or 6 failed to give a SOR, while both typable and nontypable strains with T-antigens 2 or 22 did produce a SOR. Variation in lipoproteinase production was evident only among strains with T-antigen 12. Our data would then suggest that an inverse relationship between SOR and an M-antigen is uncommon and has been documented only in types 5 and 12 strains; the production of an SOR by other types appears to be unrelated to their production of an M-antigen.

The majority of SOR positive strains examined in our laboratory (445 of 602 or 74 %) were not typable by the precipitin method. Lack of M-typability is of course not necessarily equivalent to lack of M-antigen or lack of virulence. The inability to type a strain may be due to other factors, such as: (1) loss of M-antigen production on serial transfer in standard media, (2) destruction of M-antigen by streptococcal proteinase, (3) poor antigenicity of certain M-antigens, and (4) production

of an M-antigen of an as yet undefined type. Many of the non-M-typable strains which produced a SOR were indeed clinically virulent strains. A total of 20 strains of group A streptococci isolated in pure culture from the blood of patients with septicaemia were examined in our laboratory shortly after their isolation. Sixteen of these strains gave a SOR and despite their virulence only two of them were M-typable, a type 2 and a type 48 strain. The SOR positive strains not typable by the precipitin method included 7 strains of T-pattern 28, 5 strains of T-pattern 3/13/B 3264, and one strain each of T-patterns 8/25/imp. 19 and 22. Virulence of other currently non-M-typable strains producing a SOR is suggested in that representative SOR positive, non-M-typable strains of 5 different T-patterns (4, 11, 28, 3/13/B 3264, and 5/27/44) grew well in rotated human blood and hence presumably contain an M-antigen (Lancefield, 1957; Maxted, 1956). Our data would then suggest that although lipoproteinase-producing strains are difficult to type by the precipitin method, many of these strains are clinically virulent and possibly produce M-antigens which presently are difficult to characterize.

Table 6. *Serum opacity reaction of strains of identical serotype isolated from individual patients at 3-week intervals*

	Initial isolate SOR negative		Initial isolate SOR positive	
Interval	Subsequent isolate SOR − (no. of strains)	Subsequent isolate SOR + (no. of strains)	Subsequent isolate SOR − (no. of strains)	Subsequent isolate SOR + (no. of strains)
3 weeks	67	0	0	24
6 weeks	27	0	0	21
9 weeks	18	0	0	3
12 weeks	10	0	—	—
15 weeks	5	0	—	—
18 weeks	6	0	—	—
21 weeks	1	0	—	—

Consistency of lipoproteinase production by individual strains

With the exception of the two type 12 strains previously discussed, we have not encountered a SOR positive strain which on subsequent examination failed to produce this reaction. Likewise we have not encountered a strain giving a negative SOR which later was found to give a positive reaction. Individual strains maintained a consistent SOR under laboratory conditions.

A similar consistency of SOR production was evident in strains isolated directly from patients. Two or more isolates of the same serotype as determined by precipitin test or T-agglutination which were obtained from an individual patient at one examination were tested for SOR. In all 98 patients from whom one isolate was SOR negative, the second isolate of the same serotype was also SOR negative. From all 58 patients from whom an SOR positive strain was isolated, the second isolate of the same serotype was likewise SOR positive.

In order to determine whether lipoproteinase production by a strain might vary

after prolonged colonization of a patient, strains of identical serotype isolated from patients with pyoderma or pharyngitis at intervals of three weeks were examined for SOR; the results are seen in Table 6. When the original strain isolated was SOR negative, all subsequent isolates of the same serotype gave a negative SOR, even if recovered as long as four months after the initial isolation. When the original strain produced a positive SOR, all subsequent isolates of the same serotype also gave a positive SOR. Our experience thus indicates that the production of lipoproteinase by individual strains is consistent both in strains maintained in the laboratory and in strains chronically carried by patients.

DISCUSSION

Data obtained from the examination of a large number of stock and clinical strains from a variety of sources confirm the relationship between M-typability and lipoproteinase production previously reported by Gooder (1961) and by Köhler (1963). Types of group A streptococci for which the production of M-typing antisera has been accomplished without undue difficulty have been found not to produce a SOR. On the other hand, types for which the production of M-typing antisera has been difficult, as well as the majority of non-M-typable strains, have been found to produce lipoproteinase. The reasons for the relationship between poor M-typability and SOR production in group A streptococci remains unclear. Gooder found that extracts containing lipoproteinase did not destroy the antigenicity of M-protein on streptococcal cells or in Lancefield extracts. Further investigations of the mechanisms underlying the association of these two streptococcal proteins seem indicated.

A consistent association between serotype and SOR was found in the group A strains examined. Among strains typable by the more specific M-precipitin method, this association was absolute in that all strains examined of a given type either uniformly produced a SOR or uniformly failed to produce this reaction. When strain classification could only be accomplished by the T-agglutination method, a consistent association between SOR and serotype was also apparent. Only in the more complex T-agglutination patterns such as 3/13/B 3264, 14, and 8/25/imp.19 was variation in SOR production seen among members of a serotype. Such variation may be due to the inclusion within these few serotypes of distinct, but currently unclassifiable strains, only some of which produce this enzyme.

The association between lipoproteinase production and serotype of group A streptococci is indeed so close as to suggest a relationship between them. One possibility would be that lipoproteinase and one of the proteins determining serotype—the M or T antigen—may be identical or closely related proteins. Subsequent studies have indicated that, like the M and T antigens, the lipoproteinases from different types of group A streptococci are antigenically distinct, but no direct relationship between the lipoproteinase antigens and other well recognized cellular antigens, such as the M, T, and R antigens, has been demonstrated (Top & Wannamaker, unpublished observations). The full significance of the close association between serotype and lipoproteinase production must await further investigation of these relationships.

The serum opacity reaction does not appear to be generally useful as a screening test for the presence or absence of M-antigen in individual strains of group A streptococci; 157 of the 713 M-typable strains (22 %) tested produced a SOR and 177 of the 622 non-M-typable strains (28 %) failed to produce the reaction. In view of the close association of the SOR and serotype, however, this simple test might serve as a preliminary screening test to determine which M-typing antisera to use in typing individual strains in laboratories not equipped to classify strains by the T-agglutination method. Moreover, because of the often inverse relationship between M protein and lipoproteinase production, we have found it helpful, along with the ability to survive in rotated normal human blood, as a means of screening strains which are non-typable with current M antisera in order to select those which merit further investigation as possible new M types (Top *et al.* 1967).

SUMMARY

The serum opacity reaction (SOR) is produced by some streptococci of group A, but not by streptococci of groups B, C, D, F and G. The production of this reaction was found to be closely related to serotype as determined by M and T antigens. The SOR of an individual strain was found to be consistently stable over a period of time both in strains maintained in the laboratory and in strains isolated sequentially from individual patients following streptococcal infections. Strains for which the demonstration of an M-antigen by the precipitin method was difficult or impossible in general produced a SOR, while strains more easily typable generally failed to produce this reaction. Laboratory selected variants of type 12 strains showed an inverse relationship between M protein and lipoproteinase production, whereas M positive and M negative variants of other serotypes showed no variability with respect to lipoproteinase production.

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The optimal mode of transport for swabs obtained from surfaces examined for organisms causing food-borne disease

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INTRODUCTION

It has been well established that the prevention of food-borne disease outbreaks should preferably be based on constant supervision of food-producing establishments and that, in addition to direct examination of the foods themselves, verification of the sanitary condition of the surfaces in constant contact with food products is a very essential step (Mossel, Kampelmacher & v. Noorle Jansen, 1966). Inspectors in charge of such food-borne disease prevention programmes may not always have facilities to examine such surfaces completely on the spot. In such cases they will have to ship their swabs to a central laboratory. Methods of chilled transport are troublesome and relatively costly, while shipping in selective culture media allows the receiving laboratory to detect one group of organisms only. Hence other ways of transport have quite some interest in this area.

In this study, three such methods have been studied in a strictly quantitative way, viz. the shipping of various types of swabs (i) at various water activities (Scott, 1957); (ii) in various types of saline; (iii) in Stuart type (1959) transport media. Four types of organisms of general interest in public health bacteriology have been chosen, i.e. fermentative Gram negative rods of the Enterobacteriaceae group, such as *Salmonella* and *Escherichia coli*, *Staphylococcus aureus*, Lancefield group A streptococci and spores of *Bacillaceae*. In addition a few tests were carried out with *Pseudomonas aeruginosa*, and with *Vibrio parahaemolyticus*.

MATERIALS AND METHODS

Bacteria

The strains of *Salmonella typhimurium* and *E. coli* had been recently isolated from minced meat. The *Staph. aureus* strain had been recently isolated from a furuncle, the group A streptococcus from a sore throat, the strain of *Ps. aeruginosa* from a urinary tract infection. The strain of *V. parahaemolyticus* was obtained from Dr R. Sakazaki, National Institute of Health, Tokyo, Japan. The strain of *Bacillus cereus* stemmed from an outbreak of food poisoning caused by an Indonesian rice dish.

Freshly prepared 24 hr. cultures in brain heart infusion broth of all strains were used. In the case of studies on spores of *B. cereus*, these cultures were heated for one minute at 80° C. which, in agreement with Knaysi (1951), we found entirely effective in killing the vegetative cells (Mossel, 1967) without unduly reducing the numbers of spores.

Inoculation of swabs

Cotton as well as calcium alginate swabs of approximately 25 mg. weight were applied to the tips of wooden applicators of 12 cm. length. They were sterilized in a pressure cooker for 15 min. at 121° C. in glass cylinders with cork stoppers.

Three swabs of each type were used per strain and per given simulated transport condition. Hence, when the survival of a strain at a given humidity was studied after 0, 3, 7 and 24 hr. exposure twelve swabs were prepared. Inoculation was carried out by evenly distributing over the swabs 0·02 ml. quantities of a suitable dilution of the brain heart infusion broth cultures of the test organisms. This mode of inoculation corresponded with counts of the order 10^3–10^4 per swab, depending on the viable count of the suspension used and the degree of osmotic shock to which the organisms were exposed when being transferred from a medium of a_w of *ca.* 1·00 to the very dry swabs (Mossel & Koopman, 1965).

Exposure of swabs to various transport environments

Some sets of swabs were transferred to sterile culture tubes, containing 1 ml. sterile saturated solutions of potassium nitrate and sodium bromide respectively. The latter solutions could be expected to secure a_w values of 0·95 and 0·59 respectively (O'Brien, 1948; Stokes & Robinson, 1949). The actual a_w figures for every freshly prepared and sterilized, saturated solution were verified by the direct manometric method of Legault, Makower & Talburt (1948).

Other sets of swabs were preserved in 10 ml. quantities of saline, and saline + 0·1 % peptone, according to Straka & Stokes (1957) and in the same volume of Ringer solution, with 1·0 % of sodium hexametaphosphate ('Calgon') added. This is the solution usually applied for the dispersion of calcium alginate plugs (Higgins, 1950) and found non-toxic to various bacteria before (Mossel & Büchli, 1964). Tentatively 0·1 % of peptone was also added to this fluid.

Finally, sets of swabs were kept in 12 ml. Stuart's transport medium. Because in tentative experiments we had observed some growth, particularly of Enterobacteriaceae, it was also attempted to modify Stuart's medium so that it might be a maintenance medium only. Rather than trying to use antibiotics, as Stuart himself did, for our purpose a less selective mode of inhibition of bacterial growth was investigated. Reduction of the pH of the medium to values in the range 5·0–6·4 could be considered promising (Dernby, 1921). Hence, in addition to Stuart's original medium of pH = 7·4, the same medium adjusted to pH 5·0, 5·5, 6·0, 6·7 and 7·1 by adding sufficient quantities of sterile 10 % tartaric acid solution was also used.

Conditions existing during actual transport were imitated by exposing the swabs to a temperature of 19–22° C. for up to 24 hr.

Counts of organisms after various periods of simulated transport

After 3, 7 and 24 hr. of storage at *ca.* 21° C. the tubes containing swabs in saline or Ringer-calgon were shaken for 2 min. Thereupon 0·1 ml. quantities were plated in duplicate on the surface of blood agar, incubated for about 24 hr. at 37° C. and the numbers of colonies obtained counted.

The swabs stored over the various salt solutions and those kept in Stuart's medium and its modifications were transferred to tubes containing 10 ml. saline, or Ringer-calgon. After shaking for 2 min., surface plate counts on blood agar of the dispersions thus obtained were again made.

All results were calculated as numbers of viable organisms per one swab.

RESULTS

The results obtained have been brought together in Tables 1–3. Those of Table 3 have been summarized in Table 4.

In Table 1 the fate of various types of bacteria on cotton swabs at various water activities is presented. In Table 2 similar tests on alginate swabs are reported.

Only in the case of *Staph. aureus* at $a_w = 0{\cdot}95$, could differences between survival on cotton and alginate swabs be detected during 'conditioned' dry transport.

'Conditioned' dry transport both at $a_w = 0{\cdot}58$ and $a_w = 0{\cdot}95$ lead to considerable losses in viable cells of *Salmonella typhimurium*. All liquid transport media lead to growth of *S. typhimurium*, particularly those containing peptone, as could be anticipated. In the transport medium of Stuart, generally, growth of the salmonellas tested occurred.

As could be expected *Staph. aureus* showed a higher a_w resistance than *S. typhimurium*. Particularly on alginate at $a_w = 0{\cdot}58$ almost no decrease occurred under 'conditioned' transport; although there was some decrease at $a_w = 0{\cdot}95$, especially on cotton. In Stuart's transport medium neither growth nor decrease was noticed, but some growth occurred in the presence of peptone. These observations are in agreement with the higher nutrient requirements of *Staph. aureus*.

Str. pyogenes showed rapid losses during 'conditioned' transport, at any r.h. Decreases in numbers were observed during simulated transport in saline and to a somewhat lesser extent in Ringer-calgon and peptone. There was no growth and also virtually no decrease in Stuart's medium.

Spores of *B. cereus* showed only slight losses under 'conditioned' transport on cotton. No growth in Stuart's transport medium was observed. This behaviour is typical for spores, which confirms the correctness of the procedure used for preparing the spores.

Because some organisms showed a tendency to grow in Stuart's transport medium, modifications of this fluid of various pH values were tried. The results are presented in Tables 3 and 4.

With *S. typhimurium* once more, growth in Stuart's medium occurred at pH = 7·4–7·1. When the pH was reduced, virtually no decrease in numbers of viable cells was observed.

Table 1. *Survival of various bacteria during simulated transport on cotton swabs*

The figures are logarithms to base 10 of the counts per swab, after 0, 3, 7 and 24 hr. storage at about 21° C.

Organism	Time stored (hr.)	Stored at a_w 0·58 Shaken with Saline	Stored at a_w 0·58 Shaken with Ringer calgon	Stored at a_w 0·95 Shaken with Saline	Stored at a_w 0·95 Shaken with Ringer calgon	Stored in Saline	Stored in Saline + 0·1% peptone	Stored in Ringer calgon	Stored in Ringer calgon + 0·1% peptone	Stuart normal shaken with Saline	Stuart normal shaken with Ringer calgon
S. typhimurium	0	4·19	3·93	4·19	3·93	2·33	2·11	2·45	2·58	3·55	3·59
	3	< 2·00	< 2·00	3·83	3·98	2·40	2·40	2·18	2·75	3·26	3·50
	7	< 2·00	< 2·00	3·95	4·05	2·85	4·00	2·75	3·54	3·91	4·18
	24	< 2·00	< 2·00	2·23	2·45	5·30	8·00	2·75	8·00	5·00	5·70
Staph. aureus	0	4·19	4·48	4·18	4·48	2·48	2·70	2·78	2·48	4·78	4·48
	3	4·32	4·08	3·95	4·08	2·48	2·70	2·60	2·60	4·36	—
	7	3·36	3·23	3·60	4·60	2·70	2·70	2·70	2·90	4·30	—
	24	3·89	4·00	3·60	< 2·00	2·00	3·00	2·60	4·60	4·30	—
Str. pyogenes	0	4·30	4·48	4·30	4·30	3·34	2·95	3·36	3·32	4·30	4·48
	3	< 2·00	< 2·00	3·85	4·00	< 2·00	2·30	3·45	3·43	4·30	4·48
	7	< 2·00	< 2·00	2·90	3·58	< 2·00	2·00	3·36	3·04	4·48	4·48
	24	< 2·00	2·48	< 2·00	< 2·00	< 2·00	< 2·00	3·15	2·00	4·30	4·48
B. cereus spores	0	3·65	3·83	3·91	3·91	—	—	—	—	3·87	3·85
	3	3·53	3·48	3·57	3·52	—	—	—	—	3·81	3·74
	7	3·11	2·85	3·48	3·86	—	—	—	—	3·72	3·91
	24	3·40	3·30	3·64	3·28	—	—	—	—	3·88	3·98

Table 2. *Survival of various bacteria during simulated transport on alginate swabs*

The figures are logarithms to base 10 of the counts per swab, after 0, 3, 7 and 24 hr. storage at about 21° C.

Organism	Time stored (hr.)	Stored at a_w 0·58 Shaken with: Saline	a_w 0·58 Shaken with: Ringer calgon	a_w 0·95 Shaken with: Saline	a_w 0·95 Shaken with: Ringer calgon	Stored in: Saline	Stored in: Saline + peptone	Stored in: Ringer calgon	Stored in: Ringer calgon + peptone	Stuart normal shaken with: Saline	Stuart normal shaken with: Ringer calgon
S. typhimurium	0	4·30	4·25	4·30	4·25	2·85	2·83	2·88	2·68	4·23	4·18
	3	3·57	3·25	4·30	4·15	2·88	3·20	2·68	2·76	3·82	4·08
	7	< 2·00	< 2·00	4·21	< 2·00	2·92	4·15	3·00	3·81	4·16	4·48
	24	< 2·00	< 2·00	< 2·00	< 2·00	4·18	8·00	4·93	8·00	4·84	5·00
E. coli	0	—	—	—	—	—	—	4·90	—	—	—
	7	—	—	—	—	—	—	4·85	—	—	—
	24	—	—	—	—	—	—	8·00	—	—	—
Ps. aeruginosa	0	—	—	—	—	—	—	4·30	—	—	—
	7	—	—	—	—	—	—	4·78	—	—	—
	28	—	—	—	—	—	—	8·00	—	—	—
Staph. aureus	0	4·70	4·85	4·70	4·85	2·70	2·90	2·85	2·70	—	4·60
	3	4·48	4·60	4·70	4·70	2·90	2·84	2·85	2·78	—	4·30
	7	3·48	4·48	4·70	4·30	2·00	3·40	2·90	3·11	—	4·48
	24	4·60	4·48	4·00	4·00	< 2·00	4·60	2·70	4·90	—	4·48
Str. pyogenes	0	4·48	4·30	4·85	4·85	3·90	3·85	3·60	3·60	4·48	4·30
	3	3·48	2·85	3·60	3·70	2·85	3·70	3·48	3·30	4·00	3·70
	7	2·60	3·00	2·70	3·48	< 2·00	3·48	3·11	2·78	4·30	4·30
	24	< 2·00	< 2·00	2·30	< 2 ·00	< 2·00	3·00	2·95	2·30	4·00	4·00
B. cereus spores	0	3·85	3·85	3·78	3·85	—	—	—	—	3·78	3·70
	3	3·70	3·70	3·95	3·60	—	—	—	—	3·60	3·70
	7	3·85	3·78	3·78	3·85	—	—	—	—	3·70	3·78
	24	3·90	3·78	3·85	3·70	—	—	—	—	3·85	3·78

E. coli behaved similarly. Its profuse growth in Stuart's medium at the original pH confirms Stuart's own observations.

The behaviour of *Ps. aeruginosa* was generally somewhat erratic: taking into account the accuracy of surface counts of this organism it may be concluded that Stuart's medium used at a pH range 5·5–7·4 will not lead to considerable losses.

Results obtained with *Vibrio parahaemolyticus* are somewhat different in that the pH range in which no losses occur during transport is only > 5·9 to 7·4.

Table 3. *Survival of various bacteria during simulated transport in Stuart's medium at various pH values*

The figures are the logarithms to the base 10 of the counts per alginate swab after 0, 7 and 24 hr. storage at about 21° C.

Organism	Hours of storage	pH of Stuart's medium 7·4	7·1	6·7	6·2	5·9	5·5	5·0
S. typhimurium	0	4·00	4·00	4·30	4·00	4·30	4·30	4·00
	7	—	4·60	—	—	—	—	3·90
	24	5·30	4·90	3·90	4·00	4·00	4·00	3·90
E. coli	0	4·70	4·70	—	4·60	3·95	3·95	3·85
	7	4·60	4·30	—	4·30	—	—	3·85
	24	8·00	6·00	—	4·78	3·78	3·78	3·70
Ps. aeruginosa	0	5·00	4·00	5·00	4·30	4·30	4·30	—
	7	—	—	—	—	—	—	—
	24	4·60	5·90	4·48	3·90	3·95	3·70	—
V. para-haemolyticus	0	3·87	3·85	3·48	3·81	3·85	—	3·99
	7	3·53	3·59	3·51	3·54	2·00	—	< 2·00
	24	3·52	3·59	3·36	3·02	< 2·00	—	< 2·00
Staph. aureus	0	4·00	3·78	4·00	4·48	3·90	3·90	3·60
	7	—	3·85	—	—	—	—	2·30
	24	3·90	3·30	3·60	3·60	2·60	2·30	< 2·00
Str. pyogenes	0	3·30	2·95	3·30	3·00	3·30	3·00	3·30
	7	—	3·48	—	—	—	—	3·00
	24	3·30	5·30	2·78	2·30	< 2·00	< 2·00	2·70
B. cereus spores	0	3·48	3·70	3·48	3·00	4·00	4·30	3·90
	7	—	3·78	—	—	—	—	3·85
	24	3·30	3·78	3·00	3·00	4·00	4·00	3·70

Table 4. *Summary of the data of Table* 3. *Derived from Table* 3

The figures indicate the ratio of the count at 24 hr to the count at 0 hr.

Organism	pH of Stuart's medium 7·4	7·1	6·7	6·2	5·9	5·5	5·0
S. typhimurium	20	8	0·4	1	0·5	0·5	0·8
E. coli	2000	20	—	1·5	0·7	0·7	0·7
Ps. aeruginosa	0·4	80	0·3	0·4	0·45	0·25	—
V. parahaemolyticus	0·45	0·55	0·8	0·2	< 0·014	—	< 0·01
Staph. aureus	0·8	0·3	0·4	0·13	0·05	0·025	< 0·025
Str. pyogenes	1	200	0·3	0·2	< 0·05	< 0·1	0·25
B. cereus spores	0·7	1·2	0·3	1	1	0·5	0·6

Unfortunately *Staph. aureus* showed a quite different behaviour: while in Stuart's medium of pH = 7·4 a tendency to maintenance seemed to exist, the organism did not tolerate a lower pH value; at pH < 6·2 within 24 hr. at 21° C. decreases of an order far over 1 D occurred.

Str. pyogenes showed growth in Stuart's medium of pH = 7·1; a tendency to decrease at lower pH values was observed, particularly at pH ⩽ 5·9.

Finally, spores of *B. cereus* showed virtually neither increase nor decrease in Stuart's medium at any of the pH values tested, which confirms anew the correctness of the mode of spore preparation used.

Table 5. *Fate of various bacteria stored in Ringer calgon and Stuart's medium at* 11–13° C.

The figures are the logarithms to the base 10 of the counts per 1 ml. after 0, 7 and 24 hr. storage.

	Ringer calgon log. of count after (h)			Stuart medium pH = 7·0 log. of count after (h)		
Organism	0	7	24	0	7	24
S. typhimurium	4·13	4·08	4·15	4·19	4·08	3·98
E. coli	3·41	3·45	4·01	3·32	3·30	3·13
	4·95	4·90	4·81	4·60	4·13	4·63
Ps. aeruginosa	3·97	3·87	3·92	3·75	3·59	3·47

DISCUSSION

There was no complete agreement between the numbers of viable cells of a given organism in repeated simulated transport tests, except for the spores of *B. cereus*. This demonstrates that minor differences in the condition of the organisms to be transported from the sampling site to the laboratory may also determine the fraction of surviving cells.

The usual way of unprotected transport of swabs leads to losses in viable cells of some types of bacteria often exceeding two logarithmic cycles. This confirms the observations of Ellner & Ellner (1966). Dry transport has therefore to be abandoned and to be replaced by transport in a suitable aqueous medium.

The extent of growth observed in certain experiments when cells of Enterobacteriaceae were transported in Stuart's medium at pH = 7·1 for 24 hr. showed an average of one logarithmic cycle. The fall in counts of *Str. pyogenes* and *Staph. aureus* when transported in Stuart's medium at decreased pH, for the same period of time, was often more than tenfold. Hence Stuart's medium at pH = 7·1 is to be preferred for the transport of swabs. The increases in counts to be expected when dealing with Enterobacteriaceae—and hence slight shifts in the composition of the original micro flora—can always be limited by shortening the period of transport or by reducing the temperature. We have found that chilling of the medium, to ground water temperature, i.e. 11–13° C., and subsequent transport wrapped in plastic foam ('Tempex') will completely inhibit the growth of Enterobacteriaceae in Stuart's medium of pH *ca.* 7·0 for 10–12 hr. (Table 5).

As is illustrated in Tables 1 and 2 the various bacteria tested behave virtually

in the same way as in Stuart's medium when transported in Ringer-calgon without peptone added. Here again we established that 10–12 hr. transport at 11–13° C. will prevent the proliferation of the only group of bacteria actively growing in Ringer calgon, i.e. Enterobacteriaceae (Table 5). From a purely bacteriological point of view there seems, therefore, little to choose between Ringer calgon and Stuart's medium. However, where swabs are to be transported to the laboratory by lay personnel, Stuart's medium has the obvious advantage of being solid so that it cannot be lost by spilling.

SUMMARY

An investigation was carried out on the influence of the mode of transport at *ca.* 21° C. on the fate of *S. typhimurium*, *E. coli*, *Ps. aeruginosa*, *Staph. aureus*, *Str. pyogenes* and spores of *B. cereus* collected from surfaces by swabbing with cotton and alginate.

Transport at relative humidity 0·58–0·95 leads to losses in viable counts sometimes exceeding two log cycles. In Stuart's medium and Ringer-calgon particularly Enterobacteriaceae showed growth when transported at 21° C. Reduction of the pH value of Stuart's medium led to losses of far over one log cycle in some types of bacteria and could therefore not be recommended.

Slight chilling of the medium, e.g. to 12° C., and insulation during transport appeared to maintain counts of the organisms studied at their original level in both Stuart's medium and Ringer calgon for 10–12 hr.

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Comparison of MacConkey broth, Teepol broth and glutamic acid media for the enumeration of coliform organisms in water

By the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies*

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INTRODUCTION

A chemically defined medium based on glutamic acid was first advocated by Folpmers (1948) in Holland for the enumeration of the coliform group of bacteria in water. Burman & Oliver (1952) at the Metropolitan Water Board carried out trials using some modifications of media and techniques. A trial of these techniques was carried out by the Public Health Laboratory Service (1958), which reached similar conclusions. These were that glutamic acid medium containing glucose gave good agreement with MacConkey broth in 24 hr. but too many false positive results in 48 hr. With a lactose modification more *Escherichia coli* were isolated in 48 hr. but it was too inhibitory for other coliform organisms. MacConkey broth itself was shown to have various defects in performance.

The lactose medium was then further improved by Gray (1959), who increased the pH and added sodium formate to increase gas production. Further modifications were made at the Metropolitan Water Board (MWB) (Windle Taylor, 1959–60; 1961–62). The concentration of lactose was increased and the phosphate decreased, and the modified medium was adopted in place of MacConkey broth for all routine samples examined by the multiple tube method, from January 1962.

Simultaneously and independently Gray also made further modifications resulting in the publication of an improved formate lactose glutamate medium (Gray, 1964). This was a more nutrient medium containing additional amino acids, growth factors, mineral salts and ammonium chloride instead of the unstable ammonium lactate. Ammonium chloride had already been incorporated in the MWB medium on Gray's recommendation.

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The Water Research Association (WRA) also carried out a simultaneous and independent investigation of glutamate media resulting in the publication of yet another recommended modification (Collingwood, 1964). This had been compared only with the MWB medium because Gray's improved medium was not then available. The WRA medium included one additional amino acid, two growth factors but not those used by Gray, changes in the mineral composition, a reduction in lactose and an increase in glutamic acid. The composition of the three media, MWB, WRA and Gray's improved version are given in Table 1.

Table 1. *Modifications of glutamic acid media*

	Burman MWB	Gray PHLS	Collingwood WRA
Lactose	20 g.	10 g.	5 g.
L(+)glutamic acid	5 g.	5 g.	10 g.
L-cystine	—	0·02 g.	0·05 g.
L(−)aspartic acid	—	0·024 g.	—
L(+)arginine	—	0·02 g.	—
Nicotinamide	—	—	1 ml. of 0·1 % aq. soln.
Riboflavine	—	—	1 ml. of 0·002 % aq. soln.
Thiamin	—	1·0 mg.	—
Nicotinic acid	—	1·0 mg.	—
Pantothenic acid	—	1·0 mg.	—
$MgSO_4.7H_2O$	—	0·2 g.	1 ml. of 1 % aq. soln.
KCl	—	—	9 g.
K_2HPO_4	1 g.	1 g.	1 g.
HCOONa	0·25 g.	0·25 g.	—
$HCOONH_4$	—	—	2 ml. of 25 % aq. soln.
NH_4Cl	2·5 g.	2·0 g.	—
$CaCl_2$	—	0·2 g.	—
Ferric citrate scales	—	0·1 g.	—
pH	6·7	6·7	6·7
Brom-cresol purple 1 % ethanolic soln.	1 ml.	1 ml.	1 ml.
Distilled water	1000 ml.	1000 ml.	1000 ml.

The above formulae are for single strength media. Double strength media are normally used with 50 ml. and 10 ml. water samples.

While all these glutamate media were under investigation, there was a parallel development designed to eliminate some of the variability of MacConkey broth. Jameson & Emberley (1956) recommended the substitution of Teepol for the very variable bile salts in MacConkey broth. In order to obtain a standard product it is now necessary to specify Teepol 610 (BDH Ltd). This is a 34 % aqueous solution of the sodium salts of straight chain secondary alkyl sulphates containing 8 to 10 carbon atoms in the side chain. Other workers who have compared this medium with MacConkey broth have all reported favourably (Jebb, 1959; Windle Taylor, 1959–60).

The Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies was formed in 1964. Among its terms of reference was 'to examine the application of new techniques to routine purposes'.

One of their most urgent tasks was therefore to examine the new techniques outlined above.

With this in view, a trial in eleven laboratories was carried out comparing MacConkey broth with Teepol broth and three modifications of glutamic acid medium.

MATERIALS AND METHODS

Coliform organisms

Throughout this paper the term coliform organisms refers to all Gram negative rod-shaped bacteria, capable of production of acid and gas from 1 % lactose peptone water in 48 hr. at 37° C. The term therefore includes *Esch. coli*.

Media

It was acknowledged from the start that MacConkey broth, the existing standard medium recommended in Report No. 71 (Report 1956) for enumerating coliform organisms and *Esch. coli* in water, was itself variable in behaviour because of variability in properties of both bile salts and peptone. To avoid complications due to this factor, a single batch of Oxoid dehydrated MacConkey broth was distributed to all participating laboratories for use as a standard. In addition each laboratory made MacConkey broth as required from the normal material available to them. Teepol broth was made in each laboratory by the method recommended by Jameson.

The single strength Teepol medium was prepared from peptone, 20 g.; NaCl, 5 g.; Teepol 610 (BDH Ltd), 1 ml.; lactose, 10 g.; phenol red (0·4 % solution), 2·5 ml.; distilled water to 1000 ml. The pH was adjusted to give a final pH of 7·5 after autoclaving at 115° C. for 15 min. The peptone was the same as that used for the laboratory-prepared MacConkey broth and was not the same in all laboratories. This medium differs from Jameson & Emberley's (1956) original formula by the specification of Teepol 610 as already explained and by the use of phenol red instead of bromocresol purple. Teepol modifies the pH range over which most indicators change colour, but phenol red is an exception. Furthermore, phenol red has not been shown to have any toxic effects on any organisms at normal indicator concentrations.

The three glutamic acid media outlined in Table 1 were prepared in dehydrated form especially for the Committee by Oxoid Ltd, partly to ensure standardization, partly because some laboratories had not the facilities to prepare a variety of complex media and partly because it was necessary to determine whether dehydrated media could be prepared which would give satisfactory results.

In the preparation of Gray's modified medium (Gray, 1964) a precipitate occurs on adding $CaCl_2$ solution to the double strength medium. Analysis has shown that this precipitate is composed of phosphates of calcium, iron and magnesium. It would be preferable to avoid a precipitate by adjusting the proportions of the mineral salts so that they remained in solution. This has been done in the Oxoid dehydrated medium. However, on analysis of Gray's medium prepared in the laboratory, the actual concentrations of mineral salts remaining in solution were

found to be different from those in the Oxoid medium. Furthermore, the proportions of the mineral salts remaining in solution varied widely on different occasions even though the same general preparation procedure was used. The results of some analyses are compared in Table 2.

In the method of preparation described by Gray, most of the iron and calcium are precipitated as phosphate, whereas in the Oxoid medium the original quantity of iron added by Gray is retained in solution mainly by reducing the concentration of phosphate. It is doubtful, however, whether this is the best way of avoiding a precipitate. Such a marked reduction in phosphate, in addition to reducing the buffering capacity of the medium, would probably reduce growth more than a reduction in iron and almost certainly more than a reduction in calcium. Furthermore, with all but very soft waters an appreciable amount of calcium would be added with the water sample.

Table 2. *Mineral composition of Gray's glutamate medium (double strength) in g./l.*

	Minerals originally added (Gray, 1964)	Minerals remaining in solution after precipitation by Gray's method (range)	Minerals contained in Oxoid medium
K_2HPO_4	2	1·4–1·8	0·6
$MgSO_4.7H_2O$	0·4	0·2–0·4	0·2
Ferric citrate	0·2	0·001–0·05	0·2
$CaCl_2$	0·4	0·02–0·15	0·2

In Gray's original work with a single strength medium all the minerals were retained in solution. Precipitation occurred only on preparation of a double strength medium. Furthermore, the advantage that he found from adding iron and calcium was obtained originally, using suspensions of pure cultures stored in deionized water. Calcium was, therefore, likely to have been a more significant factor than in a water sample naturally containing calcium.

It is suggested, therefore, that, in order to avoid a precipitate with the double strength medium, a mineral composition should be chosen within the analyses obtained with Gray's method of preparation, but with the highest possible phosphate concentration, followed by the highest possible iron concentration, the calcium being reduced to very low concentration if necessary to avoid a precipitate. These variations in composition were not appreciated until after completion of the trials and are still under investigation.

Water samples

Samples of water from a wide range of sources were required including samples likely to contain attenuated, resistant or damaged organisms. Earlier work had shown that marginally chlorinated water containing some surviving organisms would probably accentuate differences between the methods under review. As

such samples are not normally available, some samples were specially treated by some of the laboratories.

For this purpose, samples of polluted water likely to contain between 100 and 100,000 coliform organisms per 100 ml. were collected in Winchester quart bottles. These were kept at 4° C. overnight in the laboratory. The following morning the samples of water were filtered to remove particulate matter which might cause irregular results. Four ml. of 0·38 % solution of NH_4Cl was added to 2 l. to give an ammonia nitrogen concentration of at least 2 mg./l. Sufficient ice cubes prepared from water free from coliform organisms were added to keep the temperature as near 0° C. as possible during chlorination. Twenty ml. of a hypochlorite solution containing approximately 100 mg./l. of available chlorine was then added to give a chlorine concentration of approximately 1 mg./l. and the water was left to stand for 15–60 min. Any residual chlorine was then neutralized by the addition of 1 ml. of a 3 % solution of sodium thiosulphate sterilized in the autoclave. The chlorination time was chosen by trial and error so that the final sample of water would give some positive and some negative tubes in a 48 hr. test.

Unchlorinated water samples from various sources were examined by all participating laboratories. These included samples stored in bottles by some laboratories.

Methods of recording results

Either one 50 ml., five 10 ml. and five 1·0 ml. volumes or three five-tube ten-fold dilutions were set up for each sample with each of the six media and incubated directly at 37° C. without prior warming. They were examined after approximately 18, 24 and 48 hr. The amount of acid and gas produced was recorded as follows:

A 1 Minimum detectable acidity.
A 2 Acidity between A 1 and A 3.
A 3 Complete acidity.
G 1 Gas on tapping or shaking only.
G 2 Visible gas bubble, insufficient to fill concavity of inner tube.
G 3 Sufficient to fill concavity or more.

All tubes showing less than A 3 and G 3 in 18 and 24 hr. were subcultured and then returned to the incubator for reading at 24 and 48 hr.; they were not subcultured again. All tubes showing A 3 and G 3 in 18 and 24 hr. were assumed to remain so in 24 and 48 hr.

All presumptive positive tubes were subcultured into brilliant green bile broth (BGB) of a single batch and into peptone water, usually 2 % Oxoid tryptone, for indole production. These were incubated in a water bath at 44 ± 0·25° C. for 24 hr. *Esch. coli* was recorded as present in all tubes giving positive results in both tests. All tubes negative after culture in BGB at 44° C. were examined for coliform organisms of any kind. This was done by plating from the original positive presumptive tube to MacConkey agar and incubating at 37° C. To save time all 24 and 48 hr. presumptive positive tubes were usually subcultured in this way. A growth of typical coliform colonies in 24 hr. was accepted as confirmation; doubtful colonies were subcultured to lactose peptone water. Acid and gas production in

48 hr. at 37° C. was taken as confirmation of coliform organisms. The final results for each primary tube were recorded individually, for each acid and gas category for each time interval, so that results could be compared on the basis of numbers of tubes or most probable numbers of organisms.

RESULTS

Methods of comparison

All comparisons between media have been made on the basis of numbers of positive tubes obtained. This comparison involves a bias in favour of laboratories which examined the greatest number of samples. Results from all the individual laboratories, which examined sufficient samples to be individually compared, have therefore been discussed separately but detailed results have not been presented. As all positive reactions have been counted there is also a bias in favour of samples giving large numbers of positive tubes. This tends to mask the differences between the methods. Selection of the higher dilutions only for comparison, that is those giving some negative tubes, would have accentuated the differences.

Glutamate media

All participating laboratories soon found that there were marked differences in performance between the three modifications of glutamic acid media. The results for all laboratories are summarized in Table 3. This shows the outstanding superiority of Gray's modification over the other two glutamic acid media. This superiority was significant for all types of samples, for both coliform organisms and *Esch. coli*, for all degrees of acid and gas production and for all time intervals. In other words Gray's modification not only gave higher results than the other glutamate media, both for coliform organisms and *Esch. coli*, but it gave them in a shorter time with greater production of acid and gas, thus making positive reactions more easily recognizable.

The early recognition of the superiority of Gray's medium led to the decision to omit the MWB and WRA modifications from subsequent trials. Not only were the MWB and WRA media less productive of positive results than Gray's medium, but they were also slower and significantly less productive of coliform organisms than MacConkey broth. The yield of *Esch. coli* in 48 hr. was not significantly different. In this respect the results differed from previous trials with these media (Collingwood, 1964; Windle Taylor, 1959–60). This may be related to the use of dehydrated media.

Although Gray's medium gave more false positive results than the other glutamate media, this was not serious, since it gave fewer than the MacConkey broths. The superiority of Gray's medium was found by all laboratories. For unchlorinated waters the MWB medium gave the fewest positive results at 24 hr. but showed improvement at 48 hr. This again applied to coliform organisms and *Esch. coli*, for all degrees of acid and gas production and for all time intervals, although there was some variation between individual laboratories.

Table 3. *Comparison of laboratory-prepared and Oxoid MacConkey broths with MWB, WRA, and Gray's glutamate media*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive reactions			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
	Unchlorinated samples (no. of tubes examined 1036)												
A 1 G 1 or more	Oxoid MacConkey	0·7	2·5	10·7	4	15	64	363	485	600	277	252	266
	Lab. MacConkey	1·0	3·1	9·0	6	18	52	375	493	580	220	248	253
	MW glutamate	0·2	0·2	3·8	1	1	18	107	306	468	97	206	263
	WRA glutamate	0·0	0·6	5·7	0	3	30	147	315	525	109	212	279
	Gray glutamate	0·3	0·9	9·4	2	6	60	270	446	635	209	303	368
A 2 G 2 or more	Oxoid MacConkey	0·3	1·2	8·1	2	7	48	336	446	593	224	246	266
	Lab. MacConkey	0·9	1·6	7·1	5	9	40	327	432	560	213	247	251
	MWB glutamate	0·0	0·0	2·9	0	0	13	67	259	444	66	179	256
	WRA glutamate	0·0	0·4	3·1	0	2	15	90	261	485	73	180	274
	Gray glutamate	0·0	0·2	7·6	0	1	48	216	402	630	158	283	368
	Chlorinated samples (no. of tubes examined 1375)												
A 1 G 1 or more	Oxoid MacConkey	0·6	3·2	10·8	5	26	87	348	610	805	284	419	447
	Lab. MacConkey	2·9	6·0	15·4	24	49	126	344	616	818	279	388	428
	MWB glutamate	0·0	0·5	7·9	0	3	52	39	310	662	34	248	445
	WRA glutamate	0·0	0·1	6·1	0	1	45	35	269	678	34	232	449
	Gray glutamate	0·0	0·3	11·4	0	3	102	157	561	895	141	456	595
A 2 G 2 or more	Oxoid MacConkey	0·5	1·4	7·2	4	11	56	266	553	780	230	404	445
	Lab. MacConkey	2·4	4·4	11·4	19	35	91	287	564	801	257	381	428
	MWB glutamate	0·0	0·0	1·7	0	0	11	24	215	632	23	181	442
	WRA glutamate	0·0	0·0	2·6	0	0	16	18	183	623	17	167	446
	Gray glutamate	0·0	0·1	7·4	0	1	64	101	479	870	96	393	592

MacConkey broth

Similarly, the Oxoid MacConkey broth was compared simultaneously with laboratory-prepared MacConkey broths. Results for all laboratories are also summarized in Table 3. There is fairly close agreement between the two media. Comparison of each pair of figures shows that the Oxoid medium gave more positive results than the laboratory MacConkey broth under most circumstances, though most of these differences are not significant. The Oxoid medium also gave significantly fewer false positive results with chlorinated samples. It was found that two laboratories obtained consistently lower results with laboratory-prepared medium. One of these laboratories had deliberately used bile salts from a nationally available source, which conformed to the normal requirements for use in MacConkey broth but had given unsatisfactory results with membrane filters. Consistently better results with laboratory-prepared media were given by two other laboratories.

It can be concluded, therefore, that the Oxoid MacConkey broth was a satisfactory standard for comparison with other media, as it gave results within the range of variability encountered in laboratory-prepared media, and with numbers of positive results more often above than below those with laboratory-prepared media.

Gray's glutamate medium compared with MacConkey broth

For the remainder of the trials laboratory-prepared MacConkey broth was therefore omitted and only three media—Oxoid MacConkey broth, Teepol broth and Gray's modified glutamic acid medium—were retained for comparison. The results for all laboratories are summarized in Table 4. This shows clearly that at 48 hr. Gray's medium gave significantly higher numbers of positive results ($P \leqslant 0{\cdot}05$) for coliform organisms and *Esch. coli* than the other two methods, with both chlorinated and unchlorinated waters for all degrees of acid and gas production. This was consistent for all laboratories for *Esch. coli*. At two laboratories Gray's medium gave lower coliform counts than MacConkey broth with unchlorinated samples and at two other laboratories Gray's medium gave lower coliform counts than MacConkey broth with chlorinated samples. These results suggest that the increased numbers of coliform organisms obtained with Gray's medium are probably due mainly to increases in the counts of *Esch. coli*. In fact the increase in numbers of isolations of *Esch. coli* is greater than that of coliform organisms although the former are included in the latter. It does not follow, however, that coliform organisms other than *Esch. coli* would not be so readily isolated with Gray's medium. It merely signifies that where *Esch. coli* and other coliform organisms occur together, growth of the *Esch. coli* has not been suppressed by the other coliform organisms. This has been verified at one laboratory which examined a special series of samples containing moderate numbers of coliform organisms but very few *Esch. coli*. These results will be described later.

In 24 hr. Gray's medium gave significantly more positive results for coliform organisms and *Esch. coli* in unchlorinated waters. The differences are not significant for chlorinated samples. Similar results for *Esch. coli* were obtained by all laboratories. Four laboratories obtained lower coliform counts with Gray's medium than

Table 4. *Comparison of MacConkey Broth, Teepol Broth and Gray's glutamate medium*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive results			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
		Unchlorinated samples (no. of tubes examined 3409)											
A 1 G 1 or more	Oxoid MacConkey	0·7	2·1	10·3	11	35	172	1119	1354	1676	804	837	854
	Teepol	0·9	4·4	10·1	15	77	176	1121	1475	1736	788	869	894
	Gray glutamate	0·1	0·5	8·2	2	10	164	951	1403	1869	790	1047	1145
A 2 G 2 or more	Oxoid MacConkey	0·4	1·3	8·6	6	22	141	982	1248	1637	771	829	853
	Teepol	0·1	2·5	9·0	1	42	153	957	1360	1700	729	866	893
	Gray glutamate	0·0	0·1	6·9	0	2	127	707	1262	1830	618	1000	1140
		Chlorinated samples (no. of tubes examined 1441)											
A 1 G 1 or more	Oxoid MacConkey	0·4	2·8	10·3	3	23	85	360	632	827	290	427	457
	Teepol	0·2	1·6	6·0	2	14	51	330	660	852	268	428	456
	Gray glutamate	0·0	0·4	11·5	0	4	106	173	581	924	152	458	605
A 2 G 2 or more	Oxoid MacConkey	0·2	1·1	6·0	2	9	48	275	573	804	236	413	454
	Teepol	0·1	0·8	4·5	1	7	40	224	593	828	202	410	451
	Gray glutamate	0·0	0·1	7·9	0	1	71	116	475	899	104	413	602

with MacConkey broth with unchlorinated samples and five laboratories did so with chlorinated samples.

In 18 hr. Gray's medium gave significantly fewer positive results than the other media under nearly all circumstances. This was found consistently by all laboratories except two where Gray's medium gave more positive results than MacConkey broth for both *Esch. coli* and coliform organisms, especially where low degrees of acidity and gas production were included, with both chlorinated and unchlorinated samples.

Teepol broth compared with MacConkey broth

The Teepol medium gave significantly better results for coliform organisms in 24 and 48 hr. than MacConkey broth with chlorinated and unchlorinated samples and for *Esch. coli* in 24 and 48 hr. with unchlorinated samples. For *Esch. coli* in chlorinated samples the positive results were slightly lower but the difference is not significant. In 18 hr. the only significant differences between Teepol and MacConkey broths were with chlorinated samples at A 2 G 2, Teepol giving fewer positive results. In 18 hr. Teepol gave consistently more positive results than Gray's medium. Again some variation in performance with Teepol broth occurred in individual laboratories, particularly at 18 hr.

False positive reactions

The number of false positive reactions was negligible in Gray's medium in 18 and 24 hr. They were of greater but still low frequency in Teepol and MacConkey broth. At 48 hr. there was little difference between the three media with unchlorinated samples but with chlorinated samples Teepol gave significantly fewer false positive results than the other media.

The proportions of false positive reactions, however, varied widely in different laboratories and different media. In 48 hr. the false positive results for different media in different laboratories ranged from 0 to 16%. This, of course, reflects variation in the bacterial flora other than the coliform group, in water samples from different parts of the country. In MacConkey broth *Clostridium perfringens* (*welchii*) or mixtures of organisms have usually been reported as the most frequent causes of false positive results. In glutamate media, *Bacillus polymyxa* and *B. macerans* are the commonest causes. *Cl. perfringens* does not usually grow in glutamate media and *B. polymyxa* and *B. macerans* cannot grow in MacConkey broth; none of them is believed to be capable of growth in Teepol broth. False positive results, therefore, depend mainly on the relative frequency of occurrence of chlorine-resistant spores of *Cl. perfringens*, *B. polymyxa*, and *B. macerans* in the water samples. When chlorination is applied, sufficient to kill all coliform organisms, as in water treatment plants, the number of false positive reactions caused by chlorine-resistant spores would probably be increased as their presence would not be masked by positive reactions due to growth of coliform organisms.

Four laboratories examined a few samples of normally treated works-chlorinated waters containing no coliform organisms. All results were negative in 24 hr. The 48 hr. results are given in Table 5. This shows Gray's medium gave a large number

of false positive reactions compared with MacConkey broth, and Teepol broth gave none at all.

The third edition of Report 71 recommends that all MacConkey broth tubes showing acid and an amount of gas sufficient or more than sufficient to fill the concavity at the top of the Durham tube should be regarded as 'presumptive positives'. A lesser volume of gas may be disregarded unless visible gas appears in the liquid when the tube is lightly tapped. This means A 1 G 3 or possibly A 1 G 2 with the present notation. Since G 2 or G 3 is usually accompanied by at least A 2, it would be worth considering how many false positive results would be avoided by ignoring all reactions less than A 2 G 2 and how many coliform organisms would be missed. These results for all samples are summarized in Table 6.

Table 5. *False positive results given by samples of water containing no coliform organisms*

Number of tubes showing false positive reactions in 48 hr.*

A 1 G 1			A 2 G 2			
Oxoid MacConkey	Teepol Broth	Gray Glutamate	Oxoid MacConkey	Teepol Broth	Gray Glutamate	Total Tubes
7	0	30	2	0	26	176

* No false positive reactions were observed in 24 hr.

Table 6. *Effect of ignoring results less than A 2 G 2*

	Oxoid MacConkey broth		Teepol broth		Gray's medium	
	No. of tubes	% of positive results	No. of tubes	% of positive results	No. of tubes	% of positive results
Reduction in false positive results	68	2·7	34	1·3	72	2·5
Reduction in true coliform results	62	2·4	60	2·3	64	2·3

This table shows that some false positive results would be eliminated, but this would be offset by an approximately equivalent loss of true coliform results. The table also shows that there would be a small loss of true coliform results if the recommendation in the current 3rd edition of Report 71 were used.

It is suggested, therefore, that A 1 G 1 at 18 and 24 hr. should be accepted in the presumptive test for coliform organisms with all of these media. This will give quick results with a negligible number of false positive reactions. At 48 hr. if excessive numbers of false positive reactions are obtained with A 1 G 1 then A 2 G 2 could be accepted as a minimum with no greater loss of true coliform results than in the present recommended method.

Coliform organisms

As stated earlier a special series of twelve samples containing moderate numbers of coliform organisms and very few *Esch. coli* was examined at one laboratory. This enabled the suitability of the media to be assessed for coliform organisms without interference from *Esch. coli*. These samples were from new mains which had failed to give negative results despite repeated flushing. Although the mains were chlorinated originally, the water was not considered to contain chlorine resistant organisms, as the persistence of coliform organisms under these circumstances is usually due to growth on accumulations of dirt or other extraneous material in the mains. These results were, therefore, included in the unchlorinated series in Table 4 but they have been abstracted and shown separately in Table 7.

This shows that in 48 hr. Gray's medium was as successful for coliform organisms other than *Esch. coli* as it was in the other series for mixtures of these organisms. Their growth in Gray's medium was slow compared with that in MacConkey broth and Teepol broth and most of them gave poor acid and gas production in all media. The few *Esch. coli* that were present grew more readily in Gray's medium. The number of false positive reactions was high in all media but lowest with Gray's medium.

Adjustment of pH

In a previous trial in 27 laboratories, Gray's medium freshly prepared in his laboratory was found to give positive results equal to or greater than MacConkey broth even in 18 hr. (Gray, 1964), whereas in the current series growth was slower in Gray's medium. He suggested that this might be due to a difference in pH. Folpmers (1948) originally used media at pH 6·0 to inhibit growth of sporing bacilli, Gray (1959) found that pH 6·7 was the optimum and both Gray (1949) and Windle Taylor (1961–62) showed that less satisfactory results were obtained at pH 7·5. Gray obtained a pH 6·7 in the finished medium by adjustment of the pH to 6·8 before autoclaving. The pH of the Oxoid medium before autoclaving was 7·3 and observations at the different laboratories showed that final pH varied from 6·3 to 7·0. Various autoclaving techniques were used from 115° C. for 10 min. up to 121° C. for 15 min. and various shaped bottles and tubes either with screw caps, Oxoid caps or cotton wool plugs were used. All of these factors together with variations in sizes and types of autoclaves and hence rates of heating and cooling may effect the final pH. Gray, therefore, suggested that comparison should be made between Oxoid Gray's medium unadjusted and adjusted to pH 6·8 before autoclaving. A small number of samples was examined in this way and the results are summarized in Table 8. The final pH obtained after autoclaving these adjusted media varied from 6·1 to 6·7.

The results in Table 8 suggest that more *Esch. coli* were isolated and that coliform organisms and *Esch. coli* grew more rapidly in the pH adjusted medium. More acid and gas was also produced in the early stages. None of the differences in this table are however significant.

Table 7. *Comparison of MacConkey broth, Teepol broth, and Gray's glutamate medium on a series of new mains*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive results			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
A 1 G 1 or more	Oxoid MacConkey	0	6·7	27·1	0	4	16	22	43	59	1	2	2
	Teepol	0	5·5	23·6	0	3	13	29	41	55	1	1	1
	Gray glutamate	0	0	15·2	0	0	10	6	31	66	3	4	4
A 2 G 2 or more	Oxoid MacConkey	0	0	4·5	0	0	2	1	9	44	0	1	1
	Teepol	0	0	18·4	0	0	9	4	14	49	0	1	1
	Gray glutamate	0	0	1·9	0	0	1	0	4	52	0	2	4

Total no. of tubes examined 121.

Table 8. *Comparison of Gray's glutamate with pH adjusted and unadjusted*

	pH before auto-claving	Number of tubes yielding								
		False positive results			Coliform organisms			*Esch coli.*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
A 1 G 1 or more	7·3	0	1	10	94	143	171	81	119	125
	6·8	4	4	12	99	149	174	87	128	133
A 2 G 2 or more	7·3	0	0	4	56	121	167	54	111	124
	6·8	0	0	3	69	135	172	66	118	133

Total no. of tubes examined 300.

Chlorinated samples

One outstanding peculiarity of Gray's medium indicated by these trials was its apparent superior ability to initiate growth of organisms, especially *Esch. coli*, which had been exposed to chlorine. One laboratory studied this phenomenon further by applying the marginal chloramine treatment described earlier to suspensions of *Esch. coli* in water. The resulting chlorinated suspensions were then added to MacConkey broth, lactose broth and Gray's medium.

When suspensions initially containing 500 to 5000 *Esch. coli* per 100 ml. were used, counts between 10 and 250 per 100 ml. after chlorination were regularly obtained with Gray's medium but no growth or only occasional growth was observed in tubes of MacConkey broth, or even in lactose broth which contains no inhibitory substances. In other words some of these organisms were viable in Gray's medium but not in ordinary nutrient media. It is easy to assume that these organisms are chlorine-damaged and the results suggest selective enzyme damage, but addition of some of the main ingredients of Gray's medium to MacConkey broth, singly or together, did not permit initiation of growth of these organisms. Conversely, omission of the supplementary growth factors from Gray's medium singly or collectively did not affect its ability to initiate growth of these organisms.

It has been recognized, however, that young bacterial cells are generally more sensitive to disinfectants or any other lethal agent, than older cells, and, furthermore, that the cells in a pure culture are not all of the same age (Walters, 1965; 1967). It follows, therefore, that marginal chlorination may merely be selecting the older cells. Some of these older cells may also be in a resting stage or at least have a prolonged generation time (Quesnel, 1963). This would account for the slow growth obtained in the trials.

DISCUSSION

These trials have indicated that Gray's medium is a satisfactory alternative to MacConkey broth for the detection of coliform organisms in water. It should not, however, be regarded as a second-best substitute. It is superior because of its ability to yield greater numbers of organisms, particularly of chlorinated or attenuated *Esch. coli*.

It could be argued that ability to recover damaged organisms is not essential in a medium; on the other hand, we do not know whether these organisms are chlorine-damaged or whether they are just chlorine-resistant nor whether they are viable in the body. Their presence certainly indicates marginal treatment. In view of the known resistance to chlorination of some viruses and of *Pseudomonas aeruginosa* the ability to isolate coliform organisms which survive marginal chlorination would provide an added safety factor in water treatment.

The original purpose in devising a chemically defined medium was that it should be standard in composition in all laboratories. It is a pity, therefore, that differences in pH occur so readily. This could be overcome at each laboratory by adjustment of the pH before autoclaving so that the medium for use has a final pH of 6·7.

The initial pH required should be determined in each laboratory, as it will probably vary according to the sterilizing equipment available and its method of use and the nature of the media containers and their methods of closure.

Had Gray originally used a mineral composition that avoided precipitation, confusion would not have arisen subsequently in attempts to avoid it. For samples of hard water addition of calcium and magnesium is unlikely to be necessary.

The adoption of Gray's medium for the bacteriological examination of water should not preclude the search for still further improvements. In view, however, of its superiority to other glutamate media, it is recommended that confusion should be avoided by rejecting the other glutamate media with which it has been compared.

Teepol broth can also be recommended as a satisfactory alternative to MacConkey broth but it will not give the same recovery of chlorinated organisms as Gray's medium.

These experiments were carried out with an Oxoid dehydrated version of Gray's medium which differed somewhat in mineral composition from the original. Further experiments are being carried out using a dehydrated medium which attempts to reproduce the mineral composition finally achieved by Gray (1964).

SUMMARY

Oxoid dehydrated MacConkey broth was compared with laboratory prepared MacConkey broth, Teepol broth and three modifications of glutamic acid media, by participants in eleven laboratories. A variety of chlorinated and unchlorinated water samples were used. The Oxoid MacConkey broth was shown to be a satisfactory standard for comparison. Teepol broth was found to be a satisfactory alternative to MacConkey broth. Gray's improved glutamate medium was shown to be the best of the glutamate media and it gave results superior in most respects to MacConkey broth. In particular more *Escherichia coli* were obtained as well as more coliform organisms. Organisms surviving marginal chlorination were recovered more readily. Growth was sometimes slower than in MacConkey broth but this could possibly be improved by closer attention to pH adjustment and mineral composition.

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The role of cutaneous diphtheria in the acquisition of immunity

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INTRODUCTION

Several workers have described the carriage of *Corynebacterium diphtheriae* organisms in the cutaneous ulcers of troops stationed in the tropics during war time. Craig (1919) attributed the origin of these 'desert', 'septic', or Veldt sores to the Klebs–Loeffler bacillus, whilst other workers such as Benstead (1936) and Liebow, MacLean, Bumstead & Welt (1946) observed that the diphtheria organisms were mere secondary invaders on lesions of the skin, which had been previously caused by some trivial injury. Liebow and his colleagues, working in the Pacific Islands, presented data indicating 'the existence of a tremendous reservoir of diphtheria amongst the natives in the tropics which is largely cutaneous, which affects chiefly young children and which accounts in large measure for the remarkably accelerated acquisition of a state in which they do not react to the Schick test'. Similar inferences have been drawn by workers such as Marples & Bacon (1956) and P. J. Collard (personal communication). The present investigation carried out amongst a non-immunized population in a semi-rural area of Ceylon, confirms the findings of the above workers and suggests that cutaneous diphtheria may be the main method whereby natural immunization is acquired in tropical countries.

METHODS

The field survey was carried out in four villages situated in the Western Province of Ceylon. The villages cover an extent of about 14 square miles and have a population of about 43,000 (figures from the Medical Officer of Health, Kotte, Ceylon). Before this investigation, no active immunization against diphtheria had been carried out in this area on a large scale.

The Schick test

The Schick test toxin ('Wellcome' brand) was flown from England in small batches and stored at 4° C. Each batch was used within 2 months of arrival. Tests were read on the fourth day after injection, thus allowing time for any pseudo-reactions which may have developed to disappear.

Isolation of C. diphtheriae *from carriers*

Swabs were taken from all the children who were Schick tested to determine the carrier rate of *C. diphtheriae*. One swab was taken from both tonsillar areas

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of the throat, another was introduced into each nostril in turn and the anterior nares were swabbed, a third was rubbed against both knees and the inner malleoli of both ankle joints. Swabs were also taken from any septic lesions such as otitis and conjunctivitis and from any cutaneous ulcers present. Each swab thus taken was immediately inoculated on Downie's blood tellurite plates (Cruickshank, 1965*a*). After 24 hr. incubation at 37° C., any colonies morphologically resembling *C. diphtheriae* were subcultured on blood agar plates and Loeffler's serum slopes. When pure cultures were obtained, they were confirmed to be *C. diphtheriae* by cellular morphology, colony appearance on tellurite media, fermentation tests in serum sugars, reduction of nitrates to nitrites and the inability to hydrolyse urea.

In testing for acid production in serum sugars, 1 % peptone water containing 20 % ox serum, 1 % of each sugar and 10 % Andrade's indicator was used. The ability to reduce nitrates was tested on Cook's plates (Cook, 1950). Christensen's medium (Christensen, 1946) was used for the testing of urease production.

Tests for toxigenicity

Tests for toxigenicity were carried out by both *in vivo* and *in vitro* methods. The *in vivo* tests were carried out according to the methods described by Cruickshank (1965*b*). The *in vitro* tests were done by using Elek's agar diffusion technique (Elek, 1948) as modified by M. Glasset (personal communication). Glasset reduced the concentration of New Zealand agar in the basal medium from 1·5 to 0·7% and correspondingly reduced the concentration of antitoxin in the filter paper strip from 1000 units to 50 units per ml. This technique not only accelerates the production of lines of precipitate due to the toxin-antitoxin complex but also prevents the formation of other lines of precipitate which are not specifically due to the toxin and antitoxin. All the cultures tested gave identical results when tested by both methods.

RESULTS

The percentages of Schick negative children, the percentages of *C. diphtheriae* carriers and the percentages showing cutaneous ulcers in each age group are

Table 1. *Results of Schick survey and carrier survey*

Age groups	Total no. tested	Percentage Schick negative	Percentage *C. diphtheriae* carriers	Percentage occurrence of cutaneous ulcers
0–3 months	20	95	0	0
3–6 months	28	78·5	10·7	3·4
6 months–1 yr.	97	22·7	7·2	7·2
1–1½ years	85	23·5	8·2	14·1
1½–2 years	57	33·3	12·3	21·1
2–2½ years	44	34·1	15·9	13·6
2½–3 years	48	64·5	4·1	22·8
3–3½ years	43	60·5	16·3	18·6
3½–4 years	40	62·5	10	22·5
4–5 years	59	64·4	8·5	10·2
5–6 years	85	64·7	15·3	18·8
6–7 years	58	76	10·3	17·2

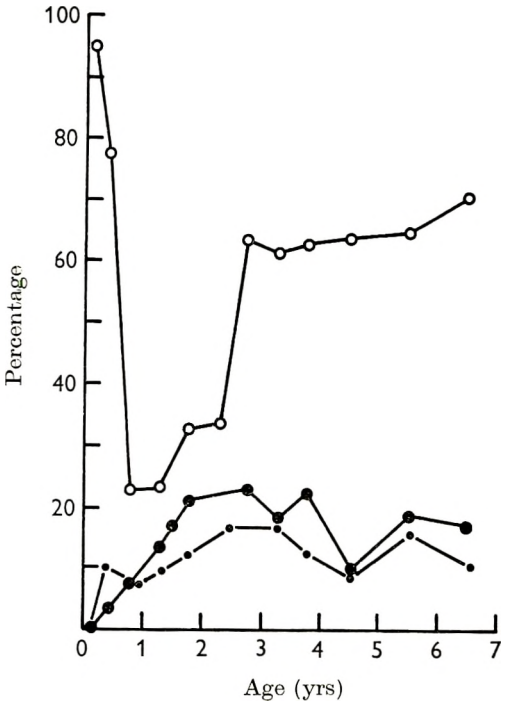

Fig. 1. Results of Schick survey and carrier survey. Schick negative children ○—○; incidence of cutaneous ulcers ●—●; total isolations of *C. diphtheriae* •—•

Table 2. *A summary of* C. diphtheriae *isolations and carrier rates*

Strains isolated

70	mitis type
2	gravis type
1	intermedius type
73	

Sites of isolation

Throat	24 (3 toxigenic)
Cutaneous ulcers	40 (9 toxigenic)
Intact skin	6
Nose	2
Ear	1 (1 toxigenic)
	73* (13 toxigenic)

* Includes isolations from five children who carried the organism in more than one site.

Carrier rates

Number of children tested	664
Number of children carrying *C. diphtheriae*	68 (10·2 %)
Number of children with cutaneous ulcers	98
Number of isolates of *C. diphtheriae* from ulcers	40 (40·8 %)
Cutaneous ulcer carriage rate of *C. diphtheriae*	6·0 %
Throat carriage rate of *C. diphtheriae*	3·9 %

tabulated in Table 1. Although about 700 children were tested altogether, only the results of 664 have been included in the series for various reasons such as not being available when the results were read, uncertainty of the age, etc. The same results are also depicted in Fig. 1. A summary of the *C. diphtheriae* isolations is given in Table 2. Included among the group classified as ulcers was a culture isolated from a swab taken from an infected smallpox vaccination site. Five children harboured the organisms in more than one site.

DISCUSSION

The pattern of immunity in the different age groups as measured by the Schick test is typical of that found in any non-immunized population where the rate of exposure to the disease is high. The findings agree partly with those of a few surveys carried out in India (Robinson & Lalitha Bai, 1964; Das, 1934; Paricha, Banerjee & Wordsworth, 1939). It is seen in Fig. 1 that 65 % of the children are Schick negative by the age of 5 years.

The incidence of diphtheria in Ceylon as judged by the notifications of clinical cases is not very high. For example, only 465 cases of diphtheria were reported from the whole of Ceylon in 1964 (Assistant Epidemiologist, Ceylon 1967) from a population of about eleven million. The carrier rate of *C. diphtheriae* as assessed by Gulasekeram, Gunaratna & Somasunderan in 1956 from a group of school children below the age of eleven was only 2·2 %. Making allowance for the fact that all cases of diphtheria are not reported, the incidence of the disease and the carrier rate mentioned above are not sufficiently high to justify a development of 65 % Schick negativity by the age of 5 years. Unless grossly apparent in other sites, clinical diphtheria is only diagnosed from lesions in the throat. Similarly in the survey mentioned above, the carrier rate was assessed only from throat and nasal swabs. The present work reveals the high incidence of diphtheria organisms in the cutaneous ulcers of these children. Whereas only 3·9 % of the children carried *C. diphtheriae* in their throats or noses, 6·0 % of them had the organisms in ulcers; 40·8 % of the ulcers examined in these children yielded *C. diphtheriae* organisms. A higher percentage of toxigenic organisms was found among the cultures isolated from the ulcers than from those isolated from other sources. Not only is there a relationship between the percentage of total diphtheria isolations and the percentage of ulcers found in each age group, but there is also a relationship between the latter and the Schick conversion rate in each age group (see fig. 1).

Although 69 % of the toxigenic *C. diphtheriae* cultures were isolated from children between 1 and 3½ years where the rate of Schick conversion is the highest, the majority of isolations represent non-toxigenic cultures. The question arises whether non-toxigenic diphtheria organisms contribute in any way towards the acquisition of immunity to diphtheria. 'Non-toxigenic' organisms cannot obviously produce an antitoxic immunity unless they produce minute quantities of toxin which cannot be detected by routine laboratory techniques, as suggested by Marples & Bacon (1956). On the other hand, the non-toxigenic diphtheria organisms may have been derived from strains which were originally toxigenic. Before the intro-

duction of antibiotic therapy, several workers reported the isolation of non-toxigenic *C. diphtheriae* organisms from convalescent patients who were originally infected with toxigenic cultures. Okell (1929) while discussing the association of virulent and avirulent strains in patients, reports several instances where toxigenic and non-toxigenic organisms existed in the same patient at the same time, or where the isolation of the original toxigenic organism was followed by the isolation of a non-toxigenic. The toxigenic and non-toxigenic organisms found in the same patient were shown to belong to the same serological type in several cases. Liebow and his colleagues in their study of cutaneous diphtheria in the tropics (Liebow *et al.* 1946) noticed that as the ulcers got older the toxigenic strains were replaced by non-toxigenic strains. Although the work of Anderson & Cowles (1958) showed that such a conversion from toxigenicity to non-toxigenicity may occur *in vivo* through the mediation of antibodies against bacteriophages, the present writers could not detect any bacteriophage-neutralizing antibodies in the sera of fifty Schick negative children tested.

A third possibility is that the non-toxigenic organisms are not descendants of the original toxigenic organisms, but that they belong to a different group which is better adapted to lead a parasitic existence upon its host. The toxigenic organisms are probably more fastidious in their requirements and need the devitalized tissue produced by their toxin for continual existence. In fact it is seen from this survey that ten out of thirteen of the toxigenic organisms isolated were from sites where there was pus or serum. Only three toxigenic organisms have been isolated from apparently healthy throats, while none were isolated from intact skin or from the nose. The fact that the non-toxigenic organisms isolated appeared to belong to a bacteriophage pattern which is different from that of the toxigenic organisms also supports this view.

Frobisher & Parsons (1943) have shown that non-toxigenic diphtheria organisms produce an antibacterial immunity in rabbits whereby the injected animals become more resistant to doses of toxigenic *C. diphtheriae* which are invariably fatal to normal animals. A similar antibacterial immunity may occur in humans, thus protecting them from the severity of the infections due to subsequent attacks by toxigenic organisms.

Cutaneous diphtheria may present a source of danger to non-immunized adults who come into the tropics for the first time from other countries where the carrier rate is low. This has been demonstrated during the two world wars by Craig (1919), Benstead (1936) and Liebow and his colleagues (1946.) But amongst the local child population which is exposed to cutaneous diphtheria while partially immune owing to the presence of maternal antibody, it does not appear to present such a serious problem. At least one of the dangers of faucial diphtheria, that of laryngeal obstruction, does not present itself. Being limited to the surface area of an ulcer, the organisms cannot spread over a large area and hence probably cannot produce much toxin. As the area for absorption of toxin is also small, it is likely that less toxin enters the system. Hence in the absence of artificial immunization, this 'live vaccination' may provide a method of acquiring a natural immunity against the occurrence of serious cases of diphtheria.

SUMMARY

A field survey conducted amongst children in a semi-rural area of Ceylon has revealed a high rate of Schick negative conversion early in life. A high carrier rate of *C. diphtheriae* in cutaneous ulcers which probably accounts for this early Schick conversion has been detected. The role of cutaneous diphtheria and that of non-toxigenic organisms in the acquisition of natural immunity to the disease is discussed.

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The immunogenicity of heat-inactivated vaccinia virus in rabbits*

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Endemic smallpox is now confined to South East Asia and the tropical regions of Africa and South America, but may be casually imported into any other region. Travellers, and particularly air travellers, from endemic areas greatly increase the population who may be exposed to the disease, though risk to any one individual may occur very infrequently, if at all, and must be set against the chances of complications from vaccination with live virus. Even in the healthy these are not negligible, and are greatly increased in eczema and hypogammaglobulinaemia. An inactivated vaccine would be free from risks due to virus multiplication and would be a preferable alternative provided it could be shown to give adequate protection.

Several workers have reported experiments with animals and man using vaccinia inactivated by various means, and they have interpreted their results differently in the absence of any agreed criteria of immunity. Usually, immunity has been assessed by intradermal challenge with live virus, and, on this basis, some workers, e.g. Amies (1961) and RamanaRao (1962) in rabbits, and Kaplan, Benson & Butler (1965) in man, regarded the immunity produced by inactivated vaccinia as unsatisfactory.

Since only multiple intradermal doses of dermo-vaccinia virus will kill a rabbit, and even then not with certainty, workers attempting to assess the degree of immunity produced have been trying to measure degrees of skin immunity and the difficulties of doing this have been reviewed by McNeill (1966). In smallpox, where a viraemia is thought to play an important part in pathogenesis (Downie, 1965), circulating antibody may be the most important factor in deciding whether an infection develops, and the susceptibility of the skin may be of secondary importance. This assumes, however, that the antibody is efficient in neutralizing virus, and though development of neutralizing antibody has been reported by most workers when enough antigen has been given, the authors have said little about the nature of the antibody produced. Appleyard (1961) did note that the antibody elicited by rabbitpox soluble antigen in adjuvant was less efficient than that which appeared following natural infection with vaccinia, but other workers refer only to the titre.

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The ultimate aim of developing a vaccine for human use has led most workers to use a small number of injections. As a result the maximum response that a rabbit (or man) is capable of making to inactivated virus has not been completely assessed. Mahnel (1961) claimed that a single dose of high titre material elicited antibody, while McNeill (1965) obtained very variable results with single doses. It is difficult to see how useful estimates of immunity can be made unless enough antigen is given to ensure as uniformly high a response as can be obtained. Dosage schedules employing fewer injections or lower titre virus can then be compared with this as a yardstick.

Various inactivating agents have been used, but no one agent has emerged as clearly the best. Collier, McClean & Vallet (1955) obtained encouraging results in rabbits using ultra-violet-irradiated virus, both in terms of antibody produced and immunity to challenge, but results in a pilot study in man (Kaplan, McClean & Vallet, 1962; Kaplan, 1962; Kaplan *et al.* 1965) were regarded as unsatisfactory, in that the immunity to challenge was not reproduced though antibody was elicited. Amies (1961), RamanaRao (1962) and McNeill (1965) showed formalin-treated virus to be immunogenic in rabbits and Herrlich (1959; 1964) has used such a vaccine for pre-immunization before vaccination with live virus without, however, completely banishing complications.

In 1924, Nakagawa reported that rabbits inoculated with very large quantities of the closely related cowpox virus heated to 98° C. for 40 min. produced a good immunity to challenge with live virus. Later workers using heat as the inactivating agent have used either too much or too little. Parker & Rivers (1936) used 100° C. for 2 hr. and produced no immunity, whereas Gordon (1925) who used 55° C. for 30 min., and Kligler & Bernkopf (1935) who used 56° C. for 2 hr. produced good immunity probably resulting from surviving live virus. Woodroofe (1960) showed that virus stored for a week at 4° C. is inactivated more slowly than fresh virus, and the use of freshly prepared virus is essential if heat treatment is intended.

The present work was undertaken to explore immunity in rabbits to heated vaccinia virus, partly to follow up Nakagawa's work and partly because heating is a simple procedure which does not require the addition of any substances to the virus. It was intended to give enough antigen to ensure a maximal response, and the nature of the antibody produced was examined in detail. Finally, these results were related to the rabbits' ability to withstand intradermal challenge with vaccinia, cowpox or the more lethal rabbitpox, an infection similar in several respects to human smallpox (Bedson & Duckworth, 1963).

MATERIALS AND METHODS

Virus

The Lister Institute strain of vaccinia was used throughout to prepare vaccines. It was propagated on the backs of rabbits by lightly scarifying it into the shaved skin to produce confluent infection (Hoagland, Smadel & Rivers, 1940). Three days after infection the animals were stunned and exsanguinated, and the infected skin was removed. The dermal pulp from each infected area was scraped into 10–

15 ml. phosphate/phosphate buffer pH 7·2, ionic strength 0·01 μ, containing penicillin (100 u./ml.) and streptomycin (100 μg./ml.), and extracted by shaking with glass beads. The extract was clarified by low speed centrifugation and the virus was partially purified by centrifuging twice at 10,000 r.p.m. for 30 min. in the Type 40 rotor of the Spinco Model L. The international type strains of cowpox and variola major (Fenner & Burnet, 1957) and the Utrecht strain of rabbitpox (Jansen, 1941) were also used. The first was propagated exclusively on the backs of rabbits and the other two on the chorioallantoic membrane (CAM) of 12-day fertile hen's eggs. Virus was extracted and partially purified as already described. Virus for use in vaccines was used as an aqueous suspension. Stock virus was stored at $-20°$ C. as a 50 % suspension in glycerol.

Rabbits

New Zealand White and California rabbits were used to prepare virus. The vaccines were tested in 6-month-old Black and White Dutch rabbits weighing approximately 1 kg.

Infectivity titrations

Virus was diluted in phosphate buffered saline without magnesium or calcium (Dulbecco & Vogt, 1954) containing 10 % bacteriological nutrient broth, penicillin (100 u./ml.) and streptomycin (100 μg./ml.), and 0·1 ml. quantities were inoculated on the CAM of four or five eggs per dilution. End-point titres were calculated from mean pock counts obtained 2 days later with vaccinia or rabbitpox, and 3 days later with cowpox or variola.

Neutralization tests

These were done in eggs and were essentially the method of McCarthy & Downie (1948). Briefly, virus diluted to contain 50–100 pock-forming units (pfu) per 0·05 ml. was mixed with an equal volume of dilutions of serum which had been heated at 56° C. for $\frac{1}{2}$ hr. The mixture was allowed to react at room temperature (19–22° C.) for 1 hr. before 0·1 ml. was inoculated into four or five eggs per dilution. After incubation for 2 or 3 days as for infectivity titrations, pocks were counted, the results plotted on graph paper and the 50 % virus reduction titres obtained by eye. They were expressed as the $\log_{10}$ of the dilution, and are the mean of duplicate experiments.

Virus agglutination. Haemagglutination and haemagglutination-inhibition tests

The methods used were those of Craigie (1932) and McCarthy & Helbert (1960) respectively.

Complement-fixation tests

The method was essentially that of Downie & Macdonald (1950) using 3 in. $\times$ $\frac{1}{2}$ in. round-bottomed glass tubes, veronal buffer, and 2 % washed sheep cells (Burroughs Wellcome) sensitized with two minimal haemolytic doses (MHD) of rabbit haemolysin prepared in the laboratory. Unit (0·2 ml.) volumes of antigen, complement

(2 MHD/unit volume) and antiserum were added in that order, and fixation carried out at 37° C. for 1 hr. and 4° C. overnight before the addition of unit volume of sensitized cells. The mixture was incubated at 37° C. for 1 hr., and the results read after the remaining cells had settled. Appropriate antigen and antiserum controls were always included. The antigen used was 'soluble antigen', the supernatant of the first high speed centrifugation used in virus preparation.

Gel diffusion

The method used was similar to that of Rondle & Dumbell (1962). Ionagar (Oxoid), 1 % in distilled water, was autoclaved, and a 4 mm. layer was poured in a 3 in. Petri dish. Wells 9 mm. in diameter with centres 14 mm. apart were cut in the agar and the reagents were added undiluted and uninactivated. They were left to diffuse at room temperature in a humidified box for as long as necessary, and the lines of precipitate were photographed by dark-ground illumination when fully formed. It was found that the addition of buffer, salt or preservative to the agar did not improve the patterns, and often gave rise to granular, snow-like precipitates. No significant contamination with bacteria or fungi occurred. The antigens used were 'soluble antigens' as used for complement fixation.

Absorption experiments

The virus yield from the confluent growth of virus on the backs of twelve rabbits was pooled, partially purified by centrifugation and suspended in 10 ml. buffered distilled water. It was used to absorb small samples of antilive and antidead virus sera, either as live virus or after heating at 65° C. for 1 hr. The method used was as follows: To 1 ml. of unheated serum at 4° C. was added 0·5 ml. heated or live virus plus 0·05 ml. 9 % NaCl to make the mixture isotonic. The virus/serum mixture was allowed to react at 37° C. for 2 hr. and then at 4° C. overnight. It was clarified by centrifugation at 4500 r.p.m. in the bench centrifuge, and a further 0·5 ml. of virus suspension and 0·05 ml. of 9 % NaCl. added. It was allowed to react as before, followed by clarification and a final third absorption. After the final absorption the mixture was centrifuged at 20,000 r.p.m. for 1 hr. in the SW 39 rotor of the Spinco model L centrifuge, the supernatant was passed through Millipore membrane filters with an average pore diameter of 450 mμ and the filtrate tested, where appropriate, for residual live virus. None was found. These procedures resulted in dilution of the original serum 1:2·5, and this was included in calculating dilutions in subsequent tests.

IMMUNIZATION

Preparation of vaccines

Freshly prepared virus was always used, either uninactivated or inactivated by heat. Virus for inactivation was sealed in glass ampoules in approx. 5 ml. quantities, and heated by total immersion in a water-bath at 65·5 $\pm$ 0·5° C. for 1 hr. The virus was then removed and aggregates broken up by brisk pipetting with a pasteur pipette. The heated virus was used as an aqueous suspension for immunization and was stored at 4° C. during use. No adjuvants were used.

Testing of heated virus vaccines

Before use, each batch of vaccine was tested for residual live virus. The following tests were carried out:

(*a*) Quantities of 0·1 ml., containing the equivalent of 2×10^7 pfu, were inoculated undiluted on the CAM of five or six eggs. After 2 days the membranes were harvested aseptically, extracts were made and passed in further eggs. No pocks were ever seen on either first or second pass. Therefore, no evidence either of virus survival or of multiplicity reactivation was obtained.

(*b*) Small quantities of live virus, so as to give < 20 pocks per membrane, were mixed with the inactivated virus, and 0·1 ml. of the mixture was inoculated on the CAM of six to ten eggs. The expected number of pocks was always obtained showing that any live virus present was capable of expressing itself in the presence of large quantities of inactivated virus, and that the presence of some live virus did not reactivate detectable amounts of the heated virus.

(*c*) Serial tenfold dilutions of the vaccine were inoculated intradermally into the shaved backs of rabbits. Neither erythema nor oedema was seen. Further passage was not undertaken.

In addition to the above direct evidence, indirect evidence of complete inactivation was given by the qualitatively different response obtained in rabbits immunized with heated virus compared with those immunized with live virus.

Immunization schedules

Just before inactivation, preparations of virus were titrated in eggs, and estimates of the quantity of virus given in immunization are based on the pre-inactivation titre.

Groups of rabbits were divided into two subgroups. One subgroup was given heated virus and the other live virus. In all, thirteen rabbits were immunized with heated virus.

Heated virus. Initially, six intradermal and two intramuscular injections were given, containing the total equivalent of 10^9 pfu.

Three weeks later, a course of six intravenous injections was given, each containing the equivalent of 10^8 pfu in 0·5 ml. volumes. The injections were given twice weekly for 3 weeks, and the animals were bled from the marginal ear vein 1 week after the last injection.

Live virus. A similar schedule was followed except that a total of 300 pfu was given intradermally and no intramuscular injections were used. Three weeks later the same schedule was followed for intravenous injection and the rabbits were bled 1 week after the last injection.

Before immunization a sample of blood was taken from all rabbits, and the serum tested for pre-existing antibody by neutralization tests. None was found.

RESULTS

Antibody response

Following immunization with heated virus all the rabbits developed neutralizing, complement fixing (CF), haemagglutination inhibiting (HI) and precipitating antibodies. The titres of CF and HI antibodies were consistently lower than those in comparable antilive-virus sera. Typical examples are shown in Table 1 (complement fixation) and Table 2 (haemagglutination inhibition).

Vaccinial haemagglutinin has been shown to be separate from the virion, and is produced as a by-product of virus multiplication (Chu, 1948*a*). The inoculation of HA-free virus into rabbits results in the development of HI antibody (Chu, 1948*b*), and the failure to develop this antibody has been taken as evidence of complete inactivation of a vaccine virus (Kaplan, 1962). Therefore it was necessary to test the heated virus antigen used in immunization for the presence of haemagglutinin. The preparation tested caused detectable haemagglutination at 1/32, and the equivalent live virus haemagglutination titre was > 1/64. The procedures used to clarify the virus did not, therefore, remove all the haemagglutinin and, since it was stable at 65° C., HI antibody could have been and was developed.

Neutralization tests reflected similar differences in the neutralization of vaccinia virus (Table 3), but were less marked in neutralization of cowpox virus (Table 4). In all rabbits, however, a high titre ($> 10^4$) of antibody was found against both viruses. The un-neutralized fraction and the slope of the neutralization curves were similar to those found with sera made against live virus. The neutralization of smallpox virus by both types of sera was similar to that of vaccinia, though the titres were about one-tenth.

Gel diffusion tests in agar, however, showed clear qualitative differences between the two groups of sera. Plate 1, fig. 1, shows the comparison of four sera made against heated virus, nos. 108, 109, 110 and 111, with two sera made against live virus (72c and 130). Plate 1, fig. 2, shows the same sera compared with another antilive virus serum no. 55. It can be seen that in all cases the antidead virus sera give a single broad line which shows complete identity with one given by antilive virus sera. In addition, 72c and 130 show two other lines; one which is not given by any of the antidead virus sera and one which may be present in small quantity in 108 but not in the others. This is also apparent with serum 55, except that it shows two lines clearly not given by any of the antidead virus sera.

When a cowpox soluble antigen is used, the pattern shown in Pl. 1, fig. 3, is found. One (serum 72c) or two (serum 130) lines are given by the antilive virus sera, but no lines by the antidead virus sera. These sera are capable of neutralizing cowpox and the 'neutralizing' system would therefore appear not to be a precipitating one. However, Rondle & Dumbell (1962) showed that not all precipitating cowpox antigens are present in untreated soluble antigen. They reported a line pattern component '*f*' in vaccinia gel-diffusion patterns which could only be demonstrated in cowpox antigen following trypsin treatment. It is possible that trypsin might have released an antigen which reacted with the antidead virus sera and which might have been identified with '*f*'.

Table 1. *Complement-fixation test*

Serum no.	Made against	Antigen*	Dilutions	Serum dilutions 1/40	1/80	1/160	1/320	1/640
55	Live virus	L	1/40	+ + + +†	+ + + +	+ + + +	+ + +	+
			1/80	+ + + +	+ + + +	+ + +	+ + +	+
		H	1/40	+ + + +	+ + + ±	+ + + +	+	±
			1/80	+ + + +	+ + + +	+ + +	+	±
	Serum controls		.	−	−	.	.	.
65	Heated virus	L	1/40	+ + + +	+ + + ±	+ +	−	−
			1/80	+ + + +	+ + + +	+	−	−
		H	1/40	+ + + +	+ + +	+ +	±	−
			1/80	+ + + +	+ + +	+	−	−
	Serum controls		.	−	−	.	.	.

* Antigens: L, soluble antigen, supernatant of first high speed centrifugation in virus purification. H, soluble antigen, heated 65° C. for 1 hr.
† Results: + + + +, complete fixation; + + +, 75% fixation; + +, 50% fixation; +, 25% fixation; ± trace of fixation; −, no fixation, complete lysis.

Table 2. *Haemagglutination inhibition test*

Serum no.	Made against	Reciprocal serum dilutions 40	80	160	320	640	1,280	2,560	5,120	10,240	20,480
55	Live virus	−	−	−	−	−	−	−	±	+ +	+ + +
65	Heated virus	−	−	−	−	−	±	+ +	ND	ND	ND
25	Unimmunized control	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	ND	ND	ND

Antigen controls Undiluted	Dilutions 1/2	1/4	1/8	1/16
+ + +	+	±	−	−

+ + +, complete haemagglutination; + +, approx. 50% haemagglutination; +, approx. 25% haemagglutination; ±, trace of haemagglutination; ND, not done.

A third group of rabbits was immunized partly to confirm the previous results, but also to obtain some information about the rise of antibody levels with successive doses. Before each intravenous injection a sample of blood was taken, with a final sample 1 week after the last injection. The antibody levels achieved were similar to those in previous groups, and a single line only was given in gel diffusion. Control rabbits receiving live virus developed almost maximum titres of neutral-

Table 3. *Vaccinia neutralizing antibody titres*

Antilive virus		Antidead virus	
Serum no.	Titre*	Serum no.	Titres*
112	4·5	108	4·8
114	4·9	109	4·7
115	4·7	110	4·4
117	4·8	111	4·3
128	5·7	113	4·6
129	4·8	119	4·3
130	4·8	131	4·8
Mean	4·9	Mean	4·6

* Log_{10} reciprocal of 50 % end-point titre.

Table 4. *Cowpox neutralizing antibody titres*

Antilive virus		Antidead virus	
Serum no.	Titres*	Serum no.	Titres*
112	4·1	108	4·8
114	4·5	109	4·5
115	3·9	110	4·1
117	4·0	111	4·4
128	4·5	113	4·4
129	4·2	119	3·9
130	4·5	131	3·7
Mean	4·2	Mean	4·2

* Log_{10} reciprocal of 50 % end-point titre.

izing and haemagglutination inhibiting antibodies after the intradermal injections alone. Precipitating antibodies in gel diffusion, however, did not reach a maximum number of lines until after two intravenous injections. In contrast, development of antibody to heated virus was more gradual with significant levels of neutralizing antibody appearing only after the second intravenous injection. The one precipitation line also appeared at the same time. Haemagglutination inhibiting antibody appeared more gradually, rising to a maximum only after five or six intravenous injections. Representative results from one rabbit in each vaccine group are shown in Fig. 4. It should be noted that all antibodies had reached a plateau of response by the time the last injection had been given.

The absorbed sera were tested for residual antibody activity. Absorption of the serum made against heated virus, using either heated or live virus, removed all detectable antibody activity, and, in gel diffusion, the preparation containing live virus as the absorbant showed *antigen* activity. In contrast, the serum made against live virus and absorbed with heated virus showed only slight diminution of activity

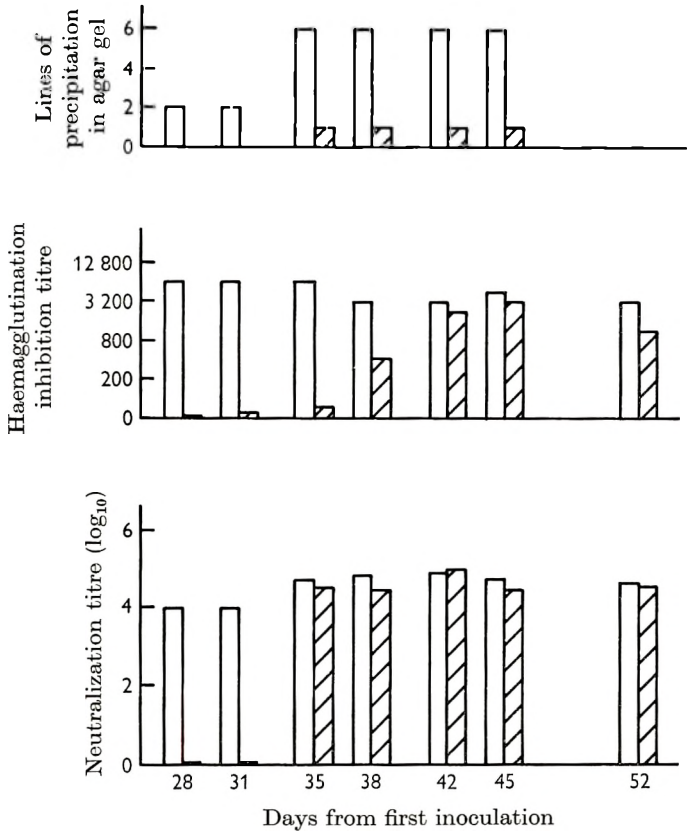

Fig. 4. Development of neutralizing, haemagglutinating and gel diffusing antibodies in two rabbits immunized with heated and live virus respectively. Rabbit 141 (□) was immunized with live virus and rabbit 144 ▨ with heated virus. Each histogram represents the titre of the antibody in a sample taken immediately before an intravenous injection of virus, the seventh sample (day 52) being taken one week after the last injection. See 'Immunization' in the text for details of injection schedules.

in neutralization, complement-fixation or virus-agglutination tests when unheated virus or viral antigen was used. In complement-fixation tests using heated antigen, only trace activity was detected and the absorbed serum failed to agglutinate heated virus at the lowest dilution tested (1 in 20). A summary of the results is given in Table 5. The neutralization results were confirmed using a different pair of sera and absorbing as before.

Table 5. *Summary of serological tests using absorbed sera*

Test	Serum no.... / Made against... / Absorbed with*...	55 Live virus Nil	55 Live virus L	55 Live virus H	65 Heated virus Nil	65 Heated virus L	65 Heated virus H
Complement fixation	Live antigen	1/480	1/120†	1/240	1/160	< 1/40	< 1/40
	Heated antigen	1/240	1/60†	< 1/40	1/160	< 1/40	< 1/40
Virus agglutination	Live virus	1/160	< 1/20	1/80	1/80	< 1/20	< 1/20
	Heated virus	1/160	< 1/20	< 1/20	1/80	< 1/20	< 1/20
Neutralization‡	Vaccinia	4·5	< 1·0	4·0	4·3	< 1·0	< 1·0
	Cowpox	3·6	< 1·5	< 1·5	3·9	< 1·5	< 1·5
	Variola	4·2	< 1·5	3·7	4·1	< 1·5	< 1·5

Test	Serum no.... / Made against... / Absorbed with*...	54 Live virus Nil	54 Live virus L	54 Live virus H	64 Heated virus Nil	64 Heated virus L	64 Heated virus H
Complement fixation	Live antigen / Heated antigen	ND	ND	ND	ND	ND	ND
Virus agglutination	Live virus / Heated virus	ND	ND	ND	ND	ND	ND
Neutralization‡	Vaccinia	4·7	1·5	4·6	3·9	< 1·5	< 2·0
	Cowpox	3·5	< 1·5	< 1·5	3·7	< 1·5	< 1·5
	Variola	4·6	< 1·5	4·2	4·2	< 1·5	< 1·5

L, absorbed with live vaccinia virus. H, absorbed with vaccinia virus heated to 65° C. for 1 hr.
† This serum was anticomplementary + + + + at 1/40.
‡ Log_{10} reciprocal of 50 % end-point titres.
ND, not done.

Challenge experiments

These were designed to test the immunity of the rabbits to challenge with live virus. The viruses used in some experiments were vaccinia and cowpox, both relatively non-lethal for the rabbit. In others rabbitpox virus, which is lethal for rabbits, was used.

Challenge with vaccinia and cowpox

The rabbits were challenged intradermally by graded $\log_{10}$ doses of live vaccinia virus into one shaved flank and cowpox virus into the other, the dose of virus used being confirmed in eggs. Equivalent doses of heated virus were given at the same time. The rabbits were examined on the fourth and seventh days after inoculation and the maximum size of any lesions appearing was recorded. Normal rabbits and rabbits immunized with live virus were included as controls.

Table 6. *Challenge with vaccinia of rabbits immunized with heated virus*

Dose in pfu...	1	10	10^2	10^3	10^4
Rabbit no.					
144	−	−	−	±	+ + N
145	−	−	±	+	+
	Controls				
(*a*) Normal rabbits					
156*	−	+	+ N	+	+ + N
158*	−	+ N	+ N	+ N	+ + N
(*b*) Immunized with live virus					
140	−	−	−	−	−
141	−	−	−	−	−

± Small papule.
\+ Lesion not more than 9 mm. in diameter.
\+ + Lesion not more than 19 mm. in diameter.
N Lesion showing necrosis.
* Secondary lesions present at 7 days.

The results of challenge of one group of rabbits with vaccinia are shown in Table 6. It can be seen that the immunity exhibited by rabbits 144 and 145 falls somewhere between the complete immunity, to the doses used, shown by the rabbits immunized with live virus and the susceptibility of the normal controls. Some lesions developed but only one rabbit (144) showed necrosis, and that only in one lesion. Necrosis was a common feature of the lesions in the normal controls. In addition, the normal controls showed secondary spread at 7 days and this was not seen in rabbits immunized with heated or live virus.

The lesions due to cowpox were very similar in extent and appearance though necrosis was more common, a feature of cowpox infection in the rabbit. The heated virus did not cause lesions on any rabbit, so that it is unlikely that hypersensitivity played a significant part in the development of the lesions due to live virus. Challenge of another group of rabbits on another occasion showed the same features.

Rechallenge with vaccinia and cowpox

These rabbits were re-challenged 1 week after complete healing of the first lesions, 35 days after the first challenge. Two fresh normal controls were included. The purpose of this rechallenge was to discover whether challenge with live virus had produced an improved immunity comparable to that produced by live virus alone. The results are shown in Table 7. Again, no lesions were produced at the site of injection of heated virus. Slightly smaller doses of virus were used in this challenge in an attempt to increase the sensitivity of the test.

Table 7. *Rechallenge with vaccinia of rabbits immunized with heated virus*

Dose in pfu...	10^{-1}	1	10	10^2	10^3
Rabbit no.					
144	–	–	–	+	++
145	–	–	–	±	+
		Controls			
(*a*) Normal rabbits					
150	–	–	++N	++N	++N
159	–	+	+	++	++N
(*b*) Control rabbits from first challenge					
156	–	–	–	–	±
158	–	–	–	–	–
(*c*) Immunized with live virus					
140	–	–	–	–	±
141	–	–	–	–	–

± Small papule.
\+ Lesion not more than 9 mm. in diameter.
++ Lesion not more than 19 min. in diameter.
N Lesion showing necrosis.

Comparison of Table 7 with Table 6 shows the following:

(*a*) The control rabbits of the first challenge (156 and 158) and the rabbits immunized with live virus show virtually complete immunity to the doses used.

(*b*) The new control rabbits show the same susceptibility as before.

(*c*) The rabbits immunized with heated virus show a similar susceptibility to rechallenge as they did to initial challenge, and had developed no increase in resistance. These results were confirmed in another group of rabbits.

Serum samples from these challenged rabbits were then examined by gel diffusion. Serum was obtained from the rabbits after immunization and before challenge (serum *a*), after challenge (serum *b*) and after rechallenge (serum *c*). At the same times sera were obtained from controls which were immunized but not challenged. The results of testing these sera are shown in Pl. 2, figs. 5–8. Rabbits 140 and 141 were immunized with live virus and were challenged twice, 144 and 145 were immunized with heated virus and were challenged twice. Rabbits 142 and 146 were immunized with live and heated virus respectively but were not challenged, and 156 and 158 were normal unimmunized rabbits which were challenged twice. The '*a*' sera from immunized rabbits gave the same appearance as found previously, the live virus sera showing 3 or more lines not present in the

heated virus sera. The post-challenge sera from the rabbits immunized with live virus showed no new lines, as expected. The same sera from rabbits immunized with heated virus showed the addition of a single faint line or zone after challenge. A comparison with vaccinia and cowpox soluble antigens suggested that this line was due to an antigen from the cowpox used in the challenge, rather than vaccinia. The complete vaccinia pattern was not developed—in contrast to the normal controls which developed 4 or 5 lines after the first challenge.

Challenge with rabbitpox virus

The purpose of this experiment was to examine whether the rabbits immunized with heated virus, though not immune to intradermal challenge, would yet possess enough immunity to protect them from a lethal virus. The lethal dose of

Table 8. *Challenge with rabbitpox*

Group	Immunized with	Lesions	Viraemia	Secondary spread	Temperature > 103° F.	Died
I	Heated virus	3/6*†	0/6	0/6	4/6	0/6
II	Live virus	0/6	0/6	0/6	0/6	0/6
III	None	6/6	6/6	6/6	6/6	5/6

* Figures in the table refer to the number of rabbits exhibiting the feature over the number in which it was sought.

† Maximum size of lesions were: rabbit 109, 58 mm.; rabbit 113, 15 mm. and rabbit 119, 8 mm. Doses received were: 109, 20 pfu; 113, 200 pfu; 119, 20 pfu.

vaccinia or cowpox viruses in a single intradermal injection is very large, and probably infinite, while that of rabbitpox is small, 0·1–1·0 pfu (Bedson & Duckworth, 1963), and it was felt that challenge with this virus would be a test of life-protecting immunity.

Three groups of six rabbits were used, six immunized with heated virus, six with live virus and six were unimmunized controls. It was decided to challenge the rabbits by intradermal injection rather than intranasal instillation because the dose can be more accurately controlled and infection can be observed more directly. Each group of six rabbits was divided into three subgroups of two, and each subgroup given a different dose of rabbitpox virus to discover whether the degree of immunity could be related to the dose of challenge virus. The doses used were 2, 20 and 200 pfu and were given as a single intradermal injection of 0·1 ml. into the shaved flank. The rabbits were observed daily for 2 weeks, and thereafter as necessary. Where lesions were observed, samples of blood were taken, prevented from coagulating with heparin, and tested for viraemia by inoculation undiluted into duplicate tube tissue cultures of RK 13 cells. The results are summarized in Table 8, in which the following can be seen:

1. No clear relationship between dose of virus and size of lesion was noted, though the only surviving control rabbit did receive the smallest dose.

2. No lesions developed in the rabbits immunized with live virus, though lesions were seen in three rabbits immunized with heated virus and one of them reached

more than 5 cm. in diameter, with marked necrosis. In none of the rabbits, however, was a viraemia detected on the ten occasions when it was sought.

3. In the unimmunized control rabbits, all developed lesions and five out of six died.

4. A viraemia was detected in all control rabbits and all of them developed secondary lesions. No secondaries were seen in either group of immunized rabbits.

5. Though the rabbits immunized with heated virus which developed lesions also developed a pyrexia, they were neither seriously ill nor anorexic.

DISCUSSION

The experiments reported in this paper were intended to establish the nature of the response of rabbits to inactivated virus, fulfilling the criteria of complete killing of the virus and administration of enough antigen to elicit maximum titres of antibody. They were not intended primarily as a pilot study of possible vaccines for human use, though this was the underlying purpose. In previously published work, rigorous proof of complete inactivation, including loss of the ability to be reactivated, has not been attempted. In the present work, no evidence of residual live virus in the heated vaccines was obtained, nor was there any evidence that the virus was capable of reactivation, either by live virus, in multiplicity reactivation or on passage. It was also shown that any live virus remaining would have been able to express itself amongst the dead. Heated virus has been shown to interfere with the replication of live virus but the ability to do so is lost in 30 min. at 60° C. (Galasso & Sharp, 1963). It might be expected that heating at 65° C. for 60 min. would also destroy the capacity to interfere. Further, since the response to heated virus was qualitatively different from that to live virus, it is unlikely that a significant quantity of live virus could have survived heating. It is probable then, that the responses were due only to the administration of heated virus, incapable of multiplication.

The experiments on dose requirements for maximum antibody production showed that enough antigen had been given to elicit a maximum response with the dosage schedule used. Neutralizing, haemagglutination-inhibiting and precipitating antibodies had reached a plateau of response, in which their titres were comparable to those produced by live virus. The antibody produced appeared to be similar to that produced by stimulation with live virus. Neutralization tests gave a similar pattern in terms of resistant fraction and the abruptness with which the end-point was reached. This is in contrast to the antibody response elicited by Appleyard (1961) using 'soluble antigen' in adjuvant, where the end-point was approached gradually with a progressive increase in the resistant fraction of unneutralized virus. In neutralization, complement-fixation, haemagglutination-inhibition and agglutination tests the antibody behaved in the same way as that to live virus. It was only in gel diffusion that qualitative differences were seen.

Here, the antidead virus sera gave only one line of precipitation with soluble antigen, three or four lines fewer than antilive virus sera. What these missing lines represent is not clear; they may be either antibodies to heat labile components of the virus or internal components not released with failure of the virus to replicate.

or they may be antibodies to by-products of virus growth, including enzyme systems. No information was obtained to distinguish between these hypotheses, but these missing antibodies do not appear to be the sole mediators of any of the serological activities measured in neutralization, complement-fixation, virus-agglutination or haemagglutination-inhibition tests. It is possible that the neutralization reaction does not precipitate and the results using cowpox antigen could support this. More information relevant to this point could be provided by using cowpox antigens treated with trypsin, etc.

The results of neutralization tests using absorbed sera showed that virus could be neutralized through heat-labile and heat-stable antigens. Sera made against live virus and absorbed with heated virus can contain no antibodies present in sera made against dead virus, particularly if all the antibody activity in antidead virus sera can be absorbed out with dead virus. Therefore, in the poxvirus system, there is no unique neutralizing antigen or antibody.

The precise mechanism of poxvirus neutralization has not yet been fully elucidated. Dales & Kajioka (1964), using the electron microscope, have shown that neutralized vaccinia is adsorbed to L cells and viropexis follows. Once within the cell the neutralized virus is gradually degraded without uncoating. They used live virus and antiserum made against it; whether antisera to heated virus would neutralize in the same way is not known, and this point might be further examined. However, these results tend to support the view that neutralization is due to antibody on the surface of the virion interfering with its replication in a relatively non-specific way.

Absorption of live virus antisera with heated virus did not reduce their ability to neutralize vaccinia but removed all detectable cowpox neutralizing antibody. This difference between the two viruses is interesting. It has been examined further and will be reported elsewhere.

The experiments on the dose required to produce a maximum response suggested that one or two more intravenous injections were used than was necessary. Nevertheless, without the use of adjuvants, multiple injections are needed in rabbits. These results throw some light on the varied responses noted by other workers who used a small number of injections. It is possible that other doses or other schedules might give the same result with fewer injections, and these require investigation. Also, the use of adjuvants would probably give a reduction in the number of injections required, though avoidance of mineral oil adjuvants when developing a vaccine for human use is probably desirable.

The results of challenge experiments showed a number of interesting features:

1. The immunity produced by heated virus was sufficient to save rabbits from death or serious illness from rabbitpox.

2. The immunity to intradermal challenge with vaccinia or cowpox was intermediate between normal rabbits and those immunized with live virus.

3. On rechallenge there had been little or no increase in immunity following the initial challenge.

Challenge by intradermal inoculation of a quantity of live virus into an avascular region is a severe test of immunity. Humoral antibody will reach the site with

comparative difficulty, and may not be present in significant quantity until the infection is established with the development of inflammation and oedema. In a disease like smallpox, where the virus is disseminated by a viraemia, circulating antibody will play a large part in resistance. The failure of any of the rabbits immunized with heated virus to develop secondary lesions, viraemia or generalized illness is an indication that such immunity can be life saving. Judged only by the skin reactions to challenge, the immunity produced by heated virus could be said to be unsatisfactory, in the sense that Amies (1961) and RamanaRao (1962) found their rabbits to be poorly resistant. Had they used a different challenge virus they might have found their rabbits to be more immune than they thought.

That the lesions produced by challenge were not due to allergy was shown by the concomitant use of heated virus as a second 'challenge'. No lesions were seen, in contrast to McNeill's (1966) results. He noted delayed-type hypersensitivity with his heated virus controls. Both McNeill (1966) and RamanaRao (1962) noted necrosis at the sites of challenge of live virus, the latter being able to distinguish by this means those rabbits which had been immunized with killed virus from those receiving live virus. In the present study the heated virus appeared to *protect* from necrosis, rather than promote it, when compared with the response of unimmunized rabbits. These conflicting results are not explicable at present, but the interval between immunization and challenge may be important. Fulginiti, Arthur, Perlman & Kempe (1966) found severe local reactions to live measles when administered four years after killed virus, but not when live virus was given as part of the immunization schedule.

Failure of the animals immunized with heated virus to develop further immunity after challenge with live virus is interesting. If development of a lesion represents virus multiplication, then new antigens must have been formed locally. The failure to develop further detectable antibody and immunity to challenge suggests that recognition of such fresh antigen by the rabbits' defences is not a peripheral one, and that circulating antibody prevented its being registered by more central mechanisms. This would certainly have to be borne in mind when considering human application.

To summarize the present series of experiments, rabbits immunized with heated virus developed a detectable immunity which was sufficient to protect them from the more severe and lethal effects of challenge, but only following a course of multiple injections that would probably not be acceptable for use in man. It might be possible to combine the injections with other immunizations, but this would require careful assessment. It is both of interest and a caveat that the immunity developed may prevent a complete response to challenge (i.e. vaccination), and no information has been obtained as to the duration of immunity. It is worth noting that, following three doses of inactivated measles vaccine, the disease can still be caught and may even be more severe (Rauh & Schmidt, 1965).

Much further animal work remains to be done before even a pilot study could be carried out in man, but enough information has been obtained to suggest that it might be possible to develop a heated vaccine without the disadvantages and hazards of virus multiplication.

SUMMARY

Rabbits were immunized by multiple intradermal injections followed by six intravenous doses of vaccinia virus inactivated by heating to 65° C. Particular attention was paid to confirming that the virus used was fully inactivated and incapable of reactivation. The immunized rabbits developed neutralizing and other antibodies to a titre comparable with those developed in response to live virus, but multiple intravenous injections were required to elicit a maximum titre. A qualitatively different response was seen only in immunodiffusion tests in agar gel where two or three fewer lines developed with antisera to heated virus than with those to live virus. The rabbits were subsequently challenged intradermally either with vaccinia and cowpox or with rabbitpox. They showed some immunity to vaccinia and cowpox, compared with normal controls, but less than that elicited by live virus. Their resistance to lethal doses of rabbitpox was life-saving though some rabbits did develop lesions. Later rechallenge of the rabbits showed that they had not developed further immunity, in distinction from the normal controls. The implications of these findings are discussed.

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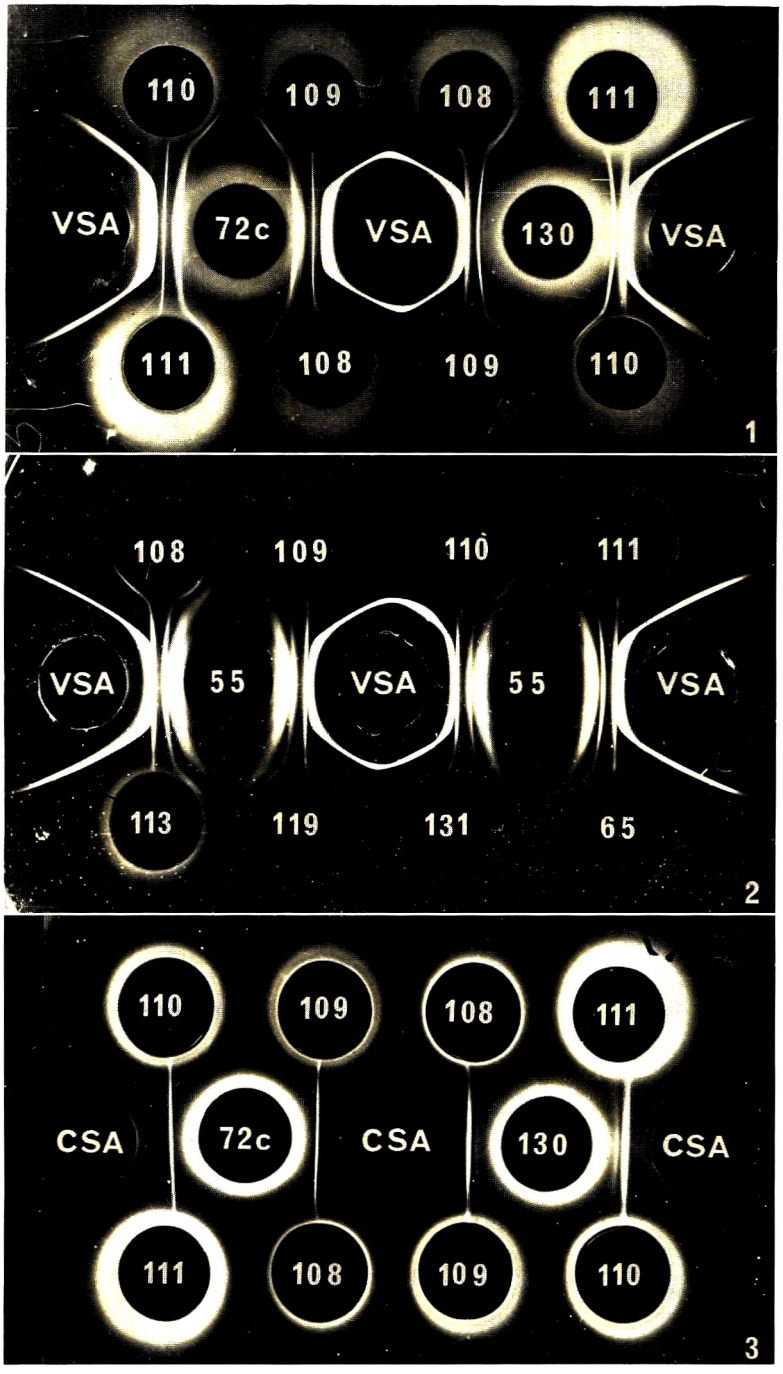
EXPLANATION OF PLATE

PLATE 1

Fig. 1. Line patterns given by antisera made against heated and live vaccinia virus. VSA, vaccinia soluble antigen. 72c and 130, sera made against live virus. 108–111, sera made against heated virus. The sera made against live virus give 1 or 2 more lines than those made against heated virus.

Fig. 2. Similar to Fig. 1. Serum 55 was made against live virus, and shows 2 or 3 more lines than those made against heated virus.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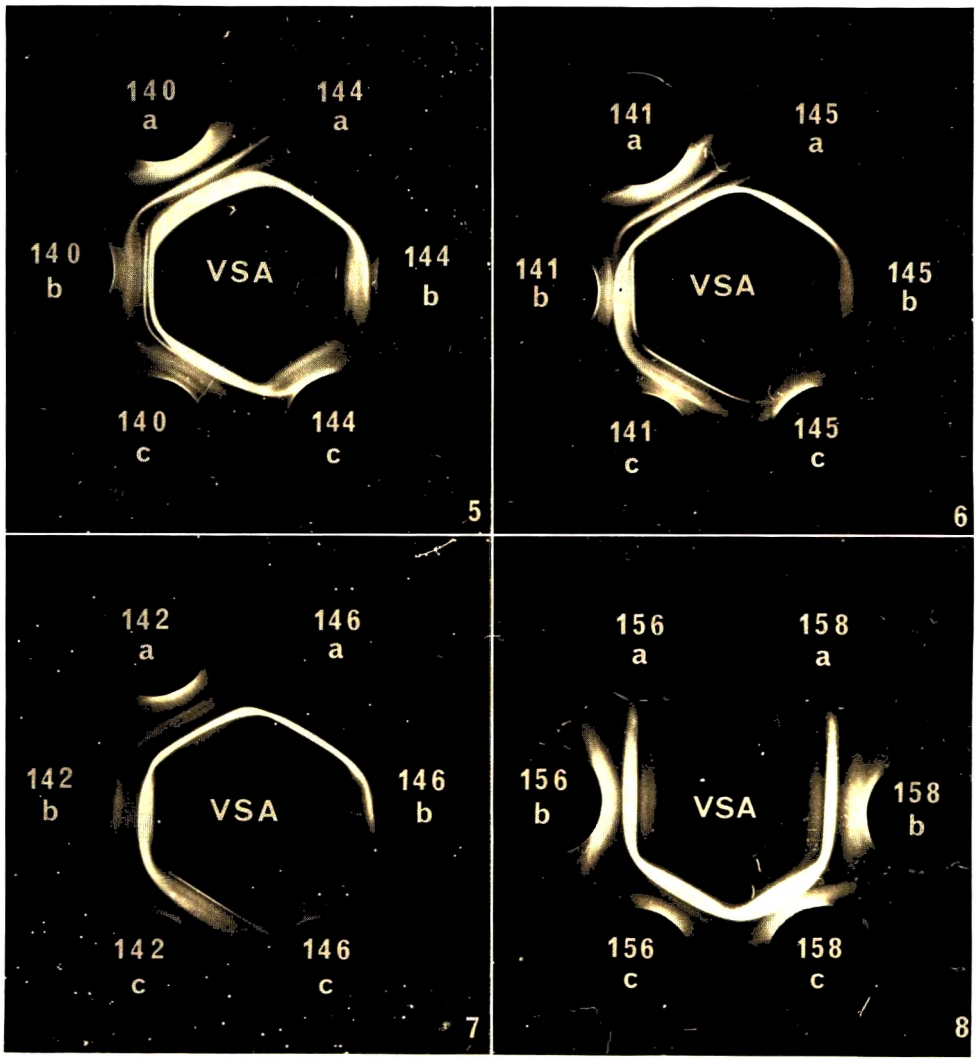
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142
b
VSA
146
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142
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146
c
7
156
a
158
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156
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VSA
158
b
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c
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8

Fig. 3. Line patterns given by antisera to heated and live virus with cowpox antigen. CSA, cowpox soluble antigen. Sera as in Fig. 1. The sera made against live virus show one or two lines of precipitate but no lines are given by the antiheated virus sera. This point is discussed further in the text.

PLATE 2

Figs. 5–8. Comparison of pre- and post-challenge antisera. The *a*, *b* and *c* sera are those taken following immunization, after first challenge and after rechallenge. Rabbits 140 and 141 were immunized with live virus, and challenged twice. Rabbits 144 and 145 were immunized with heated virus and also challenged twice. Rabbits 142 and 146 were immunized with live and heated virus respectively and were not challenged. Rabbits 156 and 158 were normal unimmunized rabbits which were challenged twice.

VSA, vaccinia soluble antigen.

Figs. 5 and 6 show that the rabbits immunized with live virus (140 and 141) did not develop fresh lines following challenge and their line systems were multiple. The rabbits immunized with heated virus developed a single line before challenge with vaccinia and cowpox and added a second faint line after challenge. This is discussed in the text. Fig. 8, the control rabbits (156 and 158) developed multiple line patterns after initial challenge.

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An Arizona serotype isolated from a case of gastro-enteritis in Britain

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Human infection with one of the Arizona group of organisms has not previously been reported in Britain, but Edwards, Kauffman & Stucki, (1960) have reported one case in Europe, of gastro-enteritis in a man recently returned from holiday in the Balearic Islands. The organism was a new Arizona serotype, biochemically atypical.

In 1962 Harvey & Price reported the presence of Arizona strains in Indian crushed bone. More recently Harvey, Price & Dixon (1966) gave an account of Arizona strains isolated from abattoirs in England and Wales, and Brookes & Fife (1966) reported twenty-two new Arizona serotypes isolated from coconut, bone-meal, dried egg and reptiles. Nevertheless, no members of the Arizona group were included in the recently published list of salmonellas isolated from human, animal or other sources and identified in Great Britain between 1951 and 1963 (Taylor *et al.* 1965) as none were isolated from human or animal, and only rarely from other sources.

It may therefore be of interest to record a recent case of gastro-enteritis in a child from whom an Arizona strain was isolated. A symptomless excretor was also found in the same family. The source of the infection was traced to pet terrapins (a type of small turtle belonging to the genus *Graptemys*) recently purchased by the family from a pet shop in Sheffield.

CASE REPORT

A 3-year-old girl, living with her parents and 5-year-old brother in Sheffield, developed gastro-enteritis on 27 May 1966. She had a fever and was quite ill, her symptoms continuing intermittently for the following 8 days. A specimen of faeces was sent to the Public Health Laboratory on the first of June. A provisional report of 'an organism has been isolated which is probably a salmonella' was made 2 days later. The patient was treated with a proprietary mixture of kaolin and neomycin and quickly responded to treatment. Food poisoning was not suspected as the

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cause of infection and she appeared to be an isolated case within the household. Neither the parents nor brother experienced gastro-enteritis, although on direct questioning the mother admitted that the brother felt sick at the time of his sister's illness.

After the organism had been provisionally identified as an Arizona, the family were asked whether they had any reptiles, tortoises or turtles as pets. They admitted that they had recently purchased two terrapins. It was ascertained that the daughter was fond of playing with them and frequently put them to her mouth. The terrapins were sent for examination to the Public Health Laboratory. Enquiries showed that these reptiles had been purchased from a Sheffield pet shop, but by the time a public health inspector visited the premises all the remaining terrapins from the particular batch (twelve in number) had been sold. This batch originated from Florida, U.S.A., and were imported via Liverpool docks, and a distributor in Knutsford, Cheshire. Other batches had been imported from Ceylon and Singapore. Terrapins do not breed in Britain because the climate is too cold. Water from the tank in which they had been kept was still available. This water together with a sample of dried flies upon which terrapins feed were examined. A week later two of the terrapins sold by the shop were traced and later examined.

LABORATORY INVESTIGATIONS AND FINDINGS

The faeces were cultured by direct inoculation on three solid media, MacConkey agar, brilliant green MacConkey and deoxycholate citrate agar. Three liquid media, tetrathionate broth, selenite F. and peptone water were also inoculated. After 18 hr. incubation at 37° C. the tetrathionate broth was subcultured on brilliant green MacConkey agar, and the selenite F. and peptone water on deoxycholate citrate agar. All the plates were incubated at 37° C. overnight.

Table 1. *Biochemical findings in the preliminary screen*

Test	Result
Fermentations of:	
Triple sugar*	—
Glucose	AG
Mannitol	AG
H_2S production	+
Indole production	−
Urease	−

* Triple sugar contains 0·5 % lactose, sucrose and salicin in peptone water. The other sugars were 1 % solutions in peptone water. AG = acid and gas production.

A few non-lactose fermenting colonies were isolated from the faeces of the daughter on the brilliant green MacConkey agar and deoxycholate citrate agar. The colonies were indistinguishable from salmonella colonies morphologically. A heavy, almost pure, growth of non-lactose fermenting colonies was obtained on each of the plates subcultured from liquid media.

The results from a small routine set of biochemical tests were consistent with the

biochemical reactions of a salmonella (Table 1). However, when further biochemical tests were performed the results were no longer typical (Table 2). Serologically the organism was not agglutinated by polyvalent salmonella 'O' antisera but was strongly agglutinated by polyvalent salmonella 'H' and by single factor salmonella 'H' c antisera.

The organism was sent to the Salmonella Reference Laboratory at Northallerton, where it was identified as a possible Arizona, and the identification was confirmed by the reference laboratory at Colindale. The organism was serotyped and had the following antigenic structure, Ar 26:32–21, the equivalent salmonella antigenic structure being S 61:c-z_{35}. Serum obtained from the daughter 5 weeks after the onset of her symptoms was titrated against Arizona O antigen 26 and was positive at a dilution of 1/16, but against Arizona H antigens 32 and 21 it was negative at 1/10 and upwards.

An Arizona of identical serotype was isolated from the water in which the terrapins belonging to the family were kept. No Arizona was isolated from the intestines of these terrapins at postmortem, but from one of them four salmonellas were isolated, *S. oranienburg* (6,7:m, t–), *S. saint paul* (1,4,5,12:e, h,–1,2), *S. bredeney* (1, 4, 5, 12, 28:1, v–1, 7) and *S. carrau* (6, 14, 24:y–1, 7). The last strain produced indole.

From one of two other terrapins obtained from the same pet shop an Arizona of identical serotype was also isolated. No isolations, Arizona or salmonella, were obtained from the tank water or dried fly food from the pet shop. No isolations were made either from the girl's mother or father, but an Arizona of similar serotype was isolated from the faeces of her brother. Four weeks after the first isolation Arizona was still present in the faeces of both children. Later specimens, however, proved negative.

DISCUSSION

The Arizona group of organisms is closely related serologically to the salmonella group but is distinguishable by biochemical methods (Table 2). An occasional strain of salmonella may also fail to ferment dulcitol and a few liquify gelatin. The malonate and the O.N.P.G. tests, however, provide a firm differentiation between Arizona and salmonella strains. The first description of an Arizona was by Caldwell & Ryerson (1939), who isolated this strain from diseased chuckwallas, horned lizards and Gila monsters. This organism was designated *Salmonella* sp (Dar-es-Salaam type, var from Arizona) because it liquified gelatin, as does the true Dar-es-Salaam type. Later Kauffman (1941) studied a culture which, although it fermented lactose and liquified gelatin, he placed in the genus *Salmonella* because of the close relationship of its H antigens to those of known salmonella types. The organism was called *S. arizona*. Soon afterwards other serologically related organisms were isolated with similar biochemical properties and it was decided that they belonged to a group of enterobacteria not previously described. Edwards & West (1945) were the first to describe phase variation in a member of the Arizona group. Previously only monophasic members had been described. The first comprehensive study was by Edwards, West & Bruner (1947), who studied 382

cultures. Later Edwards, Fife & Ramsey (1959) published another comprehensive study with a review of published work to date. The final classification of the Arizona group is still undecided, but it seems probable that they will be placed on their own as a separate subgenus (subgenus III) within the genus *Salmonella*.

Table 2. *Comparison of the properties of a typical Salmonella and a typical Arizona with the organism isolated from the case*

	Salmonella	Arizona	Organism isolated from the case
Fermentations of:*			
Lactose	−	+ or ×	+2
Maltose	+	+	+
Mannitol	+	+	+
Sucrose	−	−	−
Dulcitol	+	−	−
Salicin	−	−	−
Gas from glucose	+	+	+
Indole production	−	−	−
Methyl red test	+	+	+
Voges-Proskauer test	−	−	−
Growth in Simmon's citrate	+	+	+
H_2S production	+	+	+
Gelatin liquefaction	−	(+)	+10
Phenylalanine deamination	−	−	−
Malonate	−	+	+
O.N.P.G.† test	−	+	+

+ = Prompt positive. +2 = positive on 2nd day. × = late and irregularly positive. (+) = delayed positive.

* 1% sugars in peptone water.

† O.N.P.G. = O nitrophenyl-β-D-galactopyranoside (Lapage & Jayaraman, 1964).

There have been many previous isolations of Arizona from human faeces from countries other than Britain (Seligmann, Saphra & Wassermann, 1944; Edwards, 1945; Ferris, Hertzberg & Atkinson, 1945; Buttiaux & Kesteloot, 1948; Murphy & Morris, 1950; Seligmann & Saphra, 1951; Edwards, McWhorter & Fife, 1956*a*, *b*). The patients in these series suffered from diarrhoea, vomiting and fever. Arizonas have also been isolated from other organs of the body, the blood (Seligman & Saphra, 1951), pus in a case of otitis media (Butt & Morris, 1952), liver abscess, arthritic joints and osteomyelitic bone (Krag & Shean, 1959). This organism can spread to the blood stream and from there to all parts of the body, producing clinical pictures indistinguishable from salmonella infection.

The majority of Arizonas isolated have been from snakes, lizards and other reptiles, poults and turkeys, eggs and spray-dried egg powder. There are several reports (from countries other than Britain) where human salmonella infections were traced to turtles and tortoises (Alin, 1956; Williams & Helsdon, 1965; Rosenstein, Russo & Hinchliffe, 1965; United States Department of Health, 1964; Reardon & Wilder, 1964; Fleming & Williams, 1964;, Ager 1963; United States Public Health Service, 1963; Barr & Williams, 1964). In Britain Boycott, Taylor & Douglas (1953) and Boycott (1962) have published reports of salmonellas

isolated from tortoises and turtles and found evidence of human infection from this source. Thomas (1957) described another outbreak in which the causative organism proved to be a paracolon bacillus intermediate between the *Salmonella* and Arizona groups. There has been only little mention of Arizona strains isolated from similar sources (Dimow, 1965*a*, *b*; Dimow & Rohde, 1965). Rosenstein *et al.* (1965) isolated an Arizona from the turtle water taken from the retail store during their investigation.

In the Report on the Health of the City of Liverpool (Semple, 1965) there is an interesting account of a family outbreak of *S. paratyphi B.* infection traced to a pet boxer bitch. She also infected her eight pups, some of which died. The bitch recently dug up the remains of a tortoise which had died 2 years previously. This was thought to be a possible source of the salmonella, but bacteriologically the remains proved negative. The family also had a bowl of terrapins, the water of which the bitch and her pups frequently drank. The terrapins were later found to be excreting *S. paratyphi B.* They were therefore supposed to have been infected by the bitch or her pups, but it is possible that it was the terrapins that infected the dogs.

In the recently published list of Sources of Salmonella 1951–63 (Taylor *et al.* 1965) 443 salmonella strains were isolated from tortoises in Britain. Among these strains there were 54 different serotypes, the commonest being *S. bleadon* (45 strains), *S. kottbus* (36 strains), *S. uphill* (29 strains) and *S. taunton* (27 strains). However, tortoises and turtles seem to be an uncommon source of the four salmonella serotypes reported in this paper. Neither *S. saint-paul*, *S. bredeney* nor *S. carrau* are present in the above list and only six strains of *S. oranienburg* were isolated from this source. As mentioned previously there were no isolations of Arizona from either human or animal sources.

CONCLUSIONS

Terrapins, like tortoises and turtles, are excretors of salmonellas and Arizonas. If the habit of keeping terrapins as pets increases then these small innocuous animals may well become an important source of human Salmonella infection. This is especially so in young children. Terrapins are more likely to act as a source of human infection than tortoises for the following reasons. Terrapins are smaller and therefore easier and more tempting for young children to handle. They are usually kept indoors and in a tank of water. It has been observed that children do play with them and are in the habit of transferring them in and out of the tank at frequent intervals. If the terrapins are excretors then the water in the tank will become infected, so increasing the possibility of spread to humans or to other pets.

Members of the Arizona group of organisms are well known causes of salmonella-like infections, both of gastro-enteritis and of a more generalized infection. An Arizona has not previously been isolated from a human source in Britain: but this may well be because it has not been recognized, as this one might not have been if lactose had been fermented earlier. Arizonas are less likely to be missed on media such as Wilson and Blair, which do not rely on lactose fermentation for preliminary

differentiation. Their colonies are identical with those of other salmonellas, after overnight incubation about 1 mm. in diameter with a black centre surrounded by a clear translucent edge. The medium surrounding the colony has a metallic sheen.

The importance of knowing all possible sources of infection must be stressed. Serotyping as a method of pinpointing the source of infection, as well as in the tracing of contacts, must also be stressed.

SUMMARY

Two isolations of Arizona from humans were made in Sheffield in 1966. The first was from a 3-year-old girl with a history of gastro-enteritis; the second was from her 5-year-old brother who was a symptomless excretor. Infection was traced to pet terrapins recently purchased from a local pet shop. The relative epidemiological significance of Arizona infection and the potential hazard of pet terrapins has been reviewed.

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The growth of a virulent strain of African swine fever virus in domestic pigs

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Many isolates of African swine fever virus (ASFV), especially those of relatively recent origin in reservoir species of wild Suidae, produce an extremely high mortality in domestic pigs (DeTray, 1963; Scott, 1965*a*). Others which have been maintained for prolonged periods in domestic swine populations or, more particularly, in cell culture systems, have a reduced virulence but animals which survive infection often remain virus carriers (Botija & Jover, 1964; Scott, 1965*a*, *b*). The process of infection has not been studied quantitatively for either type of virus, however, and there are no published data on which to base an assessment of differences in their *in vivo* behaviour.

Similarly, little is known about the route of infection in ASF, although it is commonly assumed to be by nuzzling or ingestion, with primary invasion of the upper respiratory or alimentary tracts (Scott, 1965*a*, *c*). In addition, the selection of tissues from sick or dead animals for diagnosis by virus isolation should depend on a knowledge of the distribution of the virus at various stages of the disease but such information appears to be lacking.

For these reasons we have carried out a sequential and quantitative study of the development of ASF infection in pigs infected by the intranasal route with a strain of virus causing virtually 100% mortality. The results should serve as a basis for future comparative investigations with attenuated strains.

MATERIALS AND METHODS

Virus strain

The virus used was the Tengani strain, which caused an epizootic of ASF in Malawi in 1962 characterized by a very high mortality in domestic pigs and which was presumably derived from warthogs (Cox & Hess, 1962; Matson, 1960). The virus was stored at about $-70°$ C. in the form of portions of a pig spleen (EV/59) which were thawed as required and used to prepare 10% (w/v) suspensions in phosphate-buffered saline, pH 7·2 (PBS of Dulbecco & Vogt, 1954); the suspensions were clarified by low-speed centrifugation and titrated in pig bone marrow (PBM) cultures. Occasionally such suspensions were stored at 4° C. for up to 14 days before use; this procedure probably did not lead to any loss of infectivity, as no fall of titre was demonstrable after at least 8 weeks under these conditions.

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On subcutaneous inoculation in British pigs the Tengani strain of virus produced a significant rise of temperature after 2–7 days, depending on the dose of virus administered, and death followed invariably within the following 10 days.

Experimental animals

Large White pigs of about 45–55 lb. live weight were bought from commercial sources. They were housed in isolation units and their rectal temperature was recorded every morning; in the great majority of them temperatures higher than 104° F. could be regarded as definitely abnormal.

Intranasal infection of pigs

The animals were held in dorsal recumbency whilst 1 ml. of infected spleen suspension was dropped slowly into both nostrils. Part of the inoculum was undoubtedly blown out during expiration, part of it may have been swallowed but the absence of coughing indicated that fluid probably did not gain access to the trachea. Titration in pigs showed that the inoculum contained $10^{7\cdot2}$ ID 50 of ASFV.

Harvest of tissues

Two to five pigs were killed by captive-bolt pistol at intervals after inoculation which varied from 16 hr. to 7 days (Tables 1–4). They were exsanguinated as fully as possible by severing the neck vessels or by cardiac puncture with a wide-bore trocar. Blood for titration was collected into one-third the final volume of 1·5% ethylene-diamine-tetra-acetic acid:disodium salt (EDTA) in 0·7% NaCl. Two pigs, nos. FI/71 and FW/8, died of the disease but their bodies were still warm and the blood was unclotted at the time of collection of samples.

The skin was cleansed with running tap water and a range of tissues, as shown in Tables 3 and 4, was collected into small Petri dishes; separate sterile instruments were used for each tissue. Lymph nodes were carefully dissected out from the surrounding connective or adipose tissue, and the nomenclature used for those of the head and neck was that advocated by Saar & Getty (1964). Alimentary and respiratory mucosae were gently washed with copious quantities of tap water before excision; less cohesive mucosae, as in the intestines, were scraped off with a scalpel.

The 'oral' mucosa consisted of portions dissected from the folded region lying between the base of the tongue and the epiglottis. 'Nasal' mucosa was stripped from the middle and lower thirds of the dorsal turbinate bones. The 'retropharyngeal' mucosa was dissected from the dorsal wall of the pharynx lying immediately beneath the base of the skull and the ventral straight muscles of the head; this region has a median fold and the mucosa contains numerous lymphoid follicles. The mucosa of the trachea was obtained from its lower third, immediately above the bifurcation, and bronchial mucosa as a pool of scrapings from both main-stem bronchi. Caecal and colonic mucosae were always taken from the same regions, near the apex of the caecum and near the apex of the spinal coil of the colon.

Slices of kidney included both cortical and medullary tissue, whilst 'brain' consisted of parts of the grey and white matter, near the cruciate fissure of the cerebral

hemispheres. 'Bone marrow' for titration was prepared as a 10 % (10^{-1}) suspension of packed cells from a femur, processed as described by Plowright (1964).

Detection and titration of infectivity in tissues

After further washing with PBS where desirable to free them from blood (e.g. for the nasal mucosa), tissues were weighed in quantities varying from about 0·3 to 1·0 g., chopped with scissors or crossed scalpels and homogenised in Ten Broeck grinders to yield 10 % (w/v) suspensions in complete PBM culture medium, containing per ml. 300 units of penicillin, 100 units of neomycin and 150 units of nystatin. Suspensions were always prepared within about 3–4 hr. of removal of tissues from the pig.

Dilutions in a 10-fold series were prepared from these crude suspensions and a minimum of two dilutions were each inoculated in a dose of 0·2 ml. into five tubes of PBM cells. For detection of virus 10^{-1} and 10^{-2} dilutions of solid tissues were normally used, whereas blood was inoculated undiluted and at 10^{-1} dilution. Undiluted blood was removed after 4–18 hr. by washing 2–3 times with PBS in order to remove excess erythrocytes and thus allow haemadsorption to be seen; the other inocula were not removed at all. Although severe cytotoxic effects occasionally resulted with 10 % tissue suspensions or undiluted blood, enough cells were usually left attached to permit detection of minimal quantities of virus by haemadsorption.

For virus titrations four tenfold dilutions usually spanned the end-point satisfactorily but when the titre was unexpectedly high or low, samples were retitrated from 10 % suspensions kept at 4° C. for periods up to 2–3 weeks.

Fractionation of blood

Blood (40 or 80 ml.) with a final concentration of 0·5 % EDTA was centrifuged for 30 min. at about 2300 *g*. in a refrigerated machine and the supernatant was then removed and clarified by a second cycle of centrifugation under the same conditions; the upper part of the supernatant was regarded as 'plasma'. The leucocytes, mixed with red cells, were aspirated from the surface of the cellular layer of the first centrifugation and resuspended and deposited twice in 20–40 ml. Ca:Mg-free saline containing 0·02 % EDTA. The washed leucocytes were then deposited in tubes of 7 mm. internal diameter and the superficial, whitish or purple fractions removed for further washing in 40 ml. of 0·02 % EDTA. Finally, the 'leucocyte' concentrates were resuspended in one-tenth of the original volume of normal pig serum or complete culture medium to constitute the 'leucocyte fraction'.

'Erythrocyte' fractions were prepared by taking about 2 ml. of the lower part of the deposit from the primary centrifugation and washing 3–5 times in 20–40 ml. of EDTA saline. Finally, a 10 % (v/v) suspension was prepared in PBS or complete culture medium.

The packed cell volume in all original blood samples was determined by a standard method in haematocrit tubes.

Tissue cultures and infectivity end-points

Cultures were prepared from PBM cells washed out from fragments of red marrow derived from the long bones of Yorkshire-type pigs of about 25–35 lb. live weight. The cells were resuspended in medium to a concentration of 5·5–6·0 × 10^6/ml. and dispensed in 2 ml. quantities into tubes 150 × 16 mm. which were incubated for 2–4 days at 37° C. in stationary racks before inoculation. The medium used at first consisted of modified Eagle's basal medium (Macpherson & Stoker, 1962) with 10 % tryptose phosphate broth (Difco) and 25 % unheated normal pig serum; this mixture was later changed to a simpler one containing Earle's balanced salt solution with the same addition of serum. No change of medium was normally carried out during the life of the cultures but 0·2 ml. of a 1 % suspension of washed pig erythrocytes was added to each tube on the penultimate day of incubation after virus inoculation; this procedure was essential to obtain clearcut haemadsorption end-points.

Microscopic examinations for haemadsorption and cytopathic effects (Malmquist & Hay, 1960) were usually carried out on the 4th, 6th and 7th, 8th or 9th days after inoculation. End-points were recorded on the 7th to 9th days and titres per gramme of solid tissues or per ml. of blood were calculated by the method of Thompson (1947). The sensitivity of PBM cultures for the detection of the Tengani strain of ASFV was approximately the same as that of pigs.

RESULTS

Clinical reaction in pigs

In seventeen of eighteen pigs the incubation period, i.e. the time after intranasal inoculation when the rectal temperature first exceeded 104° F., was 3 or 4 days; in the remaining animal it was 5 days, the mean for the group being 3·8 days. With the Tengani strain of virus peak temperatures of 105·0 to 108·4° F. were usually attained 24–48 hr. later and death commonly occurred 2–6 days after the onset of pyrexia with a mean death time of 2·9 days in seventy-eight animals, infected by various routes with different doses of virus. At 24 hr. and later after infection it was always possible to recover virus from one or more tissues, i.e. infection never failed following intranasal instillation of virus.

The route of infection with ASFV

The upper respiratory tract

Tables 1 and 2 give details of all virus recoveries from thirteen pigs which were killed during the first 48 hr. after infection. In two cases, nos. FW/99 and FI/33 a trace of virus only was demonstrable in the nasal mucosa, whilst in a further ten animals no virus at all was detected in this tissue. This was remarkable in view of the very large inoculum (> $10^{7·0}$ ID 50) which had been employed and the high stability of ASFV at 37° C. (Plowright & Parker, 1967). In the remaining pig, no. FX/0, the titre of virus in the nasal mucosa was $10^{3·2}$ HAD 50/g, which almost certainly implied viral proliferation there before generalization had occurred, as indicated by the absence of virus in the blood, spleen and bone marrow

By 24–40 hr. virus was consistently present in the retropharyngeal mucosa, in increasing quantities which were undoubtedly associated with local proliferation. One of two pigs showed definite localization in this tissue by 16 hr., without detectable transfer to the local retropharyngeal lymph nodes. In three of four pigs killed at 24–40 hr., virus was already present in the retropharyngeal nodes but to a lower titre than in the mucosa; after 48–72 hr., however, the lymph node titre exceeded that in the mucosa (Tables 1–3, Fig. 1).

Table 1. *The distribution of ASFV in the tissues of pigs killed 16–24 hr. after intranasal infection*

	Time after infection					
	16 hr.		24 hr.			
Tissue	FW/98*	FW/99	FU/20	FU/87	FW/84	FW/85
Lymph nodes:						
Medial retropharyngeal	—	—	2·8	3·4	—	—
Lateral retropharyngeal	—	—	NT	—	3·4	—
Parotid	—	—	—	2·4	—	—
Right bronchial	—	—	—	1·6	—	—
Left bronchial	—	—	—	⩾ 4·2	—	—
Mucosae:						
Nasal	—	Tr†	—	—	—	—
Retropharyngeal	2·8	—	NT	NT	4·6	4·2
Tracheal	—	—	—	1·6	NT	—
Bronchial	—	—	—	5·8	—	—
Lung:						
Hilar	—	—	—	2·8	—	—
Diaphragmatic	—	—	—	4·2	—	—

* = pig. no.
† = Trace. Haemadsorption in one of five tubes inoculated with 10% (w/v) suspension.
NT = not tested.
The figures in each column are $\log_{10}$ HAD 50/g.

The lower respiratory tract

In two of thirteen pigs which were killed during the first 48 hr. after infection (nos. FU/87 and FI/33) there was highly suggestive evidence for a primary 'complex' of infection in the bronchial mucosa and left bronchial lymph node but the retropharyngeal mucosa was not, unfortunately, examined in either of these instances. In both of these animals, the highest recorded titre of virus was in the bronchial mucosa, with as much or more infectivity demonstrable in the left bronchial as in the medial retropharyngeal lymph node (Tables 1 and 2). Virus recovered from the 'lung' tissue of these animals could obviously have been due to the inevitable inclusion of bronchial or bronchiolar mucosae. In addition, generalisation had definitely occurred by 48 hr. in pig FI/33 since the spleen titre was already $10^{3·0}$ HAD 50/g. and virus was demonstrable in the blood; early generalization had also probably occurred in one other animal (no. FU/88) killed at 48 hr., as a trace of infectivity was found in the bone marrow but not the spleen (Table 2).

The alimentary tract

No virus was recovered from the 'oral' mucosa and tonsil of any of the first thirteen pigs or from the fundic mucosa, Peyer's patches and ileal mucosa of six of them. Hence, it can be concluded that the alimentary tract does not serve as a portal of entry for ASFV, at least following nasal instillation of virus.

Table 2. *The distribution of ASFV in the tissues of pigs killed 40–48 hr. following intranasal infection*

	Time after infection						
	40 hr.		48 hr.				
Tissue	FW/87*	FX/0	FH/55	FI/33	FU/21	FU/88	FW/86
Lymph nodes							
Medial retropharyngeal	3·2†	3·4	5·2	3·8	6·8	5·6	6·8
Lateral retropharyngeal	—	—	NT	—	—	4·4	—
Mandibular	—	—	3·0	1·8	—	2·8	—
Parotid	—	—	3·4	1·8	4·8	—	5·2
Right bronchial	—	—	—	—	—	—	—
Left bronchial	—	—	—	3·8	—	—	—
Mucosae							
Nasal	—	3·2	—	Tr‡	—	—	—
Retropharyngeal	4·6	5·0	NT	NT	NT	NT	5·0
Tracheal	—	—	—	2·0	—	—	—
Bronchial	—	—	—	4·2	—	—	—
Lung							
Hilar	—	—	—	—	—	—	—
Diaphragmatic	—	—	—	2·0	—	—	Tr
Spleen	—	—	—	3·0	—	—	—
Bone marrow	—	—	—	—	—	Tr	—
Blood	—	—	—	0·4	—	—	—

* = pig no.
† = $\log_{10}$ HAD 50/g. or per ml. (blood).
‡ = Trace. Haemadsorption in one of five tubes inoculated with 10% (w/v) suspension.
NT = not tested.

The distribution of ASFV in the blood of infected pigs

The fractionation procedures which were adopted produced plasma which was free from all cellular elements but failed to produce 'erythrocyte' fractions free of leucocytes, although the numbers of the latter were materially reduced and in one instance virtually eliminated (pig no. FW/18, Table 5). Similarly, the leucocyte fractions in three of four cases had nucleated counts which were 1·6 to 1·9 times greater than those of the original blood whilst erythrocyte counts were greatly reduced (8 to 55 times) (Table 5).

The results of virus titrations are given in Table 6. Virus free in the plasma had a very high titre varying from $10^{7·0}$ to $10^{7·4}$ per ml. and constituted 4–18% of the total infectivity. The titre of 'leucocyte' fractions was invariably lower than that of the original blood, by 0·8–1·4 log units, in spite of the greater concentration of

leucocytes. The total recovery of virus in 'erythrocyte' fractions was calculated from the titre of 10% red cell suspensions and the packed cell volume; it varied from 20 to 100% with a mean of 45·6%, the highest figure being recorded for pig no. FW/18 in which the leucocyte count had been reduced about 170 times.

The generalization of ASFV in pigs

General

Tables 3 and 4 give figures for the virus content of thirty-one tissues harvested from eighteen pigs which were killed 3–7 days after infection. The behaviour of

Table 3. *The distribution of ASFV in the tissues of pigs killed 72–96 hr. following intranasal inoculation*

	Time after infection								
	72 hr.				96 hr.				
Tissue	FI/61*	FI/68	FU/92	FW/2	FI/59	FI/62	FI/69	FU/93	FV/90
Lymph nodes									
Medial retropharyngeal	8·0†	8·0	9·0	8·2	6·2	8·0	8·6	8·4	9·0
Lateral retropharyngeal	—	5·2	—	3·0	5·2	7·2	7·0	—	8·8
Mandibular	6·0	6·8	7·2	7·2	6·6	5·4	5·8	7·0	9·2
Parotid	6·0	6·6	6·4	4·6	5·4	7·2	6·0	5·8	8·4
Right bronchial	3·8	3·0	—	4·0	6·0	5·6	6·8	3·6	7·2
Left bronchial	2·8	2·6	—	3·0	4·8	6·0	6·8	2·4	7·0
Dorsal sup. cervical	1·6	—	—	3·0	5·8	4·0	5·8	—	7·6
Prefemoral	—	NT	—	2·6	4·6	4·8	5·6	—	7·8
Gastro-hepatic	3·0	NT	—	3·4	5·2	5·8	6·0	—	7·2
Mesenteric	1·6	2·4	—	3·4	5·0	4·8	5·6	—	6·8
Caecal	3·0	3·0	—	2·8	5·2	5·8	6·2	—	6·2
Pharyngeal tonsil	5·4	5·4	—	6·6	5·0	6·2	6·0	—	7·2
Peyer's patches	2·6	NT	—	—	NT	5·2	5·2	—	6·0
Spleen	6·4	6·6	4·0	7·2	7·0	8·8	7·4	5·2	9·0
Bone marrow	4·8	4·2	1·8	4·2	6·4	7·0	6·6	4·6	8·8
Blood	5·6	4·2	—	5·4	5·8	7·6	6·2	2·0	7·0
Nasal mucosa	2·6	4·2	4·6	3·6	4·6	5·2	5·0	3·0	7·2
Retropharyngeal musoca	NT	NT	NT	7·2	NT	NT	NT	—	6·2
Tracheal mucosa	3·4	2·4	—	Tr‡	4·0	4·6	5·2	—	5·6
Bronchial mucosa	3·2	2·4	—	4·2	5·2	5·4	4·6	Tr	7·4
Hilar lung	5·4	3·4	1·6	5·6	6·0	7·2	8·4	2·4	8·4
Diaphragmatic lung	5·6	3·8	2·0	5·6	5·4	7·2	8·0	2·2	8·8
Oral mucosa	3·2	2·4	—	—	4·4	5·0	5·2	—	5·6
Fundic mucosa	2·2	2·4	—	3·2	NT	NT	NT	—	6·2
Ileal mucosa	—	—	—	—	4·8	5·0	5·0	—	6·0
Caecal mucosa	2·6	2·4	—	3·4	5·0	5·2	5·0	—	5·8
Colonic mucosa	2·6	—	—	2·6	4·8	5·6	5·6	—	6·2
Liver	4·8	5·0	—	5·4	3·8	6·2	7·0	2·6	8·6
Kidney	4·2	3·2	—	4·0	4·2	6·2	5·2	—	7·0
Myocardium	3·0	2·8	—	3·6	4·4	5·2	5·4	—	6·6
Brain	—	—	—	2·8	NT	4·4	4·0	—	6·2

* = pig no.
† = $\log_{10}$ HAD 50/g. or ml.
NT = not tested.
‡ = Trace. Haemadsorption in one of five tubes inoculated with 10% (w/v) suspension.

individual animals in a group was reasonably regular with three exceptions which will be discussed later; these were nos. FU/92, FU/93 and FW/7 and figures for the two last were excluded in the calculation of means used to prepare Figs. 1–6 inclusive. Dissemination and growth of virus in the pig will now be described with reference to tissue and organ systems, omitting the three animals mentioned above.

Table 4. *The distribution of ASFV in the tissues of pigs killed 5–7 days following intranasal infection*

	Time after infection								
	5 days				6 days			7 days	
Tissue	FI/70*	FI/73	FV/21	FW/3	FI/71	FW/7	FW/16	FW/8	FW/17
Lymph nodes									
Medial retropharyngeal	7·6†	7·6	8·8	8·4	8·8	8·6	9·2	8·4	8·8
Lateral retropharyngeal	7·6	8·2	9·0	8·6	8·2	8·2	9·0	9·2	9·0
Mandibular	6·4	8·0	9·2	8·2	8·2	8·2	8·6	9·0	9·2
Parotid	7·0	7·8	8·6	9·2	8·2	8·4	9·4	9·6	8·8
Right bronchial	7·5	7·8	8·6	8·4	8·2	4·8	8·4	9·4	9·0
Left bronchial	6·4	7·4	8·2	8·8	7·6	4·0	8·4	9·2	8·8
Dorsal sup. cervical	7·0	7·2	8·6	8·6	8·2	7·8	9·0	9·0	8·8
Prefemoral	6·8	7·4	8·2	9·0	8·2	4·8	8·8	9·2	8·8
Gastro-hepatic	6·4	6·4	8·8	9·2	8·2	4·8	8·4	9·0	9·2
Mesenteric	6·0	5·6	7·4	8·0	7·2	5·6	9·2	9·2	9·0
Caecal	7·2	6·8	7·6	8·0	8·2	4·8	8·0	9·0	8·6
Pharyngeal tonsil	6·6	6·0	7·6	8·2	8·0	4·2	8·2	9·0	9·0
Peyer's patches	4·0	5·0	6·2	6·4	NT	4·2	6·2	NT	7·6
Spleen	7·4	NT	8·6	9·0	8·4	8·0	8·8	9·2	9·0
Bone marrow	6·2	5·4	8·2	8·8	7·8	7·2	8·8	8·2	8·8
Blood	8·0	8·2	7·4	8·2	7·4	7·8	8·6	8·8	8·4
Nasal mucosa	6·2	NT	7·2	8·0	7·2	4·6	8·0	8·4	8·0
Retropharyngeal mucosa	NT	NT	5·6	6·8	NT	6·8	8·4	7·2	8·6
Tracheal mucosa	3·8	NT	5·8	6·2	6·0	2·6	6·4	8·2	6·0
Bronchial mucosa	4·4	NT	6·2	6·8	5·6	5·8	6·6	NT	6·6
Hilar lung	5·2	NT	8·8	8·0	8·4	5·8	8·4	8·8	8·4
Diaphragmatic lung	5·9	NT	8·2	7·8	8·2	6·0	8·6	8·6	8·8
Oral mucosa	3·2	NT	5·8	6·0	5·0	3·6	5·8	7·4	6·3
Fundic mucosa	4·6	NT	5·8	6·4	5·6	4·4	5·8	7·2	7·0
Ileal mucosa	2·7	NT	6·4	6·2	6·6	4·4	6·0	NT	7·8
Caecal mucosa	3·7	NT	6·2	5·8	5·2	4·4	6·4	NT	7·8
Colonic mucosa	5·2	NT	6·2	5·4	6·4	4·4	6·6	NT	7·8
Liver	7·2	NT	8·2	8·0	8·0	4·4	8·4	8·8	8·4
Kidney	5·0	NT	6·8	7·6	7·2	5·0	6·6	7·4	8·0
Myocardium	4·6	NT	6·2	7·0	6·2	5·2	6·8	6·6	6·0
Brain	3·6	NT	6·2	6·8	5·6	4·6	6·0	6·8	6·6

* = pig no. † = $\log_{10}$ HAD 50/g. or ml. NT = not tested.

Lymphopoietic tissues

Following on the early localization of virus in the retropharyngeal nodes, especially the medials, it was found that maximal infectivity titres ($10^{8·0}$–$10^{9·0}$ HAD 50/g.) were present in these structures at 72 hr. (Table 3) and that the mean titre had reached a plateau by that time which was maintained to death, with

possibly no further significant increase (Fig. 1). In two other cephalic nodes, the mandibular and parotid, the virus was also well established at 72 hr., but mean titres continued to rise a further 2 $\log_{10}$ units by the end of the experiment; during the period 48–72 hr. after infection there was much more virus in these nodes than in the blood (Fig. 2). The lateral retropharyngeal nodes were not consistently infected until the 4th day (Table 3) but the majority of the bronchials, alimentary

Table 5. *Fractionation of the blood of infected pigs*

		Original blood		Erythrocyte fraction		Leucocyte fraction	
Pig no.	Day of disease	RBC count*	WBC count†	RBC count	WBC count	RBC count	WBC count
FW/16	3	ND	33·3	ND	2·0	ND	52·0
FW/18	2	5·0	18·0	ND	0·03	0·09	33·9
P 91	4	4·2	24·6	0·8	1·1	0·5	47·5
P 100	3	2·6	19·0	1·4	1·0	0·1	21·8

* = $\times 10^6$/mm.^{3} † = $\times 10^6$/ml. ND = not done.

Table 6. *The distribution of ASFV in the blood of infected pigs*

		Infectivity in:*			
Pig no.	Day of disease	Whole blood	Plasma	Erythrocyte fraction	Leucocyte† fraction
FW/16	3	8·6	7·4 (4 %)	7·6 (37 %)	7·4
FW/18	2	7·8	ND	7·4 (100 %)	6·4
P 91	4	8·0	7·4 (18 %)	6·8 (20 %)	7·0
P 100	3	7·8	7·0 (12 %)	6·8 (26 %)	7·0
Mean recovery (%)			11·3	45·6	

* = $\log_{10}$ HAD 50/ml. Figures % in brackets refer to total estimated recovery in fraction.
† = total virus recovery not calculated owing to poor recovery of leucocytes.
ND = not determined.

and superficial body nodes already showed small quantities of virus at 72 hr. The mean titres of the prefemoral and dorsal superficial cervical (prescapular) nodes are shown in Fig. 2; they continued to rise until the 7th day when they eventually exceeded that of the blood.

Virus growth was always well established in the spleen at 72 hr., the mean titre reaching a plateau by the 4th day, with only a slow rise subsequently; throughout the experiment there was more virus in the spleen than in the blood (Fig. 3). With the exception of pig FU/92 virus had begun to proliferate in the pharyngeal tonsil at 72 hr. but titres were generally lower at first than those in the cephalic nodes (Table 3). Peyer's patches did not contain a considerable quantity of virus until the 4th day and titres were lower than those in the blood, following very closely those in the intervening ileal mucosa (Fig. 5).

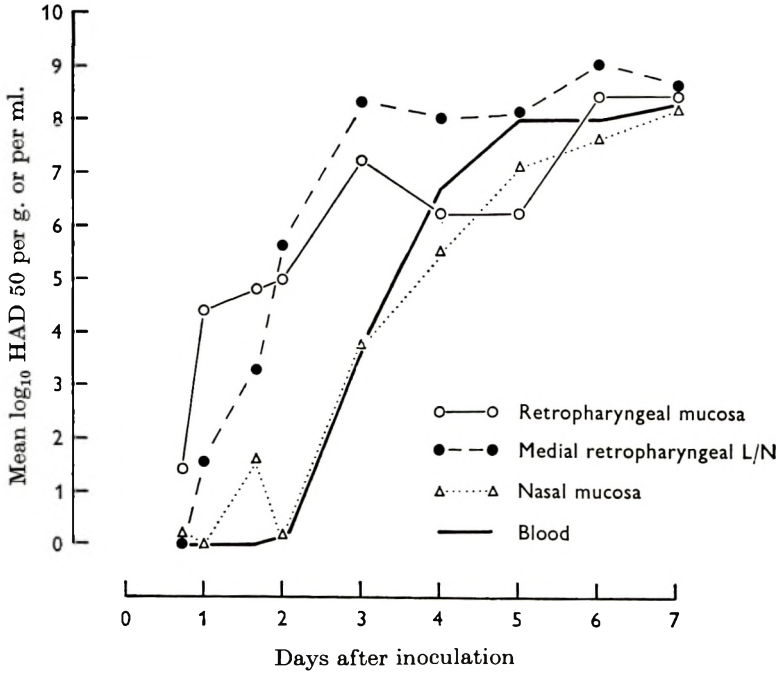

Fig. 1. Upper respiratory tract.

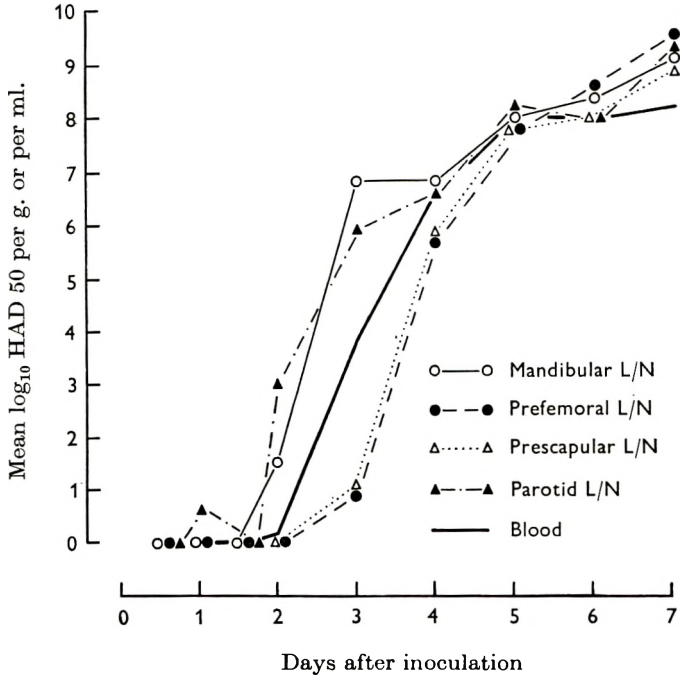

Fig. 2. Lymphoid tissues.

Blood and haemopoietic tissues

Large quantities of circulating virus were present at 72 hr. and the mean titre continued to rise to a plateau commencing on the 5th day: from this point onwards mean titres varied between $10^{8\cdot0}$ and $10^{8\cdot2}$ HAD 50/ml. It was evident from these figures that the virus content of many tissues after generalization could be largely influenced by their blood content and hence the mean blood titre was used as a 'reference' line in Figs. 1–6.

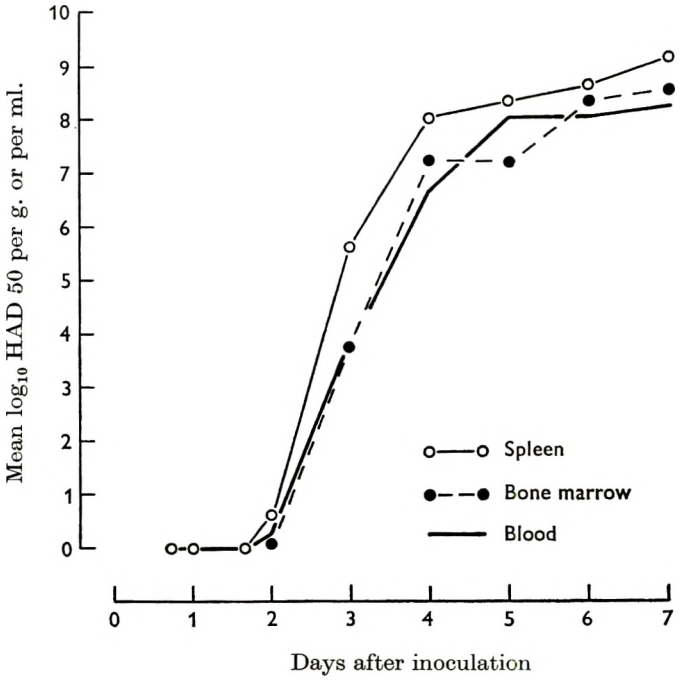

Fig. 3. Haemopoietic tissues.

The bone marrow was consistently infected from 72 hr. onwards but in only two of eighteen animals was the titre significantly greater than that in the blood; these were pigs FV/20 and FU/93 (Table 3); in two other cases (FI/70 and FI/73) the bone marrow titre was 1·8 and 2·8 $\log_{10}$ units lower than that of the blood. The mean lines for virus in the blood and bone marrow were approximately the same throughout the infection (Fig. 3).

Respiratory tract

The titre of virus in the retropharyngeal mucosa was exceeded by that in the associated retropharyngeal nodes from 48 hr. onwards though virus presumably continued to multiply in the former (Fig. 1). The bronchial and tracheal mucosa, after also probably being involved in early viral penetration in a minority of animals, never subsequently assumed an important role in supporting virus growth (Fig. 4). The titre of virus in the nasal and retropharyngeal mucosae was usually high from the 5th day onwards (Tables 3 and 4) and this, together with any contri-

butions from the lower respiratory tract, could presumably result in a high rate of nasal excretion.

The lungs contained virus consistently on the 3rd day and titres considerably in excess of those in the blood were seen in 2/5 pigs on the 4th day (nos. FI/69 and FU/20; Table 3) but not thereafter; the mean line followed very closely that of the blood, being about 2 log units higher than those of the tracheal and bronchial mucosae and continuing to increase until death or destruction on the 7th day.

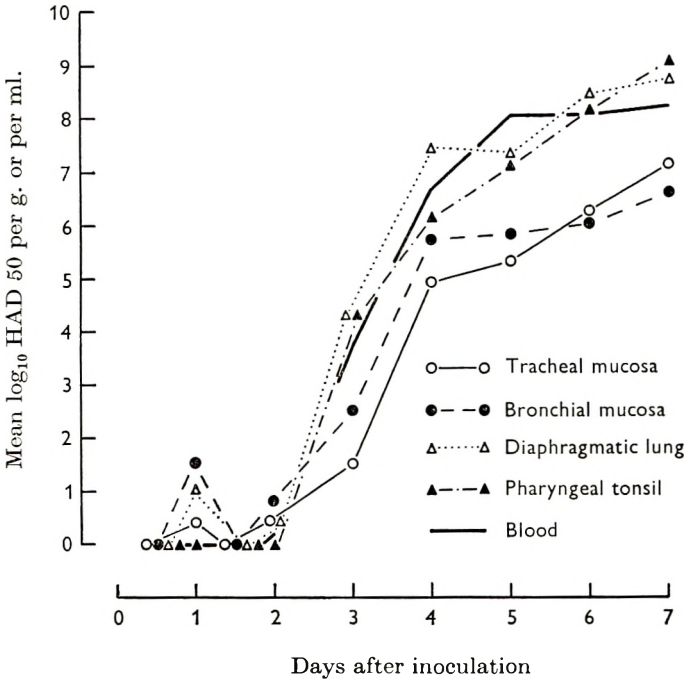

Fig. 4. Respiratory tract.

It was, incidentally, of interest to observe the close agreement between titrations of 'diaphragmatic' and 'hilar' lung; differences were never greater than 0·7 log units, the mean difference for eighteen pairs of observations being 0·20 log units (Tables 3 and 4). Assuming that there was no real difference between the viral content of tissue from these two locations, the reproducibility gives an indication of the accuracy within cell batches of the titration technique.

Alimentary tract

Virus had generally localized in the oral, fundic, caecal and colonic mucosae by the 3rd day but it was not present in the ileum until the 4th day. Virus titres in all these tissues were of approximately the same magnitude in individual pigs, with the possible exception of no. FI/70 (Table 4). The mean titres ran almost parallel, increasing rapidly to the 4th day and thereafter more slowly to death. There was always much more virus in the blood than in the alimentary tract, the difference often being of the order of 1 to 2 or more $\log_{10}$ units in individual pigs, as well as in the mean (Tables 3 and 4; Fig. 5).

Parenchymatous organs and central nervous system

On the 3rd day the liver usually contained much virus, which subsequently increased in an almost identical manner to that in the blood; this was true both of individual pigs and of the mean line (Tables 3 and 4; Fig. 6). Exceptionally, however, pigs such as no. FV/20 (Table 3) did show more virus in the liver than in the blood; it can probably be concluded that the liver, like the bone marrow, is a site of virus production particularly when its behaviour is compared with that of other highly vascular tissues such as kidney, myocardium and brain.

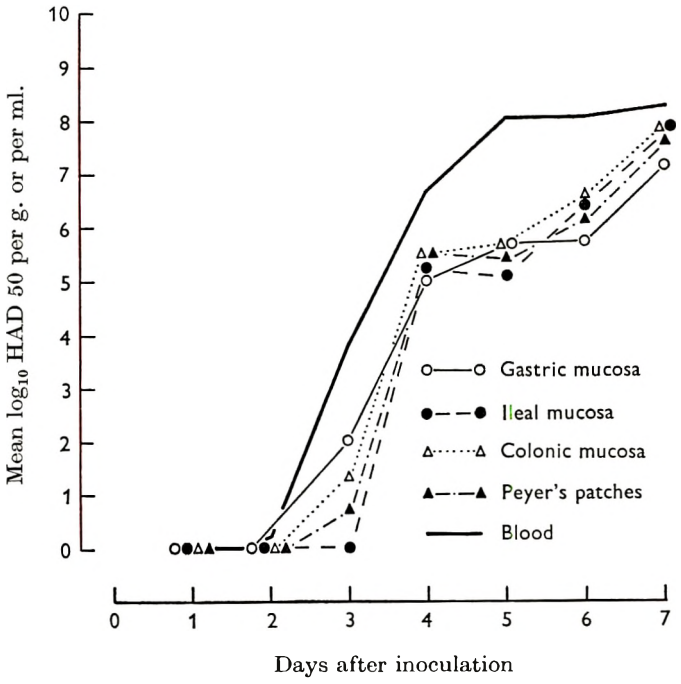

Fig. 5. Alimentary tract.

Vitrus titres in the kidney were almost invariably lower than those encountered in the liver but often exceeded those in the myocardium, which in turn usually contained more virus per gramme than did the brain (Fig. 6). There was no evidence of viral proliferation in the kidney, myocardium or brain, if titres in these tissues were compared with the degree of viraemia.

Irregular behaviour of a minority of pigs

Of eighteen pigs for which details are given in Tables 3 and 4, three apparently behaved in an irregular manner in that generalization was delayed, as in nos. FU/92 and FU/93 killed on the 3rd and 4th days, or that virus multiplication in many tissues was suppressed, as in pig FW/7.

The first two animals were infected at the same time, with the same inoculum, and neither had shown a temperature reaction by the time of sampling. Both had a severe portal cirrhosis, probably induced by Ascaris infection, but they were not

clinically abnormal. It was possible that the delay in generalization could have been attributed to a smaller effective inoculum but this explanation was contra-indicated by the very high titres of virus ($10^{7\cdot0}$–$10^{9\cdot0}$ HAD 50/g.) found in the retropharyngeal and mandibular nodes; these titres were comparable to those found in other pigs killed at 72 or 96 hr. and considerably in excess of those determined in animals killed at 48 hr. The cultures used for the titration of all tissues from 'aberrant' pigs were of very high sensitivity, as shown by their simultaneous use for materials from other pigs, giving titres in excess of $10^{8\cdot0}$ HAD 50/g.

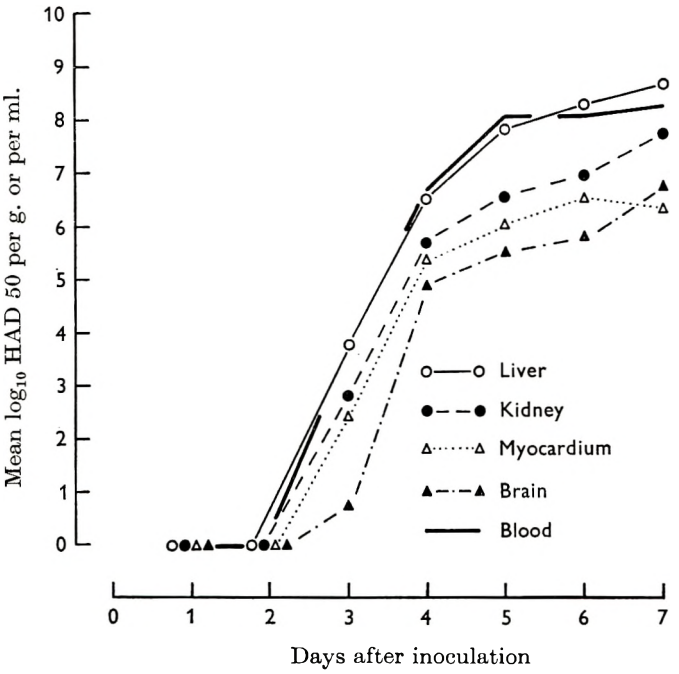

Fig. 6. Parenchymatous organs and brain.

A marked feature, in both pigs FU/92 and FU/93, was the absence of virus from many lymph nodes, even those of the head and neck; in addition no infectivity was detected in the tonsils or any part of the alimentary tract, including the oral mucosa, tonsil, Peyer's patches and mucosae of the stomach or small and large intestines. Low titres of virus were found in the lungs but no significant quantity in the mucosae of the respiratory tract, including in one case (no. FU/93) the retropharyngeal mucosa. Virus in the spleen was reduced at least 100-fold and whilst pig FU/92 was non-viraemic, a low-level viraemia ($10^{2\cdot0}$ HAD 50/ml.) was found in no. FU/93 (Table 3).

The third pig which behaved irregularly, no. FW/7, had shown a fever ($\geqslant$ 105° F.) for 3 days at the time when it was killed; it was infected simultaneously with no. FW/8 and showed some focal portal cirrhosis at autopsy. Its tissues were titrated in the same batch of cultures as those used for the animal FW/8, i.e. they were of maximal sensitivity. Again, virus titres were high in the cephalic nodes but about 3–4 $\log_{10}$ units lower than usual in the bronchial, alimentary and body nodes.

Spleen, bone marrow and blood virus had reached the expected titres but the respiratory mucosae, alimentary mucosae and parenchymatous organs showed titres which were often depressed by as much as 2 $\log_{10}$ units or more.

While no explanation can be offered for the unusual features of the infection in pigs FU/92, FU/93 and FW/7, with the exception of some possible association with the portal cirrhosis, it was decided to omit the figures for the last two animals in calculating the mean values shown in Figs. 1–6 inclusive. In the case of FU/92 the figures were included as it was considered possible that a 24 hr. delay in generalization could not definitely be regarded as abnormal.

DISCUSSION

Data obtained in this study (Tables 1 and 2) provided clear evidence that when ASFV was instilled into the nasal cavity of pigs it adsorbed to and began to proliferate in the retropharyngeal mucosa within 16–24 hr.; it did not persist, at least in infectious form, adsorbed to the turbinate mucosae and evidence for primary proliferation there was obtained in only one of thirteen pigs (no. FX/0). Virus was rapidly transported from the retropharyngeal mucosa to the retropharyngeal nodes, nearly invariably the medials, where it increased exponentially to reach virtually peak titres within 72 hr. (Fig. 1).

Efferents from the medial retropharyngeal nodes give rise to the tracheal duct, which discharges directly into the brachiocephalic vein (Saar & Getty, 1964); this must have facilitated the rapid dissemination of virus, presumably via the blood stream, to lymphopoietic tissues throughout the body, especially the spleen and lymph nodes. It would account, also, for the early involvement of the bone marrow, alimentary and respiratory mucosae as well as the parenchymatous organs (Table 3). The sequence of events in the great majority of pigs probably resembled that described in rabbits infected intranasally with vaccinia (Yoffey & Sullivan, 1939) or rabbit-pox viruses (Bedson& Duckworth, 1963); in both these instances primary proliferation occurred in the nasal mucosa, followed by spread to the superior, deep cervical lymph nodes, which in turn discharged virus after 12 hr. into the cervical lymph ducts (Yoffey & Sullivan, 1939).

The primary establishment of virus in the bronchial mucosa and associated lymph nodes of two pigs, with or without the involvement of the retropharyngeal route, suggests that infection by aerosols of small particle size can probably occur via the *lower* respiratory tract. This could be an alternative route of natural infection, as shown already for rabbit-pox (Bedson & Duckworth, 1963) and rinderpest in cattle (Taylor *et al.* 1965). It is necessary to observe, however, that transmission of ASFV by expired air could not be demonstrated by Montgomery (1921), even over a distance as short as 6 in.; he found, in addition, that muzzling was sufficient to prevent contact transmission and that feeding virus did not produce infection if the material was enclosed in a 'bait'.

Although the results of blood fractionation experiments were not entirely satisfactory they did show that about 90 % of the circulating virus was associated with the cellular fraction and that only 11 % was, on average, free in the plasma. The

finding that mean virus titres in the cephalic lymph nodes and spleen exceeded those in the blood throughout the infection and particularly during the earlier phases, strongly suggested that circulating virus was derived in large measure from these tissues. There was no evidence, however, that an important part of the viraemia was associated with intact leucocytes and the question therefore arises as to whether the virus was released in cell-free form from the producer 'target' tissues and subsequently adsorbed to erythrocytes in the circulation or whether 'carrier' erythrocytes were produced in the bone marrow.

Some evidence for the first of these alternatives was obtained in an experiment in which washed pig erythrocytes (25 %, v/v) were mixed *in vitro* with culture-propagated virus at 37° C., and sampled at intervals by washing twice with chilled PBS and titrating in PBM cultures; the infectivity of the exposed erythrocytes rose from $10^{4\cdot4}$/ml. at 30 min. to $10^{5\cdot4}$/ml. at 3 hr., the titre of the original virus being $10^{5\cdot8}$/ml. The second alternative, i.e. the production of infected red cells in the bone marrow, was not supported by the relatively low titres of virus in packed cells derived from this tissue, which seldom exceeded those in the blood at the same time. Virus bound to erythrocytes would probably, as pointed out by Mims (1964), be cleared less rapidly from the circulation than plasma virus, which would be more readily taken up by macrophages; this would help to account for the attainment and maintenance of the very high levels of viraemia which were observed throughout the later course of the infection.

The predominant association of virus with the erythrocytes of the blood in ASF recalls a similar state of affairs in the pathologically-similar hog cholera. In this disease, caused by a completely unrelated virus, infectivity is also firmly adsorbed to the erythrocytes and could be demonstrated in large quantities in washed stroma; virus was also adsorbed by normal erythrocytes *in vitro* (Powick, 1937).

The distribution of virulent ASFV in the tissues of infected pigs shows striking correlations with the predominant lesions; these include a severe, necrotic and haemorrhagic lymphadenitis with karyorrhectic destruction of lymphocytes and also a fibrinoid angiitis, affecting particularly capillaries and arterioles (De Kock, Robinson & Keppel, 1940; Maurer, Griesemer & Jones, 1958). The high virus content of lymphopoietic tissues certainly does not conflict with the hypothesis that the virus grows in lymphocytes *in vivo*. Maurer *et al.* (1958) suggested that the vascular lesions, including those of the endothelium, were also a direct effect of the virus and, if this were confirmed, it could help to account for the high level of viraemia; similar considerations apply to hog cholera (Mims, 1964).

Heuschele, Coggins & Stone (1966) used the fluorescent antibody technique to examine smears from the tissues of pigs acutely infected with ASFV. They had difficulty with non-specific fluorescence of leucocytes but found specific, fluorescent globules in impression smears of spleen, gastric lymph nodes or, especially, liver and suggested that the cells containing them were macrophages. No precise information on the types of cell supporting the growth of ASFV can be obtained until difficulties in the application of the fluorescent-antibody technique to pig tissues have been overcome.

Our data may provide a partial explanation for the observation of Montgomery

(1921), repeated on several occasions by ourselves, that infected pigs during the first 12–24 hr. of fever do not transmit ASFV to other animals in pen contact with them. Thus, in addition to high titres in the retropharyngeal mucosa, virus was consistently present in appreciable quantities in the nasal mucosa at 72 hr after intranasal infection, i.e. at the beginning of or 24 hr. before pyrexia; it was also present at this time, albeit in smaller quantities, in the intestinal mucosae. But, apart from the primary site of entry, where proliferation could have been either in surface epithelium or in the associated lymphoid follicles, it is probable that much of the infectivity in the other locations mentioned was due to their blood content (see Tables 3 and 4); excretion would not begin, therefore, until the surface epithelium had allowed passage of virus from the circulation or produced virus itself. These considerations would not, of course, apply to excretion from the plasma by the urinary route, assuming that the virus could pass the glomerular barrier.

SUMMARY

Pigs were infected by the intranasal instillation of a large dose (*ca.* $10^{7\cdot0}$ ID 50) of a highly virulent strain of African swine fever virus (ASFV) and the progress of the infection was studied by the 'routine titration approach' (Mims, 1964) using pig bone marrow cultures.

Virus growth was established within 16–24 hr. in the retropharyngeal but not in the alimentary or nasal mucosae or the tonsils. By 24–40 hr. the virus was consistently present in the retropharyngeal lymph nodes, almost invariably the medials; titres in these nodes exceeded those in the associated mucosa by 48–72 hr. Generalization, presumed to have occurred via the tracheal lymph ducts and the blood stream, was generally demonstrable after 72 hr., i.e. by the time of the onset of pyrexia or 24 hr. prior to this.

On average 11 % of the total infectivity in the blood was present in the plasma, with the rest assumed to be cell-associated. A mean of about 45 % of the total infectivity was recovered in erythrocyte fractions in which the concentration of leucocytes had been materially reduced; fractions with increased leucocyte counts contained relatively little virus and it was concluded that the great majority of circulating virus was closely associated with the erythrocytes. Adsorption of ASFV to normal pig erythrocytes was demonstrated *in vitro*.

The greatest concentrations of virus were recorded in the lymph nodes, especially those of the cephalic region, and in the spleen, where titres commonly attained $10^{8\cdot0}$ to $10^{9\cdot0}$ HAD 50/g. and exceeded those in the blood. They were, therefore, thought to be the source of much circulating virus, although there was some evidence that the liver, lungs and bone marrow may also have contributed, at least in some animals. There was no evidence that the mucosae of the alimentary and respiratory tracts or the kidney, myocardium and brain were a source of significant amounts of virus. The virus demonstrable in Peyer's patches did not exceed that in the intervening ileal mucosa.

Although contact transmission of ASF does not normally occur during the first 12–24 hr. of fever, considerable amounts of virus were usually present in the nasal

and intestinal mucosae at 72 hr. It was probable that this infectivity was due to the blood content and that excretion did not occur until the epithelium was breached.

Three pigs, all of which had lesions of a portal cirrhosis, showed a delayed or restricted generalization of virus, in comparison with the other twenty-eight animals which behaved according to a regular pattern.

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Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks

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INTRODUCTION

The earliest reports linking *Clostridium welchii* with food poisoning were by Klein (1895) and Andrews (1899). In both instances the patients suffered mild diarrhoea but no vomiting. The investigations made in both these outbreaks were, however, not sufficient to establish with certainty the causative role of the organism.

McClung (1945) in the U.S.A. reported four outbreaks of food poisoning from chicken dishes. The symptoms, appearing 8–12 hr. after the patients had eaten the meal, were intestinal cramp and diarrhoea with little vomiting. Examination of the remains of the chickens revealed the presence of large numbers of *Cl. welchii*. Hobbs *et al.* (1953) published the results of the epidemiological and bacteriological studies of several outbreaks of food poisoning occurring in the U.K. during the period September 1949 to February 1952. In all these incidents the causative organism was a strain of *Cl. welchii* that was non-haemolytic on horse blood agar, produced only trace amounts of α-toxin and formed spores capable of withstanding 100° C. for periods of 1–4 hr. Since then this heat-resistant variant of *Cl. welchii* has come to be regarded as a major cause of food poisoning in the U.K. (Hobbs, 1965), and reports from other countries (Sutton, 1966; Dauer, 1961, Hayashi, Kugita, Tawara & Yamagata, 1961) have increasingly incriminated heat-resistant *Cl. welchii* as a cause of food poisoning.

Heat-sensitive strains of *Cl. welchii* have been reported as the cause of food poisoning on only a few occasions. McKillop (1959) described an outbreak in which a β-haemolytic, heat-sensitive strain was thought to be the causative organism; while Taylor & Coetzee (1966) reported an outbreak due to non-haemolytic, heat-sensitive *Cl. welchii*. In the U.S.A. Hall, Angelotti, Lewis & Foter (1963), after examining the characteristics of many *Cl. welchii* isolated from contaminated foods and food associated with food poisoning outbreaks, concluded that heat-sensitive strains of *Cl. welchii* had been responsible for food poisoning outbreaks in that country.

This paper describes five outbreaks of food poisoning due to heat-sensitive *Cl. welchii* investigated at the Food Hygiene Laboratory, London, in conjunction with regional Public Health Laboratories in England, between September 1966 and May 1967, together with four outbreaks in which both heat-sensitive and heat-resistant strains are thought to have been involved.

MATERIALS AND METHODS

Media used

Neomycin blood agar was prepared by allowing 5–10 ml. of 1 % peptone water agar to set in a Petri dish and covering it with a layer of 10 ml. 5 % horse blood agar. When set 3–5 drops (0·06–0·1 ml.) of 1 % neomycin sulphate were spread evenly over the surface with the aid of a bent glass spreader. The plates were then dried before use. This medium inhibits the growth of coliform bacilli and most other organisms occurring in the faeces and allows the ready isolation of *Cl. welchii*. It is important to incubate for only 24 hr., as prolonged incubation permits other organisms to grow.

Egg yolk medium was prepared by the method of Willis & Hobbs (1958); one half of the plate was spread with 3 drops of *Cl. welchii* type A antitoxin (Burroughs Wellcome) immediately before use in order to demonstrate the inhibition of the lecithinase reaction.

Cooked meat medium (C.M.M.) made with veal was used throughout.

Sporulation medium. The sporulation medium was made according to Roberts (1968) with added glucose and thioglycollate (TPAY–CT).

Serology

Hobbs type antisera 1–17 of the Food Hygiene Laboratory collection were used. Where necessary a new antiserum was prepared (see below).

Examination of faeces

All faecal samples were examined using the following three techniques; (1) Direct viable cell count on an unheated faecal suspension. (2) Spore count on a faecal suspension heated at 80° C. for 10 min. and (3) Enrichment culture following heating in C.M.M. for 60 min. at 100° C.

Viable cell and spore counts of Cl. welchii

A 1/100 suspension of faeces was made by emulsifying 1 g. of faeces in 1–3 ml. of quarter strength Ringer's solution; the volume was made up to 100 ml. in a 4 oz. bottle. With this suspension serial tenfold dilutions were made to give a final dilution range of 10^2 to 10^5. Viable cell counts were carried out on neomycin blood agar using the technique of Miles & Misra (1938).

The 1/100 faecal suspension was heated at 80° C. for 10 min., and the viable count repeated. In this way the number of viable spores present could be estimated.

Isolation of heat-resistant Cl. welchii

A small portion (approximately 1 g.) of faeces was emulsified in each of two 1 oz. bottles of cooked meat medium and immediately placed in a water bath at 60–70° C. The water was boiled and one bottle was removed after 30 min., and the other after 60 min. at 100° C. The bottles were incubated at 37° C. overnight and subcultured the following day on neomycin blood agar.

All plates were incubated anaerobically at 37° C.

Identification of Cl. welchii

Colonies showing the typical appearance of *Cl. welchii* (Cruickshank, 1965) were further examined by Gram stain, lactose fermentation and inhibition of the lecithinase reaction on egg yolk medium by *Cl. welchii* type A antiserum.

The organisms were serotyped using Hobbs's sera 1–17, by the method of Hobbs *et al.* (1953). When the strains could not be typed in this way, an antiserum was prepared against the suspected organism, preferably selected from food, by the method of Henderson (1940).

Determination of heat resistance of Cl. welchii *spores*

The heat resistance of the organisms responsible for outbreaks 1 to 5 was further investigated. Spore suspensions were prepared by the method of Roberts (1968). The yield of spores was not as good as that obtained by Roberts, but was, nevertheless, satisfactory for semi-quantitative studies of heat resistance.

One ml. amounts of the washed spore suspension were inoculated into nine 1 oz. bottles of C.M.M., which were held at 100° C., three for 10 min., three for 20 min. and three for 30 min. All were removed, cooled and incubated at 37° C. for 48 hr. They were then subcultured on blood agar medium and incubated anaerobically at 37° C. for 24 hr. Results were recorded as presence or absence of *Cl. welchii*.

RESULTS

The heat resistance of an organism is generally expressed in one of two ways.

1. *Quantitatively.* By the use of such terms as D and Z values it is possible to express quantitative data on the heat resistance of an organism over a wide temperature range. *D* values are usually determined by suspending the organisms in buffer solution in sealed ampoules, heating at various temperatures and doing viable cell counts in order to ascertain the number of survivors after a known heat treatment. Heat-resistant spores of *Cl. welchii* have a D_{100} of 5 min. or longer (Roberts, 1968). The technique, although the most accurate, is somewhat laborious and is often replaced by the following technique.

2. *Semi-quantitatively.* The semi-quantitative results of heat resistance studies are usually expressed as 'Thermal death times'. They are determined by inoculating the test organism (often in unknown numbers) into tubes of medium, heating for varying periods of time and testing for the presence or absence of survivors. In the case of 'typical food poisoning strains', it has been well established that even small numbers of organisms will survive 100° C. for at least 1 hr. when heated in C.M.M. (Hobbs *et al.* 1953; Collee, Knowlden & Hobbs, 1961); whereas even a large inoculum of classical β-haemolytic *Cl. welchii* will not yield survivors after 15 min. at 100° C. when heated in C.M.M.

As this paper deals with the isolation of *Cl. welchii* from faeces, the term heat-resistant will imply that the organism could be isolated from a faecal suspension after heating in C.M.M. for 1 hr. at 100° C. Heat-sensitive will imply that the organism could not be isolated from faeces in this way. More detailed information on the heat resistance of the causative organisms is given in Table 2.

Outbreak 1

The incident occurred in October 1966 at a school in the Liverpool area.

On the morning of 18 October, 77 lb. of meat were cut into fourteen pieces of approximately equal size and cooked for 3–4 hr. in a steamer in 3 cylindrical pans approximately 18 in. in diameter and 12–15 in. deep. They were then allowed to cool from 12.30 p.m. to 9.30 p.m. The pieces were removed from the pans and refrigerated until about 10.45 a.m. on the 19th, when they were sliced and eaten cold.

Of 400 persons at risk 50 developed abdominal pain and diarrhoea within 12–15 hr. No vomiting occurred. The illness lasted 12–24 hr.

Bacteriology

The incriminated meat and faeces from seventeen persons affected were examined for *Cl. welchii*. Beta haemolytic *Cl. welchii* were found in the food, and in large numbers ($7{\cdot}5 \times 10^5 - 2{\cdot}5 \times 10^7$/g.) in fifteen of the seventeen specimens of faeces. Beta haemolytic strains were isolated after 30 min. but not 60 min. boiling from six specimens of faeces, but on re-examination of the faeces only 1 strain survived 30 min. boiling. In no case were non-haemolytic, heat-resistant strains isolated after 30 or 60 min. at 100° C. None of the strains isolated was typable by Hobbs type 1–17 antisera. An antiserum was therefore prepared against the strain isolated from the meat and it agglutinated 14/15 β-haemolytic strains isolated from the faeces. Toxicologically this strain behaved as a typical type A strain, producing α, κ and θ toxins.

Outbreak 2

This outbreak occurred in the London area in October 1966 following a harvest supper. Two frozen turkeys, each approximately 21 lb. in weight, were delivered to the premises on Friday evening. They were thawed for 36 hr. in the polythene bags before being stuffed; the stuffing contained sausage meat.

They were cooked on Sunday afternoon for 6 hr.—1 hr. at 195° C. followed by 3 hr. at a lower temperature and finally 2 hr. at 195° C. They were then removed and allowed to cool on top of the oven for 2 hr. before they were put back into the 'warm' oven for storage overnight. The next day the meat was sliced, piled on an unknown number of plates and rewarmed in the oven before serving.

Symptoms of diarrhoea and abdominal pain occurred 8–12 hr. after the ingestion of the food, in 27 of 120 persons who ate the meal. One patient, a 62-year-old woman, died 48 hr. after eating the meal. She was suffering from Pott's disease at the time. The cause of death was certified as 'acute enterocolitis due to food poisoning'.

Bacteriology

Unfortunately no food was available for examination and faeces were not collected until 7–9 days after the onset of the illness. Faeces from twenty-one patients were examined, and of these, twenty contained β-haemolytic and fourteen non-haemolytic *Cl. welchii*, generally in relatively small numbers on direct culture.

In no case were heat-resistant *Cl. welchii* isolated. Only two strains, both β-haemolytic, were typable. Antisera were, therefore, prepared against three of the non-haemolytic strains but no serological relationship between the fourteen strains isolated could be established. On the other hand an antiserum prepared against a β-haemolytic strain agglutinated 11/19 β-haemolytic strains tested.

As the faeces were not collected until 7–9 days after the onset of symptoms it must be expected that the direct counts would have dropped to within the limits obtained. These findings therefore strongly suggest that the outbreak was caused by a β-haemolytic, heat-sensitive strain of *Cl. welchii*.

Outbreak 3

The outbreak followed a meal, eaten by four persons, in a Chinese restaurant. Common items of food were grilled ox liver and ice cream. Symptoms of abdominal pain and diarrhoea occurred in all four persons 10–14 hr. after eating the meal.

Deep frozen South American ox liver was received at the Chinese restaurant 24 hr. before it was cooked. It was kept frozen for 12 hr. before it was transferred to a 4° C. refrigerator, and grilled 1–2 hr. before the meal.

Bacteriology

Faeces were collected from all four persons 24–48 hr. after the symptoms occurred. All four faeces contained large numbers of non-haemolytic *Cl. welchii* ($1{\cdot}7 \times 10^7 - 5{\cdot}0 \times 10^7$/g.) but *Cl. welchii* could not be isolated from the faeces after 30 min. at 100° C. Serologically all four strains were Hobbs type 5.

Unfortunately none of the incriminated liver was left, but the examination of similar samples yielded large numbers of haemolytic *Cl. welchii* (not typable), indicating that the liver was a potential source of *Cl. welchii*.

Outbreak 4

The outbreak occurred in April 1966 in a school canteen. On 5 April 200 children ate a mid-day meal of cold, rolled roast beef, vegetables, jam tart and custard. The beef was cooked the previous day—how long it had been cooked, and at what temperature, could not be ascertained, but samples of meat examined appeared to be adequately cooked (in a culinary sense); it was said to have been refrigerated immediately after cooking, sliced the following morning and immediately refrigerated until lunch time, when it was served.

Symptoms of diarrhoea and abdominal pain occurred in thirty of the 200 persons 10–11 hr. after the meal. There was no vomiting and the illness was of brief duration.

Bacteriology

A direct Gram stain of the beef showed fairly large numbers of organisms, mainly Gram-positive bacilli and some cocci. A moderate growth of both haemolytic and non-haemolytic *Cl. welchii* was obtained from the beef.

Six samples of faeces were collected from persons with symptoms. In all samples haemolytic *Cl. welchii* were present in large numbers ($6{\cdot}0 \times 10^6 - 1{\cdot}0 \times 10^8$/g.) in

direct culture. In no case were *Cl. welchii* grown after heating faeces at 100° C. for 30 min.

Serologically the organism was not agglutinated by Hobbs types 1–17 antisera, and an antiserum was prepared against the β-haemolytic strain isolated from the beef. This antiserum agglutinated all the six β-haemolytic strains isolated from the faeces.

Table 1. *Viable cell counts of heat sensitive* Cl. welchii *in faeces of persons associated with food poisoning outbreaks 1–5*

Outbreak no.	No. faeces examined (1)	No. containing causative org. (2)	Median v.c.c. of *Cl. welchii*		Ratio S:T (5)*
			Total (T) (3)	Spores (S) (4)	
1	17	14	$4{\cdot}0 \times 10^6$	$4{\cdot}0 \times 10^5$	1:10
2	21	11	$1{\cdot}0 \times 10^3$	$2{\cdot}5 \times 10^2$	1:5
3	4	4	$5{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^7$	1:5
4	6	6	$3{\cdot}5 \times 10^7$	$1{\cdot}5 \times 10^7$	1:3
5	11	11	$9{\cdot}5 \times 10^6$	$2{\cdot}0 \times 10^6$	2:3

* The values in this column do not indicate value of column 4/column 3. They indicate the median value of the individual S:T ratios calculated for each faeces of the outbreak involved.

Table 2. *Results of the heat resistance tests on spore suspensions grown in laboratory medium of the strains of* Cl. welchii *responsible for the food poisoning outbreaks 1–5*

Outbreak no.	Initial spore count	Number of tubes showing survivors after heating at 100 °C. for		
		10 min.	20 min.	30 min.
1	$1{\cdot}5 \times 10^4$	1/3	0/3	0/3
2	$3{\cdot}5 \times 10^3$	0/3	0/3	0/3
3	$5{\cdot}0 \times 10^4$	1/3	0/3	0/3
4	$1{\cdot}0 \times 10^3$	0/3	0/3	0/3
5	$4{\cdot}5 \times 10^3$	1/3	0/3	0/3

Outbreak 5

An outbreak occurred in May 1967 in a school canteen in the Salisbury area. On 1 May three uncooked 'frozen' tongues were delivered to the canteen, but one of the staff noted that the tongues were not truly frozen. They were cooked in a steamer for 4 hr., each in a different container, removed, skinned and pressed. They were said to have been refrigerated within ½ hr. of removal from the steamer. At 9.15 a.m. the following day they were removed from the refrigerator, sliced and prepared for the mid-day meal, which was eaten cold by 206 children and nineteen members of the staff.

Eighty-eight persons suffered symptoms of abdominal pain and diarrhoea, which began 9–10 hr. after the meal and continued into the early hours of the next morning. Recovery was fairly rapid that day.

Bacteriology

The remains of the tongue were cultured and found to contain 10^7 β-haemolytic *Cl. welchii* per gram. Faeces were collected from eleven persons 48 hr. after onset of symptoms and β-haemolytic *Cl. welchii* were isolated by direct culture in large numbers ($2{\cdot}0 \times 10^5 - 3{\cdot}0 \times 10^7$/g.) from all specimens. No *Cl. welchii* were isolated after heating at 100° C. for 30 min. All strains isolated from food and faeces gave a weak agglutination with Hobbs type 5 antiserum but as the reaction was only weak an antiserum was prepared against the strain isolated from the tongue. This antiserum agglutinated all the eleven strains isolated from the faeces.

The results from outbreaks 1–5 together with the results of the studies of heat resistance are summarized in Tables 1 and 2.

Outbreaks due to both heat-resistant and heat-sensitive Cl. welchii

In addition to the five outbreaks described, four incidents have occurred in which both heat-resistant and heat-sensitive *Cl. welchii* have been considered significant. A brief outline of each is given below.

(i) Heat-sensitive, non-haemolytic *Cl. welchii* type 7 was isolated by direct culture in large numbers (*c.* 10^7 orgs/g.) from 7/7 persons who had symptoms of diarrhoea and abdominal pain after eating boiled beef and mince. Heat-resistant, non-haemolytic *Cl. welchii*, all type 4, were isolated from 4/7 faeces, but presumably only in small numbers as they could not be isolated by direct culture after heating at 100° C. for 30 min. in cooked meat. This can be readily accomplished if the heat-resistant strains are present in numbers of 10^4/g. or greater. Unfortunately none of the incriminated meat was available for examination.

(ii) This outbreak occurred following a mid-day meal at a teachers' training college. Symptoms of diarrhoea and abdominal pain occurred in 123 of 320 persons at risk 8–24 hr. after eating the meal of roast pork. Heat-sensitive, non-haemolytic *Cl. welchii*, not type 1–17 were isolated directly in large numbers from 14/15 specimens of faeces. An antiserum prepared against one strain agglutinated 11/14 strains tested. In addition all fifteen faeces contained large numbers of heat-resistant non-haemolytic *Cl. welchii* type 3. No pork was available for examination.

(iii) In this outbreak symptoms of diarrhoea and abdominal pain occurred in 60 of 180 geriatric hospital patients 10 hr. after they had eaten a meal of minced meat. Heat-sensitive and heat-resistant strains of *Cl. welchii* (both non-haemolytic) were isolated in large numbers (*c.* 10^6/g.) from the beef. Antisera were prepared against both strains. The antiserum against the heat-resistant strain agglutinated a non-haemolytic, heat-resistant strain isolated in large numbers from 9/9 faeces. Similarly the antiserum against the heat-sensitive strain agglutinated 8/9 heat-sensitive strains isolated from the faeces.

(iv) This outbreak occurred in a hospital and followed a meal of cold roast pork. Twenty seven of 300 persons who ate the pork suffered symptoms of mild diarrhoea 8–12 hr. after the meal. Eleven samples of faeces were collected, and both heat-resistant, non-haemolytic *Cl. welchii* type 13, and heat-sensitive, non-

haemolytic *Cl. welchii* Type 1 were isolated in large numbers from all eleven faeces. No *Cl. welchii* could be isolated from the sample of meat examined.

DISCUSSION

Hauschild, Niilo & Dorward (1967) and Hauschild & Thatcher (1967), in feeding experiments carried out with human volunteers and lambs, used heat-sensitive non-haemolytic *Cl. welchii* and were able to produce symptoms of food poisoning similar to those associated with heat-resistant *Cl. welchii*. This, with earlier reports of McKillop (1959) and Taylor & Coetzee (1966), and the results described in the present paper, demonstrates that heat-sensitive strains of *Cl. welchii* are capable of causing food poisoning in man.

Heat-sensitive *Cl. welchii* are present in nature far more frequently than the heat-resistant strains; it is therefore pertinent to ask why food poisoning due to heat-sensitive *Cl. welchii* has not been reported more frequently in the past. There are two probable explanations; the natural variation in the heat resistance of the spores which enables the heat-resistant strains to survive the cooking process far more frequently, and the difficulty of isolating the heat-sensitive strains by the techniques used in the past.

It has been shown (Hobbs, 1965) that *Cl. welchii* food poisoning is due to the survival during cooking of the organism already present in the raw meat followed by germination of spores and multiplication of the organism in the non-refrigerated meat dish. The heat-resistant nature of the spore had always been considered an integral part of this process. For this reason heat-sensitive strains of *Cl. welchii* have only occasionally been considered as a cause of natural outbreaks of food poisoning. The work of Hauschild & Thatcher (1967) clearly demonstrated that the organism could cause food poisoning, but it did not demonstrate in any way that the organism could survive the cooking process and cause food poisoning under conditions similar to those occurring in a natural outbreak.

Sylvester & Green (1961) investigated the temperature gradient within and on the surface of meat samples during slow and conventional roasting. They demonstrated that the usual conventional method of roasting was bacteriologically safer than slow overnight roasting. In the conventional method the temperature within the meat reached 85–90° C., while in the overnight roasting the temperature reached only 65° C.

More recently Woodburn & Kim (1966) have shown that heat-sensitive *Cl. welchii* inoculated into the stuffing of turkey could survive a normal roasting procedure, in fact, the temperature in the deep tissue of the turkey never rose above 82° C. They were readily able to detect survivors from turkey stuffing subjected to a long slow roast (oven temperature 94° C. for $16\frac{1}{2}$ hr.) and a short fast roast (oven temperature of 232° C. for $2\frac{3}{4}$ hr.). They concluded that in meats the temperature at the end of cooking for a well-done product is about 85° C.

In outbreak 2 the temperature of the inner turkey meat probably never rose above 75–80° C. and must have been at this temperature for only a short time. The size of the turkey/time of cooking ratio leads one to this conclusion.

Although the 'classical' strains of *Cl. welchii* are considered to be heat-sensitive in contrast to the marked heat resistance of the 'typical food poisoning' strains, it must still be remembered that the spores of the heat-sensitive strains are much more heat-resistant than normal vegetative cells and a limited number of quantitative studies in this laboratory (unpublished) have shown that in meat medium they can withstand a temperature of 80° C. for relatively long periods of time (D 80 meat = 60–200 min.).

It is therefore reasonable to conclude that heat-sensitive *Cl. welchii* may, under favourable conditions, survive a cooking process, particularly if a large piece of meat is being cooked for a relatively short period of time, as occurred in outbreak 2. This likelihood is increased if the meat was previously frozen, as the three outbreaks due to frozen meats reported here demonstrate. The finding that three of the five outbreaks described in detail were due to frozen meats is particularly interesting. The duration and method of thawing have a critical effect on the temperature that might be reached within the meat during cooking, and further investigations should pay attention to these points.

The role of contamination after cooking is not clear. McKillop (1959) clearly indicates that the dust in the air and in vessels used for storage may play a prominent part in *Cl. welchii* food poisoning, although much of the epidemiological evidence to date (admittedly concerned primarily with heat-resistant *Cl. welchii*) assumes that the causative strain of *Cl. welchii* is almost always present in the meat before cooking. Much work is still to be done to clarify this point, particularly in regard to β-haemolytic *Cl. welchii*. It should be remembered that, in contrast to the non-haemolytic heat-resistant strains, the spores of classical strains germinate fairly readily without prior heat treatment (Roberts, 1968) and that both heat-sensitive and heat-resistant strains require anaerobic conditions for growth.

Until recently most workers in the U.K. may have considered *Cl. welchii* food poisoning to be caused only by the heat-resistant non-haemolytic strain of *Cl. welchii* type A. So much so, in fact, that the term 'typical food poisoning' as apart from 'classical' *Cl. welchii* has been developed to describe this variant. As a result a standardized technique of heating the suspected faeces in C.M.M. for 60 min. at 100° C. before overnight incubation has been adopted in examining faeces from suspected outbreaks of *Cl. welchii* food poisoning. Direct culture of faeces on neomycin blood agar incubated anaerobically has not been routinely carried out. This technique therefore precludes the possibility of incriminating heat-sensitive *Cl. welchii* as the cause of food poisoning outbreaks.

It therefore appears likely that heat-sensitive *Cl. welchii* is the cause of food poisoning in far more instances than present reports indicate. It may in fact be responsible for some of the incidents of food poisoning in the past for which no cause has been found. Future reports of food poisoning should pay particular attention to finding out whether the method of examination used was satisfactory for the detection of heat-sensitive *Cl. welchii*. Only in this way can a true indication of the ratio of outbreaks due to heat-sensitive and heat-resistant strains be obtained, and the significance of this heat-sensitive strain of *Cl. welchii* as a cause of food poisoning be assessed.

Sutton (1966) investigated one outbreak of *Cl. welchii* food poisoning and reported large numbers of the causative organism in the faeces of persons involved. This finding has been confirmed in this work, and also in investigations of many outbreaks due to heat-resistant *Cl. welchii* (unpublished), provided the specimens of faeces are collected within 3 days of the onset of symptoms. The viable cell count by the Miles and Misra method is generally of the order of *c.* 10^5–*c.* 10^7 *Cl. welchii*/gram; there is tendency for the count to decrease with time, so that specimens collected 1 week after symptoms have occurred may be within normal limits (median count approximately 10^3 *Cl. welchii*/gram). The increase in count may be useful in investigating outbreaks of food poisoning due to heat-sensitive *Cl. welchii*. As haemolytic, heat-sensitive *Cl. welchii* occur (mostly in small numbers) in almost all faeces (Collee *et al.* 1961), some idea of the numbers present is of importance. The finding of large numbers of *Cl. welchii* in a group of suspected faeces would certainly warrant full serological investigation, with the possible preparation of an antiserum. In some of the outbreaks investigated (due to either heat-sensitive or heat-resistant *Cl. welchii*) it has been noted that as well as multiplication of the causative organism, multiplication of strains of *Cl. welchii* already present in the intestine has occurred. Freshly collected faecal samples may therefore contain large numbers of the causative strain along with other strains of *Cl. welchii*. Thus there may be difficulties in the interpretation of serological results.

It would not be practical for busy hospitals to carry out viable cell counts on all faeces from patients suspected to be suffering from *Cl. welchii* food poisoning. The following technique, however, is suggested as an alternative measure:

1. A thick emulsion of faeces (1/5–1/10) is made in quarter-strength Ringer's solution.

2. With this emulsion a semi-quantitative direct count is carried out on neomycin blood agar using a calibrated loop in a manner similar to that now routinely used in many hospitals for urine culture. This count may be done either on unheated faeces or on the emulsion heated to 80° C. for 10 min. (spore count). The heating method has the advantage that it activates spores (which is more important in outbreaks due to heat resistant *Cl. welchii*) and makes the isolation of *Cl. welchii* a little easier, but it has the disadvantage of being time consuming. The plates are incubated anaerobically at 37° C. for 16–24 hr. (no longer). Using this technique it is at least possible to tell whether *Cl. welchii* are present in the faeces in relatively small or large numbers.

3. One–two ml. of the emulsion is inoculated into a tube of C.M.M. and heated at 100° C. for 60 min. It is then incubated overnight at 37° C. and subcultured on neomycin blood agar. This detects the presence or absence of heat-resistant spores of *Cl. welchii*.

Media routinely used for the isolation of *Salmonella*, *Shigella* and *Staphylococcus* can be inoculated using this emulsion although this must be done before heating.

SUMMARY

Details of confirmed outbreaks of food poisoning due to heat-sensitive *Cl. welchii* are given. In 4/5 incidents heat-sensitive *Cl. welchii* were isolated in large numbers from the majority of the faeces. In the remaining outbreak the faecal samples were not collected until 7–9 days after the illness. The causative organism was isolated from the food in 3/5 instances.

In addition four outbreaks of food poisoning in which both heat-sensitive and heat-resistant *Cl. welchii* were isolated are described.

The role of heat-sensitive *Cl. welchii* in food poisoning outbreaks is discussed and a suggested method of examining faeces for *Cl. welchii* is given.

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One of us (R.G.A.S.) is attached to the Commonwealth Department of Health, Australia, and is at present the holder of a Public Service Board Postgraduate scholarship.

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A comparative trial of norbormide* and zinc phosphide against *Rattus norvegicus* on farms†

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INTRODUCTION

The occurrence and spread of rats resistant to warfarin and related anticoagulant poisons in two counties of England and Wales since 1959 have stimulated interest in possible alternative rodenticides. One such alternative, norbormide first described by Roszkowski, Poos & Mohrbacher (1964) has already been compared with warfarin against small urban infestations (Drummond & Taylor, personal communication). It proved to be less effective, even at its lowest and most successful concentration, 0·5%, and eradicated only about 50% of the infestations treated. Nevertheless, norbormide could still play a useful role against warfarin-resistant rats if its effectiveness could be shown to compare favourably with that of currently recommended acute poisons such as zinc phosphide and arsenious oxide (Davis, 1967).

Norbormide has already been compared with zinc phosphide under laboratory conditions against normal and warfarin-resistant strains of wild rats by Greaves (1966). He found no significant difference in the response of the two strains to either poison and suggested that 0·5% or higher concentrations of norbormide might give results in the field as good as those normally obtained with 2·5 or 5·0% zinc phosphide, but refrained from drawing any firm conclusions until the two rodenticides had been tested under field conditions.

The field trial, now described, was planned with the dual purpose of comparing norbormide and zinc phosphide and of finding out to what degree of accuracy the laboratory results had predicted the effectiveness of the rodenticides in practice. Some of the methods, therefore, replicated those used in laboratory tests, while others were dictated by the inferences drawn from the results of those tests.

The fact that normal and warfarin-resistant rats had responded similarly to the two poisons in the laboratory meant that there could be no objection to siting the trial in an area where warfarin-resistant rat populations occurred and where, perhaps for that reason, a large number of infested farms were available. An added advantage was that acute poisons were commonly used in this area, and farmers understood and accepted the risks involved.

* 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene) norborn-5-ene-2,3-dicarboximide.

METHODS

Choice of infested properties

The search for infested farms was started by visiting holdings up to 50 acres in size, on which pigs or poultry were kept, in the Montgomeryshire parishes of Meifod and Guilsfield Without. Pig and poultry farms were chosen as not only the most likely to be infested, but also because they comprised a high proportion (85 %) of the smaller-sized holdings that could be treated by the staff available and with the amount of norbormide that could, at first, be budgeted for. Twenty-nine of the forty-eight farms treated in the trial were so found: the remaining nineteen, which came to our attention as the search progressed, were just inside the neighbouring parishes of Llanfair Caereinion, Llangyniew, Llandrinio and Guilsfield Within. It was possible in the second half of the trial to treat infestations on properties of any size because a free supply of norbormide was made available by the manufacturers; but with 50–60 % of all the registered holdings under 50 acres, most of the farms treated remained within the original limits.

Poisons and baits

Treatments were done with 0·5 and 1·0 % norbormide, the two most promising concentrations in the laboratory tests (Greaves, 1966), and with 2·5 and 5·0 % zinc phosphide, the concentrations recommended against *Rattus norvegicus* (Davis, 1967) that had also been used in the laboratory tests. Each concentration of poison was applied in four cereal baits; dry sausage rusk, Scomro,* medium grade oatmeal, and damp coarse oatmeal† with 5·0 % sugar. The first three baits had been used in the laboratory tests, and under those conditions the sausage rusk had been significantly less palatable and, when containing poison, less lethal than the other two (Greaves, 1966). Damp coarse oatmeal was included in the field trial because it was potentially a better vehicle for poison (Thompson, 1954) than the other three.

All the baits used in the field were mechanically mixed in the laboratory. To maintain similarity of consistency, as much fine oatmeal was included in each as was necessary to raise the proportion of fine ingredient to that present in the baits containing 1·0 % norbormide, which were made by mixing 1 part of 'Raticate Concentrated Rat Killer'‡ with 3½ parts of plain bait. The addition of both the fine oatmeal and the poisons was allowed for by reducing the proportion of the major cereal constituent in each formulation.

Methods and organization of treatments

There was evidence in the laboratory tests to suggest that rats discriminated less against norbormide than zinc phosphide. The corollary to this was that rats might continue to take the norbormide baits more readily and for longer periods in the field, with better results, if the baits were left down for several days. Each bait

* Sugar 5%, corn oil (5%), maize meal (<65%), rolled oats (25%).

† Two parts by weight of pinhead oatmeal: one part water.

‡ Trade mark. The concentrate contains 4·5% norbormide in a fine cereal with dye.

containing zinc phosphide or norbormide was, therefore, applied in three ways: for either 1 day or 7 days after prebaiting, and directly (i.e. not preceded by baiting with unpoisoned bait) for 10 days.

On the first day of treatment, after a survey of the infestation, plain or poison baits were distributed in numbers and in the situations calculated to be most effective. The farms were re-visited every day, except at week-ends, to replenish the baits and record the number of spoonfuls of bait eaten at each point.

When direct poisoning was practised, two dessertspoonfuls of bait were put initially at each bait point, and this was increased or topped up, if necessary, at every visit. When poisoning after prebaiting, poison bait was laid only at the bait points where prebait had been taken. At each of these, one teaspoonful of poison bait was laid for every dessertspoonful of prebait taken on the day when most had been taken, except where this meant laying less than three teaspoonfuls, in which case three teaspoonfuls were laid. This baiting system, based partly on the finding that rats usually consume only about one-tenth as much poison bait as they do plain bait in a day (Thompson, 1954), achieved the necessary aim of providing a surplus of poison bait at each point whilst being fairly economical in the use of poison. Even with this system two-thirds of the total poison bait used was uneaten by rats, but it is doubtful whether the proportion of poison bait laid to prebait taken (1:2·3) could be decreased without running the risk of leaving too many points insufficiently poison-baited.

Assessment of success of treatments

Two of the authors census-baited each infestation with whole dry wheat before and after treatment. The pre-treatment census-baiting, which lasted only 2 days and started 10 days before prebaiting or 13 days before direct poisoning, was so conducted, it was hoped, as not to condition the rats to eating the treatment baits laid later. The post-treatment census, which also lasted 2 days, began 5 days after each treatment had finished.

The census baits, weighing approximately 120 g., were measured out onto wooden trays placed in and around farm buildings, and where there were associated external infestations. The bait points were marked so that they could be used again in the post-treatment census and so that the staff doing the poison treatments could avoid baiting in the same places if alternative sites could be found.

It was known from previous work (unpublished) that the total weight of bait consumed in a given period by rats is often highly correlated with the number of bait points from which it is taken. To reduce the volume of work involved in weighing baits at individual points, only the occurrence of a take (or not) was recorded daily for each point, instead of the weight of the remaining wheat. The total wheat consumption during each census was, however, calculated by measuring the baits out from pre-weighed amounts and subtracting the weight of both the unused wheat and the wheat recovered at the end of the census.

Before the results of the trial were examined, the relationship between the total weights of wheat eaten and the total numbers of bait points with rat-takes was tested. Linear regressions of the total weights on the total numbers of rat-takes

per census, separately calculated for the pre- and post-treatment data, fitted significantly well in each case ($P < 0{\cdot}01$). The slopes and intercepts of the two regressions were compared and found to differ only insignificantly. The relationship between the weights of wheat and number of rat-takes could, therefore, be regarded as being the same in both types of census and expressable by a single equation derived from the undifferentiated data. This was calculated to be $W = 107N - 540$, when W is the total weight, in grams, of wheat consumed and N the total number of rat-takes recorded in a census lasting 2 days.

Allocation of poisons, baits and methods of treatment

The poisons used were allocated to plots of three farms according to a 4×4 randomized latin square, as in Table 1, in which the columns and rows are the cereal baits and operating staff respectively. The operating staff comprised two to poison and two (L.E.H. and G.L.J.) to do the census baiting before and after poisoning. They worked, in effect, as four pairs, one member census baiting, and the other poisoning, all the plots in one row, and each then changing partners before working on the properties in the next row. By census baiting and treating farms plot by plot in two rows simultaneously they were able to complete the treatments in the first two rows of Table 1 between 13 April and 1 July and in the third and fourth rows between 20 July and 30 September. They treated the plots in a different order in each row to reduce the chance of using the same bait on two plots at the same time in a period of adverse weather.

The three farms constituting each plot were simultaneously treated with the same poison bait; on two the infestations were prebaited for 6 days (Friday to Thursday) before one was poison baited for 1 day and the other for 7 days. Treatment of the third farm, by direct poisoning for 10 days, began 3 days later (on a Monday) than the prebaiting of the first two. This delay was necessary to avoid putting poison down for the first time just before the week-end, during which time no one would be visiting the farm to pick up poisoned rats.

RESULTS

The number of pre- and post-treatment takes of wheat and the differences between them, expressed as percentages of the pretreatment number (i.e. percentage success), are given for each farm in Table 1, but because percentages are binomially distributed, they were transformed to arcsin $\sqrt{}$(percentages) for analysis.

The variances for cereal baits and operating staff were, respectively, smaller and only insignificantly larger than the main error variance. The type of bait used and differences in practice between pairs of staff did not, therefore, significantly affect the outcome of treatments. The variance for the four concentrations of poisons was, on the other hand, significantly larger ($P < 0{\cdot}05$) and the breakdown into independent (orthogonal) components, to separate the variance due to rodenticides from the variance contributed by using two concentrations of each, shows that it was the rodenticides and not their concentrations that caused differential success. Zinc phosphide was more effective than norbormide ($P < 0{\cdot}01$).

Table 1. *The design and results of the farm treatments with 5·0 and 2·5 % zinc phosphide or 1·0 and 0·5 % norbormide in four cereal baits, applied for 1 or 7 days after prebaiting and as direct poisons, in which success was measured by the reduction in the number of takes of wheat baits put down before and after treatment*

Row		Cereal baits: Sausage rusk			Medium oatmeal			Scomro			Damp coarse oatmeal + 5 % sugar		
1	Farm number	4	5	6	9	7	8	10	11	12	1	2	3
	Stock hazard	Hp	p	p	Hp	Hp	p	p	p	H	H	Hp	Hp
	Poison conc.	5·0 % zinc phosphide			2·5 % zinc phosphide			1·0 % norbormide			0·5 % norbormide		
	Method of baiting	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
	No. precensus takes	41	38	49	28	38	33	71	14	26	33	11	11
	No. postcensus takes	9	17	36	9	12	20	47	9	22	20	8	4
	Success (%)	78·0	55·3	26·5	67·8	68·4	39·3	33·8	35·7	15·4	39.4	27·3	63·6
2	Farm number	13	14	15	22	23	24	19	20	21	18	16	17
	Stock hazard	Hp	Hp	Hp	Hp	Hp	Hp	Hp	Hp	Hp	Hp	Hp	Hp
	Poison conc.	1·0 % norbormide			0·5 % norbormide			5·0 % zinc phosphide			2·5 % zinc phosphide		
	Method of baiting	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
	No. precensus takes	57	29	29	8	18	10	20	28	11	48	16	7
	No. postcensus takes	48	24	23	4	13	7	5	19	9	15	0	0
	Success (%)	15·8	17·2	20·7	50·0	27·7	30·0	75·0	32·1	18·2	68·8	100·0	100·0
3	Farm number	34	35	36	25	26	27	28	29	30	31	32	33
	Stock hazard	p	H	p	p	Hp	Hp	—	H	Hp	p	H	p
	Poison conc.	2·5 % zinc phosphide			5·0 % zinc phosphide			0·5 % norbormide			1·0 % norbormide		
	Method of baiting	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
	No. precensus takes	16	9	40	42	17	22	22	92	17	24	18	16
	No. postcensus takes	11	3	25	4	13	11	17	64	19	23	18	20
	Success (%)	31·2	66·7	37·5	90·4	23·5	50·0	22·7	30·4	0	4·2	0	0
4	Farm number	43	44	45	40	41	42	37	38	39	46	47	48
	Stock hazard	p	Hp	Hp	Hp	p	p	Hp	p	p	H	H	H
	Poison conc.	0·5 % norbormide			1·0 % norbormide			2·5 % zinc phosphide			5·0 % zinc phosphide		
	Method of baiting	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
	No. precensus takes	28	51	20	37	24	12	56	6	16	29	39	33
	No. postcensus takes	24	36	14	31	12	6	8	4	6	19	13	6
	Success (%)	14·3	29·4	30·0	16·2	50·0	50·0	85·7	33·3	62·5	34·5	66·7	81·8

Analysis of the arcsin √(percentage) successes (Z = zinc phosphide: N = norbormide)

Source of variation	D.F.	Variance	F
Cereal baits	3	234·3	< 1·0
Operating pairs (rows)	3	652·3	1·5
Poisons	3	2367·7	5·6*
2·5 % Z + 5·0 % Z *vs.* 0·5 % N + 1·0 N	1	6320·4	15·0**
2·5 % Z *vs.* 5·0 % Z	1	463·8	1·1
0·5 % N *vs.* 1·0 % N	1	319·0	< 1·0
Main plot error	6	422·3	—
Methods of treatment	2	83·1	< 1·0
Methods × cereal baits	6	288·0	1·4
Methods × operating pairs	6	146·2	< 1·0
Methods × poisons	6	105·7	< 1·0
Subplot error	12	201·8	—

* Significant at $P < 0·05$

** Significant at $P < 0·01$.

Key: i = 6 days' prebaiting and 1 days' poison baiting; ii = 6 days' prebaiting and seven days' poison baiting; iii = 10 days' direct poison baiting.
H = pigs; p = poultry.

It is also apparent from the smallness of the variance attributable to methods of treatment, compared with the subplot error, that the results achieved by poison baiting for 1 or 7 days after prebaiting were not significantly better than those obtained by direct poisoning for 10 days. The insignificant variance for the interaction between methods and poisons confirms this and also shows that zinc phosphide was consistently better than norbormide however it was applied.

DISCUSSION

Greaves (1966) concluded, from his laboratory tests, that neither of the poisons was likely to give a complete kill in the field, having failed to do so under favourable laboratory conditions. He also suggested that, while zinc phosphide might prove better than norbormide in the field, as in the laboratory, the safety of norbormide might help to offset the not very marked difference between them by allowing better and more liberal baiting. The first of these conclusions has been verified by the results of the present trials, in that only two treatments (farms 16 and 17) ended in the eradication of all rats. In one of these (farm 17) success was largely due to the fact that the hens had been removed from the deep litter that had been the focus of the infestation at the time of the preliminary survey. The reason was less obvious on farm 16, but it was probably because the small infestation was confined to a deep litter house and did not extend to adjacent piggeries, which could not have been so efficiently and safely poisoned.

The extent to which the safeness of norbormide offset the otherwise greater effectiveness of zinc phosphide could not be measured: but generally, infestations in or close to piggeries were more difficult to poison effectively with zinc phosphide because of the risk of secondary poisoning. Piggeries were encountered, with detrimental results in some cases, on sixteen of the properties treated with zinc phosphide. The treatment on farm 26 was the worst affected because a heavy infestation in and around an old wooden shed was, unfortunately, not poisoned at all, because of the owner's fears for the safety of the young pigs housed in it.

The risk of primary poisoning of livestock also had to be considered when using zinc phosphide, particularly on farms with free-range pigs and poultry. It is unlikely, though, that this gave any advantage to norbormide in practice because, safety apart, all baits had to be protected from being eaten by livestock if they were to be available to the rats.

In spite of the advantages of safety, norbormide proved to be significantly less effective than zinc phosphide in the field, although it caused only insignificantly fewer deaths among rats under the controlled conditions of the laboratory tests referred to above. On the other hand, though the type of bait used had little effect on the outcome of the farm treatments, it resulted in significantly different mortalities among captive rats. One reason for these apparent contradictions between the laboratory and field results may be because Greaves based his conclusions about relative toxicity on the number of deaths in the choice tests that had occurred after 4 days, including, by so doing, rats that had initially discriminated against norbormide and survived the first 2 days. It may be more realistic

Table 2. *The number of dessertspoonfuls of prebait eaten by rats on the sixth day of prebaiting and the number of teaspoonfuls of poison bait eaten the following day, the first day of poison baiting, on the thirty-two farms poisoned after prebaiting*

Poison	Sausage rusk		Medium oatmeal		Scomro		Damp coarse oatmeal and sugar		Total		Mean take poison tsp.	Adjusted mean take poison tsp.
	Prebait dsp.	Poison tsp.	Prebait dsp.	Poison tsp.	Prebait dsp.	Poison tsp.	Prebait dsp.	Poison tsp.	Prebait dsp.	Poison tsp.		
Zinc phosphide (5·0 %)	118	48	144	67	12	8	94	50	755	321	40·1	41·4
	205	102	33	9	47	19	102	18				
Zinc phosphide (2·5 %)	77	26	89	48	115	50	172	141	617	312	39·0	51·1
	20	5	91	26	20	6	33	10				
Norbormide (1·0 %)	80	36	78	42	301	130	64	31	846	333	41·6	35·9
	101	21	79	32	60	14	83	27				
Norbormide (0·5 %)	88	28	9	4	90	40	98	78	870	505	63·1	55·5
	97	18	26	19	429	274	33	44				
Totals	786	284	549	247	1074	541	679	399	3088	1471	—	—
Mean take	—	35·5	—	30·9	—	67·6	—	49·9	96·5	46·0	—	—
Adjusted mean take	—	34·4	—	48·2	—	44·1	—	57·1	—	—	—	—

Analysis of variance of prebait and poison takes

Source	D.F.	Prebait M.S.	Prebait *F*		Poison bait M.S.	Poison bait *F*	
Poisons	3	1641	< 1·0	NS	1056	< 1·0	NS
Baits	3	6241	< 1·0		2191	< 1·0	
Poisons × baits	9	9341	1·3		3346	1·1	
Error	16	7224	—		3046	—	

Analysis of covariance of poison takes adjusted for infestation size by takes of prebait

Source	D.F.	M.S.	*F*	
Poisons	3	637	2·5	NS
Baits	3	698	2·7	
Poisons × baits	9	399	1·6	
Error	15	256	—	

NS = not significant.

for the present purpose to compare mortalities among naïve rats after the second day, thereby allowing for the probability that the shy rats would, in practice, have survived. Thus, considering the laboratory mortalities after 2 days (Greaves, 1966, Table 4, groups 1–6), seven out of twenty-four rats were killed by 0·5 % and 1·0 % norbormide compared with thirteen out of twenty-four by 2·5 and 5 % zinc phosphide. The χ^2 for this comparison, which is 2·14 ($P < 0{\cdot}20$) instead of 0·08 ($P < 0{\cdot}80$) for the mortalities on the fourth day, is of an order that might have become significant if more rats had been available for the tests.

Table 3. *The average weights of plain and poisoned medium oatmeal eaten per rat in free feeding tests in the laboratory, in which individually caged rats were given plain bait for one day and then poison bait for one day*

(From data supplied by J. H. Greaves)

Poison	Number of rats in test	Grams plain bait eaten per rat	Standard error	Grams poison bait eaten per rat*	Standard error	Number of rats killed
Norbormide (0·5 %)	28	18·3	1·5	1·2	0·2	18
Norbormide (1·0 %)	17	18·6	1·5	1·5	0·6	15
Zinc phosphide (2·5 %)	4	17·0	1·5	0·4	0·2	4

* The variances of the mean takes of poison bait were significantly different by Bartlett's test (Snedecor, 1956) so that the significance of the difference between their means cannot easily be tested.

However, excluding 'discriminators' from the mortalities in the laboratory data may not explain all the difference between the laboratory and field results. To examine the situation further, the takes of prebait and poison bait in the thirty-two prebaited treatments were compared to see if the intake of poison had been deficient in any of them.

The recorded numbers of dessertspoonfuls of plain bait taken on the sixth day of prebaiting and the numbers of teaspoonfuls of poison bait taken on first day of poisoning are given in Table 2. This shows that the error variances in the separate analysis of the two sets of data were of the same order as those due to poisons and baits and that this was partly due to differences in the size of the infestations. When the takes of plain bait were used to adjust the poison takes for infestation size, in an analysis of covariance, the residual variance of the poison data was considerably reduced. It did not, however, become significantly smaller than either of the reduced variances attributable to poisons or bait bases. Differences between the adjusted mean takes of the baits containing zinc phosphide or norbormide, also shown in Table 2, were not, therefore, significant although they indicated a possible inverse relationship between the amount of a poison bait eaten and the concentration of poison. Likewise the insignificantly different adjusted means of the four bait bases demonstrated, to the same limited extent, the possible advantage of a damp bait.

Zinc phosphide was superior in this field trial, in spite of the fact that the rats ate only insignificantly different quantities of each bait. This may seem surprising on considering the potential killing power, calculated from the acute toxicity tests, of equal weights of each bait. Measured in acute 50 or 95 % lethal doses, the killing power per gram of 1·0 % norbormide was nearly the same as that of 5·0 % zinc phosphide and therefore double the killing power of the same weight of 0·5 % norbormide or 2·5 % zinc phosphide.

The average weights of plain and poison bait eaten by the rats in the laboratory free feeding tests are given in Table 3. Rats in the three groups given 0·5 and 1·0 % norbormide or 2·5 % zinc phosphide ate uniformly of the plain prebait, but when given poison bait, those on norbormide ate three to four times more than did those given zinc phosphide. Differences between the proportions subsequently dying in each group were only insignificant. This result could have occurred if differences in the palatability or mode of action of the poisons was such as to allow the rats to eat three to four times more than the estimated acute lethal dose of norbormide, before the flavour or toxic symptoms produced an aversion to the bait. The result did not suggest, as it now does in the light of the result of the field trial, that in the field it may be necessary for rats to eat more 50 % or higher acute lethal doses of norbormide (as measured in the laboratory) than of zinc phosphide, to achieve the same percentage mortality. If this is so, it is a matter for further investigation, since it could be a disadvantage of norbormide that would be hard to overcome in the field.

Rats tolerant to high doses of norbormide, like some of those in the laboratory tests (Greaves, 1966) may also have influenced the field treatments. Large poison bait takes by a few tolerant individuals may have raised the average level of the norbormide takes sufficiently to mask the fact that most rats ate fewer 'acute lethal doses' than they appear to have done. Their presence in significant numbers might have accounted for most of the difference in the results with the two rodenticides.

The results of the farm treatments as a whole appear disappointing. However, direct comparisons between these and similar results recorded by Chitty & Southern (1954, Chs. 4 and 10) are not possible because the latter were assessed by census baiting, which continued until the weight of wheat consumed per day had risen to a steady level for 3 days. Furthermore, Chitty & Southern (or their collaborators) started prebaiting on the day on which they stopped census baiting, or poisoned directly only 3 days after. And even though census and treatment bait points were different, the prolonged census baiting immediately before treating may easily have conditioned the rats to taking baits as effectively as deliberate prebaiting. The standard 4–5 days' prebaiting advocated as a result of their work by these authors might not, in fact, be sufficient to obtain a maximal response except after census baiting. Our experience, supported by records of numerically increasing daily takes of bait, was that, after minimal censuses, 6 days' prebaiting was inadequate on at least fourteen farms. These treatments, eight with zinc phosphide and six with norbormide, would probably have been more succesful if prebaiting had continued until the takes had reached a peak. As it was, they

Table 4. *The number of takes of poison bait recorded on farms being treated with zinc phosphide or norbormide, used directly or for 7 days after prebaiting*

Poison		Day of direct poisoning										Day of poisoning following prebaiting						
		1	2	3	4	5*	6*	7	8	9	10	1	2*	3*	4	5	6	7
5·0 % zinc phosphide	Farm no. 6	25	12	6	11	—	—	9	5	0	0	Farm no. 5	24	—	—	1	0	0	0
	21	3	0	0	0	—	—	0	2	0	0	20	18	—	—	1	0	0	0
	27	7	4	0	0	—	—	0	0	0	0	26	6	—	—	2	0	0	0
	48	16	6	6	0	—	—	0	0	0	0	47	16	—	—	2	0	0	0
2·5 % zinc phosphide	Farm no. 8	8	4	1	2	—	—	0	0	0	0	Farm no. 7	21	—	—	3	0	0	0
	17	2	0	1	0	—	—	0	0	0	0	16	4	—	—	0	0	0	0
	36	18	7	2	7	—	—	7	1	0	0	35	2	—	—	—	0	0	0
	39	1	2	0	2	—	—	0	0	0	0	38	11	—	—	1	0	0	0
1·0 % norbormide	Farm no. 12	7	8	4	4	—	—	2	0	1	0	Farm no. 11	9	—	—	4	2	1	3
	15	8	10	5	10	—	—	7	0	4	6	14	15	—	—	15	4	5	9
	33	8	4	2	2	—	—	0	0	0	0	32	13	—	—	6	6	2	—
	42	9	11	10	10	—	—	10	12	9	9	41	25	—	—	11	6	5	3
0·5 % norbormide	Farm no. 3	10	5	2	1	—	—	3	3	2	1	Farm no. 2	12	—	—	12	7	8	6
	24	10	4	1	1	—	—	1	1	1	3	23	10	—	—	7	3	0	1
	30	13	5	4	4	—	—	5	0	5	0	29	63	—	—	26	8	2	1
	45	6	5	5	7	—	—	3	0	0	0	44	18	—	—	10	7	2	0

* The farms were not visited on Saturdays and Sundays.

probably contributed to the poor average results and, to a lesser extent, to the insignificant difference between the results by prebaiting and direct poisoning. It is also possible that the restriction imposed on the siting of the bait points, by reserving what may have been the best sites for the census baits, affected the treatments detrimentally.

The lack of any significant difference between direct and prebaited treatments in this trial was probably mainly caused by direct poison baiting for 10 days, instead of for a single day as Chitty and Southern did (1954, Ch. 4). The records in Table 4 show that although the maximum number of takes of zinc phosphide occurred on the first day of direct poisoning, the total number of takes doubled before the treatments finished. When, on the other hand, prebaited infestations were poisoned for 7 days, between 75 and 100% of all the zinc phosphide takes were recorded on the first day of poisoning (Table 4). Apart from showing why nothing significant was gained by prolonging the period of poisoning after prebaiting, the rapid acceptance of poison bait suggests less shyness and a take by a higher proportion of rats than would be attracted to directly laid baits.

The records of takes in the corresponding norbormide treatments have also been shown in Table 4, although they cannot be compared with the zinc phosphide records, because with the former poison it was impossible to distinguish takes by mice, which presumably continued to feed, unaffected by the concentration of norbormide used (Roszkowski *et al.* 1964). It is, therefore, possible, in the light of Drummond's and Taylor's records of persistent takes of norbormide for 3–4 weeks in urban treatments (personal communication) and of Greaves' evidence of rats surviving prolonged feeding on norbormide, that some of the takes recorded in Table 4 may have been by rats tolerant to its effects.

Non-toxicity to man and domestic animals remains the major advantage that norbormide has over zinc phosphide. This advantage was not enough to make up for its deficiencies as a rodenticide under the conditions prevailing on the farms between April and September, but it may be in the winter, when sheep and cattle are housed. Under most conditions the standard of acute poison treatments could probably be improved by using 0·5 or 1·0% norbormide and zinc phosphide together, employing the former in situations where the less specific rodenticide would be unsafe.

SUMMARY

Norbormide at 1·0 and 0·5% and zinc phosphide at 5·0 and 2·5% were each tested in four types of cereal bait, after prebaiting and as direct poisons, against infestations of *R. norvegicus* on forty-eight farms in Montgomeryshire.

The relative success of treatments was measured by the reduction in the number of takes from wheat baits put down for 2 days, 13 or 16 days before, and 5 days after, poisoning.

Treatments with zinc phosphide were significantly more successful than those with norbormide, irrespective of the cereal bait, concentration of poison or method of treatment used, and in spite of conditions on many farms that partially restricted the distribution of the baits containing zinc phosphide.

Differences between these results and results of laboratory tests with the same rodenticides are discussed.

Norbormide is recommended for use in situations where zinc phosphide cannot be used efficiently without risk to livestock.

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The effect of warfarin on plasma clotting time in wild house mice (*Mus musculus* L.)*

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INTRODUCTION

The results of studies of the toxicity of warfarin [3-(α-acetonyl)-4-hydroxycoumarin] to wild house mice (*Mus musculus* L.) have been reported earlier (Rowe & Redfern, 1964; 1965). The mice used were so-called 'normal' animals that had been hand-caught in corn-ricks not treated with warfarin, and suspected warfarin resistant animals that had been live-trapped in premises where prolonged but ineffective warfarin treatments had been carried out. In comparative feeding tests over 10 and 21 days with a cereal bait containing 0·025% warfarin it was found that mortality was lower and the time to death longer for the allegedly resistant mice.

The present experiments were designed to extend these findings by determining the clotting times of the plasma of normal and suspected resistant mice receiving warfarin and thus the changes in the combined level of three blood clotting factors—factor II (prothrombin), factor VII (proconvertin) and factor X (Stuart-Prower factor)—normally depressed by anticoagulants.

MATERIAL AND METHODS

Adult wild house mice only were used. Normal mice (N) were hand-caught in neighbouring corn-ricks. Suspected warfarin resistant mice were drawn from two sources: home-bred resistant animals (HBR) were 4-month-old animals whose parents had survived 21 days feeding on 0·025% warfarin in an oatmeal/mineral oil/sugar bait-base, and field resistant (FR) mice were taken in premises where extended treatments with 0·025% warfarin in medium oatmeal had been unsuccessful in eliminating infestations.

Warfarin was administered to the test animals either mixed in one of two cereal-type baits or by subcutaneous injection in a solvent, dimethyl sulphoxide. Mice given repeated warfarin doses by injection were dosed at intervals of 24 hr.

Blood samples, 0·15 ml., were withdrawn from the retro-orbital sinus of the mouse (Riley, 1960) and collected into 0·015 ml. of 3·8% sodium citrate. The blood was centrifuged, and the combined level of factors II, VII and X determined by a modification of the Quick one-stage prothrombin test, using 'Two Seven Ten' reagent (supplied by Diagnostic Reagents Ltd., Thame, Oxfordshire) and normal saline (0·85%) as diluent. Plasma that failed to clot after 15 min. was recorded as uncoagulable.

RESULTS

The clotting times of diluted plasma from normal mice

The relationship between clotting time and plasma dilution was first established for normal mice. For this purpose blood samples were taken from twelve males and twelve females. After centrifuging, the plasma from mice of the same sex was pooled and diluted by adding varying amounts of 0·85 % saline. The clotting time of the diluted plasma was then determined as in Table 1, where '100 % plasma' refers to plasma and saline in the ratio of 60:40, '50 % plasma' to plasma and saline in the ratio of 60:140, etc. It can be seen from Table 1 that increases in clotting time and hence falls in the combined level of factors II, VII and X became more marked with increasing plasma dilution.

Table 1. *Mean clotting times of normal male and female mouse plasma at various dilutions*

Percentage of 60:40 plasma/saline	Clotting time (sec.) and number of determinations: Male Mean	Male Range	Female Mean	Female Range
100	17·4 (4)	17·1–17·6	16·9 (3)	16·7–17·3
50	22·4 (4)	20·5–23·4	22·1 (4)	21·8–22·3
25	30·2 (4)	29·1–31·2	28·9 (4)	27·5–30·2
12·5	49·7 (4)	46·8–51·7	43·5 (4)	41·2–46·0
6·25	91·0 (4)	86·8–92·9	65·9 (4)	64·2–68·3
3·13	144·3 (4)	115·8–178·8	123·2 (4)	115·0–176·1

Table 2. *Mean plasma clotting times of untreated mice*

Source of mice (see text)	Sex	Number	Clotting time (sec.) Mean	Range
N	M	75	16·8	13·7–29·5
	F	81	16·6	14·0–22·3
HBR	M	63	16·7	14·6–25·7
	F	65	16·3	14·3–18·9
FR	M	19	16·3	13·5–21·6
	F	19	16·2	14·2–19·3

Variation in the clotting times of untreated mice

Table 2 shows the mean clotting times determined for mice from the three sources before administration of warfarin. Considerable individual variation in resting clotting times was observed but the differences between the mean values for mice of the same sex from the different sources and between those of males and females from the same source were not significant, the probability level being greater than 0·1 in each comparison.

Blood samples were also taken from twelve N mice daily for 1 week. The clotting times were found to vary daily in all individuals and tended to decline slightly over the period (six males averaged 15·3 and 14·1 sec.; and six females averaged 16·2 and 14·8 sec. on days 1 and 7 respectively).

Table 3. *The clotting time response of mice given a single dose of warfarin*

	Dose (mg./kg.)	Sex	Clotting time (sec.) after warfarin administration: 12 hr. Mean	12 hr. Range	24 hr. Mean	24 hr. Range	36 hr. Mean	36 hr. Range	48 hr. Mean	48 hr. Range	72 hr. Mean	72 hr. Range
N	1	M	32·1 (5)	26·3–36·8	41·6 (5)	24·3–64·5	20·1 (5)	15·2–31·6	17·5 (5)	14·8–25·2	15·1 (5)	13·0–16·2
		F	35·9 (5)	22·7–43·8	35·9 (6)	18·3–55·4	20·7 (6)	14·9–36·5	16·4 (5)	14·9–18·2	15·0 (5)	14·0–16·0
	5	M	—	—	97·5 (5)	49·5–140·3	—	—	17·7 (5)	14·8–24·5	—	—
		F	—	—	167·8 (5)	30·1–462·0	—	—	17·9 (5)	14·9–25·1	—	—
	50	M	—	—	124·7 (4)	72·6–177·0	—	—	24·8 (4)	18·3–34·1	—	—
		F	—	—	174·9 (5)	134·7–225·4	—	—	18·5 (5)	15·6–23·4	—	—
HBR	1	M	31·0 (5)	25·6–42·6	45·5 (5)	25·2–83·4	72·4 (5)	15·3–271·0	31·3 (5)	16·2–77·1	16·1 (5)	14·3–20·8
		F	22·9 (5)	17·7–34·6	17·2 (4)	16·3–17·7	20·0 (4)	14·3–34·3	14·5 (4)	13·9–15·1	13·6 (4)	13·1–14·4
	5	M	—	—	57·1 (5)	21·3–107·7	—	—	16·8 (5)	14·6–19·7	—	—
		F	—	—	26·4 (4)	14·3–44·8	—	—	14·9 (4)	13·5–15·4	—	—
	50	M	—	—	87·8 (4)	33·3–142·1	—	—	18·8 (4)	15·3–24·8	—	—
		F	—	—	41·3 (4)	26·8–68·3	—	—	15·9 (4)	14·2–16·9	—	—
FR	1	M	33·0 (6)	25·0–42·2	32·2 (7)	18·0–59·4	20·8 (7)	16·8–26·2	18·9 (6)	17·0–20·8	16·4 (7)	15·2–20·0
		F	17·0 (6)	16·4–18·2	25·0 (6)	16·2–33·5	18·8 (6)	16·5–27·2	19·9 (6)	16·2–28·5	14·8 (6)	14·0–15·2
	5	M	—	—	76·6 (6)	29·7–137·6	—	—	19·8 (6)	16·3–27·4	—	—
		F	—	—	23·2 (6)	17·8–33·2	—	—	19·9 (6)	15·4–35·5	—	—
	50	M	—	—	137·3 (7)	48·1–175·8	—	—	26·1 (7)	17·4–39·4	—	—
		F	—	—	24·3 (5)	17·8–40·2	—	—	18·5 (5)	17·5–19·9	—	—
	100	M	—	—	117·6 (7)	88·4–166·6	—	—	—	—	—	—
		F	—	—	24·0 (5)	16·9–36·1	—	—	—	—	—	—

Figures in paretheses indicate number of animals observed.

The effect of a single dose of warfarin on the clotting time of mouse plasma

Normal

A single dose of 1 mg. of warfarin/kg. body weight was administered by injection to each of eleven N mice and clotting time determinations made 12, 24, 36, 48 and 72 hr. later. After 2–3 week recovery periods the survivors were given further doses of 5 and 50 mg./kg. and clotting time determinations made after 24 and 48 hr. in each case.

Mean clotting times for the three doses of warfarin are given in Table 3. A marked increase in clotting time was found 12 hr. after the administration of the lowest dose of warfarin and clotting times were still prolonged after 24 hr. They then decreased and in six of the eleven animals investigated they had returned to normal levels after 36 hr. One mouse that still had an extended clotting time after 36 hr. died later. After 72 hr., the individual clotting times in eight of the ten survivors were slightly shorter than those determined before the administration of warfarin.

Longer clotting times were also apparent 24 hr. after the administration of the two higher warfarin dosages but as with the lowest dosage given the clotting times, with one exception, were nearly back to normal after 48 hr.

Home-bred resistant

In a similar study, ten HBR mice were given, at intervals, injected doses of 1, 5 and 50 mg. warfarin/kg. body weight. Clotting times were determined between 12 and 72 hr. after each dose. As with N mice, individual variation in response was observed and clotting times similarly increased with increasing warfarin dosages. The overall response was less marked however particularly in the case of females (Table 3).

Field resistant

The response of FR males given single injected doses of 1, 5 and 50 mg. of warfarin/kg. body weight was observed to be as marked as that found to occur in similarly treated N males (Table 3). FR females however, like HBR females, were markedly less responsive to the same three warfarin doses. After 24 hr. the mean clotting times of FR females (25·0, 23·2 and 24·3 sec. respectively) were considerably lower than the comparable values obtained with N females (32·2, 76·8 and 137·2 sec. respectively). Furthermore, increasing the dose of warfarin administered to female FR mice from 50 to 100 mg./kg. body weight did not result in any further increase in clotting times.

The effect of two standard doses of warfarin at 3 weeks interval on the clotting time of normal mouse plasma

Three groups of six N mice (three males and three females) were given by injection two identical doses of either 1, 5 or 50 mg. of warfarin/kg. body weight with an interval of 3 weeks between doses. Clotting times were determined before and after the administration of each dose.

Individual mice differed in their response to the same first dose of warfarin. The greater prolongation in clotting times that occurred with increasing warfarin dosage was evident 24 hr. after dosing. Most individuals responded similarly to the two identical warfarin doses (Table 4).

Table 4. *The clotting time (sec.) of normal mice given two doses of warfarin with an interval of 3 weeks*

Dose		Sex	Time after dose: 12 hr.	Mean	24 hr.	Mean	48 hr.	Mean
1 mg./kg.	1st	M	24·7		52·0		14·2	
			26·8	30·2	18·3	34·3	16·3	15·2
			39·1		32·5		15·1	
	2nd	M	36·4		20·3		16·9	
			29·4	35·0	28·7	25·4	16·4	16·5
			39·2		27·3		16·2	
	1st	F	28·4		20·0		17·2	
			29·3	29·2	19·6	20·4	14·7	15·9
			29·9		21·6		15·7	
	2nd	F	30·7		23·4		15·7	
			32·3	33·3	18·0	23·9	14·7	15·8
			37·0		30·3		17·1	
5 mg./kg.	1st	M	29·8		31·6		15·5	
			31·6	32·7	33·7	31·6	15·3	15·6
			36·7		29·5		15·9	
	2nd	M	27·8		71·5		20·4	
			36·2	33·2	21·6	57·7	15·6	17·2
			35·6		78·8		15·5	
	1st	F	27·3		54·4		14·3	
			30·4	32·8	135·7	111·9	550·0	194·1
			40·6		145·5		18·1	
	2nd	F	31·4		51·2		16·0	
			29·0	31·6	62·9	79·8	15·4	16·8
			34·4		125·3		19·1	
50 mg./kg.	1st	M	30·3		93·7		15·8	
			37·2	35·6	78·0	122·7	13·9	16·0
			39·3		196·5		18·3	
	2nd	M	27·3		88·8		15·8	
			30·6	27·8	80·2	79·2	27·6	22·7
			25·6		68·5		24·7	
	1st	F	30·4		93·6		15·7	
			31·2	31·6	103·3	98·5	16·5	16·2
			33·2		98·7		16·3	
	2nd	F	30·0		110·0		15·9	
			31·6	31·7	104·7	110·0	16·2	16·3
			33·4		115·4		16·9	

The effect of daily standard doses of warfarin on the clotting time of mouse plasma

Normal

Three groups of ten N mice were given daily for 10 days single doses of either 1, 5 or 50 mg. of warfarin/kg. body weight (Table 5). Clotting times were determined before the administration of the first dose (not shown in Table 5) and at 24 hr. intervals thereafter.

Table 5. *Mean clotting times (sec.) of normal and home-bred resistant mice given daily doses of warfarin*

		Dose of warfarin					
		1 mg./kg.		5 mg./kg.		50 mg./kg.	
	Days after first dose	M	F	M	F	M	F
Normal	1	34·5	37·4	84·3	154·1	125·0	89·9
	2	29·8	34·3	108·8 (1)*	164·2 (2)	141·0 (2)	458·4 (2)
	3	17·7	23·0	87·5 (2)	107·1 (2)	138·6 (1)	205·9 (2)
	4	21·7	25·9	105·0	— (3)	135·6 (1)	175·5 (3)
	5	19·6	28·6	39·2 (1)	— (2)	128·0 (1)	284·2 (1)
	6	22·1	29·9	34·9 (1)	— (1)	44·3 (2)	— (3)
	7	18·5	19·5	40·0 (1)	—	105·7 (1)	— (2)
	8	20·6	23·4	63·2	—	79·0 (1)	— (2)
	9	16·4	20·2	54·9	—	315·5	— (1)
	10	17·4	22·6	124·4	—	— (2)	— (1)
	Mortality	0/3	0/7	3/5	5/5	3/5	4/5
	Days to death	—	—	4,6,8	3,4,5,6,7	4,7,8	5,5,7,9
Home-bred resistant	1	28·0	17·4	48·2	27·1	119·7	46·5
	2	43·7	18·0	194·4	46·9	214·9	46·7
	3	53·8	16·9	175·0 (1)	43·6	58·0	31·6
	4	45·4	17·3	80·8	33·2	41·9	22·2
	7	74·0	15·6	97·5	26·5	57·9	18·0
	8	31·3	16·5	36·6	19·8	57·8	18·5
	9	24·2	16·4	35·5	23·9	48·9	17·3
	10	37·7	16·7	84·9	19·1	83·5	22·5
	Mortality	0/3	0/3	0/2	0/3	2/3	0/3
	Days to death	—	—	—	—	3,7	—

* Figures in parentheses indicate no. of animals with uncoagulable plasma.

At the lowest dosage only one mouse (a female) of the ten examined failed to respond. Maximum elevation of clotting times occurred from 24 to 48 hr. after the first dose. Clotting times then fell and the final values were similar to or less than those obtained before the administration of warfarin in all but two of the mice. At the two higher dosages, clotting times after 24 hr. were more elevated. The duration of the response was also greater, and after the second dose of warfarin the plasma of some individuals was found to be uncoagulable. Furthermore, fifteen animals died and the clotting times of the survivors (four males and one female) were still elevated after the tenth and final dose of warfarin.

Home-bred resistant

In a similar study with HBR mice, marked daily fluctuations in clotting times occurred in two of the three males given daily doses of 1 mg. of warfarin/kg. body weight (Table 5). The mean response of all three however was greater than that of the similarly treated N males. In contrast the response shown by the three HBR females to 1 mg./kg. was slight over the whole test period. Males were also clearly more affected than females when they were given either 5 or 50 mg./kg. doses of warfarin daily and two of the three males receiving the highest dose died. An initial prolongation in clotting time was observed when HBR females were given the two higher doses of warfarin, but after 10 days the clotting times obtained approached the pre-treatment values. No HBR females died.

The effect of 0·025 % warfarin in medium oatmeal on the clotting time of mouse plasma

Table 6 shows the results of feeding mice from the three different sources on 0·025 % warfarin in oatmeal and the proportion of animals having uncoagulable plasma at varying intervals during the test periods.

Table 6. *Comparative mortality and plasma coagulability of wild mice from three different sources when fed baits containing 0·025 % warfarin*

		Bait					
		Medium oatmeal			POFOSO		
Source		N	HBR	FR	N	HBR	FR
No. of mice		27	62	44	32	67	8
Proportion of mice with uncoagulable plasma on day	3	27/27	11/19	—	27/27	20/20	—
	4	—	13/15	8/12	9/9	—	—
	5	—	8/16	—	—	44/45	—
	7	4/4	9/12	3/29	5/5	—	—
	10	1/1	11/16	0/4	—	6/6	—
	14	—	3/13	1/25	—	—	—
	21	—	3/22	1/34	1/1	5/5	0/1
Mortality		27/27	40/62	10/44	31/32	62/67	7/8
% mortality		100	64·5	22·7	96·9	92·5	87·5
Range of days to death		4–12	6–17	5–11	1–10	4–19	7–20

N = Normal. HBR = Home-bred, warfarin-resistant. FR = Field-caught, suspected warfarin-resistant.

Normal

Twenty-seven N mice were treated. The clotting times of a group of three males and four females were found to be elevated on day 1 (range 44·7–70·7 sec.), had risen further by day 2 and the plasma of all seven individuals was uncoagulable by day 3. By day 7 three mice had died and the plasma of the other four was still uncoagulable. The plasma of the remaining twenty mice (ten males and ten females) was similarly found to be uncoagulable on day 3. All twenty-seven animals were dead after 12 days.

Home-bred resistant

Sixty-two HBR mice were similarly fed for 21 days. Clotting times were found to be elevated in all the thirty-one mice examined on day 2. In contrast with N mice, however, the plasma of eight mice examined on day 3 was coagulable (Table 6). Furthermore on days 10, 14 and 21 some individuals still had plasma that was coagulable. Again, in comparison with the complete kill obtained with N animals, mortality in HBR mice was low (64·5 %).

Examination of the clotting time values determined in the surviving animals (three males and twelve females) from which blood samples were taken repeatedly showed that they declined considerably during the test period and in some individuals were approaching near normal values by day 21 (range 15·0–146·0 sec.). The clotting times of the seven survivors (four males and three females) from which blood was taken on day 21 only, were comparatively higher, ranging between 51·4 and 308·8 sec. (four mice) and uncoagulable (three mice).

Field resistant

Forty-four FR mice drawn from four different localities were used in further comparative 21-day feeding tests. Eight of the twelve mice (seven males and five females) which were drawn from two localities and examined on day 4 had uncoagulable plasma on that day and died later. On the same day the clotting time values of the four female survivors were 52·6, 21·6, 30·0 and 31·5 sec. Lower values were determined on days 7, 10 and 14 and by day 21 the values had declined to 15·2, 17·3, 15·4 and 17·1 sec. respectively. Only three of twenty-four mice (thirteen males and eleven females) from the other two localities that were examined on day 7 had plasma that was uncoagulable and two, both males, were the only animals that died. Clotting times were found to be prolonged in all but one of the remaining twenty-one mice, but they then declined and on day 21 they were either below or approaching (range 14·4–36·7 sec.) the pre-treatment values.

A further eight individuals (six males and two females) from one of the latter localities all survived the feeding test and on day 21 the plasma of each mouse was found to be coagulable (range of clotting times 16·4–26·8 sec.). The overall mortality of FR mice was very low (10/44, 22·7 %).

The effect of 0·025 % warfarin in an oatmeal/mineral oil/sugar bait-base on the clotting time of mouse plasma

Normal

Thirty-two N mice (sixteen males and sixteen females) were fed 0·025 % warfarin in a bait-base comprising 85 % pinhead oatmeal, 5 % fine oatmeal, 5 % mineral oil and 5 % sugar (POFOSO) for up to 21 days and blood samples were taken daily. As with N mice fed 0·025 % warfarin in medium oatmeal, plasma withdrawn on day 3 failed to coagulate (Table 6). All but one of the thirty-two mice investigated died within 10 days. The survivor, a female, had uncoagulable plasma at the end of the test period.

Home-bred resistant

Two groups of forty-nine and eighteen HBR mice were fed 0·025 % warfarin in POFOSO bait for 21 and 26 days respectively. All twenty mice examined on day 3 had uncoagulable plasma and the overall mortality was high (62/67, 92·5 %). The plasma of the survivors (one male and four females) was found to be still uncoagulable at the end of the test periods.

Field resistant

In a single test, seven out of eight mice died after feeding on 0·025 % warfarin in POFOSO for 21 days. The plasma of the survivor (a female) was still coagulable at the end of the test period (clotting time 40·5 sec.).

Table 7. *Clotting times of seven mice following prolonged exposure to various warfarin baits*

		Sex of mice						
	Day	M	F	F	F	F	F	F
Before exposure	−1	16·5	18·1	17·7	16·4	15·5	17·8	16·5
0·025% warfarin in oatmeal	2	115·4	121·3	281·0	890·0	181·0	345·0	183·0
	21	41·6	25·2	27·9	34·6	70·0	87·1	39·8
	84	28·7	22·7	17·7	24·6	17·6	33·3	25·5
0·025 % warfarin in POFOSO	86	214·8	244·7	104·4	271·0	46·3	270·6	—
	89	UC*	UC	UC	UC	420·0	UC	UC
0·025 % warfarin in oatmeal	92	780·0	188·5	26·1	D(90)†	18·6	72·4	30·8
	96	32·2	18·5	17·9	—	18·4	23·8	18·1
0·1% warfarin in oatmeal	99	279·1	129·7	38·3	—	156·5	331·9	148·6
	124	UC	44·3	38·2	—	84·8	153·5	51·3
Diet 41 b	131	14·9	14·0	15·1	—	12·5	14·2	13·7
0·025 % warfarin in oatmeal	133	125·4	26·4	33·8	—	36·6	36·9	33·9
	138	34·7	25·0	17·6	—	15·4	21·8	27·8
0·025 % warfarin + 0·025 % sulphaquinoxaline in oatmeal	141	69·8	40·4	23·1	—	16·6	31·7	540·0
	152	107·6	28·5	20·8	—	27·2	76·5	65·4
0·025% warfarin in oatmeal + 5 % corn oil + 5 % sugar	155	281·0	26·8	22·1	—	50·4	90·1	226·2
	159	94·7	18·8	20·5	—	16·2	22·2	39·7
0·2 % warfarin in oatmeal	163	UC	145·5	47·8	—	UC	UC	UC
	209	D(197)	D(209)	UC	—	D(186)	UC	200·7

* UC = Uncoagulable. † D(90) = died on day 90.

The effect of prolonged exposure to various warfarin baits on the clotting time of mouse plasma

Seven of the twenty-two HBR mice (one male and six females) that had survived 21 days feeding on 0·025 % warfarin in plain oatmeal (column 2 of Table 6) were maintained on the same diet for an additional 9-week period. Clotting time determinations made on a further fifteen occasions showed that individual clotting

times varied irregularly during this extended feeding period. However, in all seven mice, clotting times were observed to be lower on day 84 than on day 21, and no mice died (Table 7).

The same animals were then subjected to other feeding tests. After 2 days feeding on 0·025 % warfarin in POFOSO, clotting times were found to be markedly elevated. Three days later (day 89) the plasma of six of the mice was found to be uncoagulable and the clotting time was prolonged in the case of the seventh animal. All seven mice were then once more fed on 0·025 % warfarin in plain medium oatmeal. One mouse died on day 90 but blood samples taken from the other six on two occasions during the next 7 days showed that clotting times were again declining or had returned to normal values. When the concentration of warfarin in the medium oatmeal bait was increased from 0·025 to 0·1 % (day 96) an increase in clotting time occurred. Determinations made on five occasions during the next 28 days showed that individual clotting times fluctuated irregularly. The plasma of one animal failed to coagulate at each of three successive samplings taken over a period of 14 days but on day 124 the clotting times of the other five individuals had again declined from peak values.

The mice were then transferred to a laboratory diet, diet 41*b*, (Bruce & Parkes, 1949) for 1 week, at the end of which (day 131) individual clotting times were below the pre-treatment values. The response of the mice to a further feeding period on 0·025 % warfarin in oatmeal was next investigated. After 2 days (day 133), prolonged clotting times in all six individuals were observed but the response was less marked than at the corresponding time in the first 21-day test period, when the same warfarin bait was used. Clotting times also tended to return to near normal values earlier than in the initial test period. The addition of 0·025 % sulphaquinoxaline to medium oatmeal bait containing 0·025 % warfarin in a further 14-day feeding period (beginning on Day 138) resulted in irregular but elevated clotting times. When the mice were fed warfarin in an oatmeal/corn oil/sugar bait-base, a further initial increase in clotting times occurred but on the seventh and last day (day 159), the clotting times had again declined considerably. In a final feeding period lasting 50 days, the mice were fed on 0·2 % warfarin in plain medium oatmeal. A marked rise in clotting times occurred and at some time during the test period the plasma of all individuals was rendered uncoagulable. Three mice died on days 186, 197 and 209 respectively.

The effect of a single dose of warfarin by injection and 0·025 % warfarin in medium oatmeal on the clotting time of mouse plasma

Normal

Clotting times of twelve N mice (six males and six females) were determined 3 days before and 24 and 48 hr. after each animal was injected with 5 mg. warfarin/kg. body weight. After a recovery period of 15 days the mice were fed on 0·025 % warfarin in medium oatmeal for 21 days. One animal, a female, survived the feeding period. In the females but not in the males (mean clotting time 59·3 sec.) there was some evidence of a negative correlation between the clotting time following

the injection of warfarin and the survival time during the subsequent feeding test. Female clotting times determined after 24 hr. were 103·8, 46·3, 50·5, 33·9, 25·8 and 26·5 sec. (mean 47·8 sec.) corresponding to deaths on days 6, 8, 10, 17, 19 and survival respectively.

In a similar test employing a dose of 10 mg. warfarin/kg. body weight, the clotting times of six males (mean 90·1 sec.) and four females (mean 71·5 sec.) were more elevated and all but one animal, a male, died during a subsequent feeding test. There was no obvious relationship between clotting and survival times in either males or females.

Home-bred resistant

In a single test with HBR mice, seven males and eleven females were injected with 10 mg. warfarin/kg. body weight before they were fed on 0·025 % warfarin in oatmeal for 21 days. Clotting times were determined 24 hr. before and after warfarin was injected. One male and four females survived the feeding test. Again with male animals, no obvious relationship was evident between clotting time (range 20·5–82·1 sec.) after the injection of warfarin and survival time during the feeding period. The response of the four female survivors, however, to the injected dose of warfarin (range of clotting times 15·2–21·4 sec.) was less than that of those that died (range of clotting times 30·9–168·1 sec.).

Table 8. *The clotting and survival times of field resistant mice receiving successive doses of warfarin and fed on* 0·025 % *warfarin bait*

	Clotting time after 24 hr. (sec.) Dose (mg./kg.)				Day to death during 21-day feeding period
Sex	1	5	50	100	
M	18·0	60·9	175·8	166·6	8
M	22·1	121·2	175·0	163·8	7
M	29·0	40·5	110·1	88·4	5
M	40·6	—	172·8	104·3	10
M	34·0	70·5	145·3	105·4	7
M	22·6	29·7	48·1	101·1	11
M	59·4	137·6	133·7	93·8	8
F	33·5	18·7	17·8	25·1	Survived
F	16·2	17·8	17·8	16·9	Survived
F	25·0	19·4	21·5	23·2	Survived
F	31·0	30·0	24·2	18·8	Survived
F	16·2	33·2	40·2	36·1	11

Field resistant

A shortage of test animals prevented a straightforward comparative test using FR animals. However, the FR mice referred to in Table 8 were also fed 0·025 % warfarin in medium oatmeal for 21 days after recovering from injected doses of warfarin. The individual clotting times 24 hr. after each of these mice had been given an injected dose of 1, 5, 50 and 100 mg. of warfarin/kg. body weight and the survival times in the feeding test given later are shown in Table 8. All seven

males died during the feeding test but, as with N and HBR males, no relationship was found between clotting time after dosing and the survival period. With females, however, the general lack of response even to the higher warfarin doses was in keeping with the low mortality (1/5) obtained during the feeding period.

DISCUSSION

Investigations into the changes in prothrombin times following the administration of coumarin and indanedione anticoagulants have been made in man and various laboratory and domesticated animals. It is clear from these studies that there is considerable inter-specific and intra-specific variation in response to these drugs. Using dicoumarol (3,3′-methylene-bis-4-hydroxycoumarin), Millar, Jaques & Henriet (1964) and Chandrasekhar, Hickie & Millar (1965) were able to classify rabbits and rats respectively into broad groups—non-reactors, poor reactors, reactors and hyper-reactors—on the basis of the change of prothrombin time. The wide range of days to death and percentage mortality that occurred when wild house mice were fed on bait containing warfarin (Rowe & Redfern, 1964) and other anticoagulants (Rowe & Redfern, unpublished) for fixed periods ranging from 4 to 28 days showed that this species also varies considerably in its susceptibility to anticoagulants. A further study (Rowe & Redfern, 1965) suggested that resistance to warfarin in mice was under genetical control.

As shown above, while untreated wild mice in the present study differed somewhat in their resting blood clotting factor levels, the mean clotting time values of untreated male and female mice from three different sources, or of males and females from the same source did not differ significantly. Nor was there any evidence of a clear-cut relationship between pre-treatment clotting time and the prolongation in clotting time that occurred when warfarin was administered.

The fluctuations and overall fall in clotting times that occurred when blood samples were taken daily from untreated mice is in keeping with the daily changes observed in rats following successive samplings (Pyorälä, 1965). Pyorälä comments that the stress imposed on experimental animals through repeated blood samplings may increase the activity of the sympathetic nervous system and lead to an increased production of blood clotting factors. In the present experiments therefore it is possible that the clotting factor levels of the individual mice from which blood was withdrawn regularly may have been abnormally increased and the effect due to warfarin reduced. Even so, such an effect cannot account for the marked differences in response to warfarin described in this paper both between mice derived from the same source and from the different sources.

All N mice responded to a single dose of warfarin at 1 mg./kg., and at higher doses (5 and 50 mg./kg.) clotting times became even more prolonged (Table 3). The overall response of HBR and FR mice given a single dose of 1 mg. of warfarin per kg. body weight (Table 3) was less marked than that found to occur in N individuals and was mainly due, in both cases, to a high degree of tolerance displayed by females. At the higher doses the increase in clotting time observed in HBR and FR male mice was not appreciably different from that seen in N males.

Chandrasekhar *et al.* (1965) point out that there are conflicting opinions concerning the development of tolerance to dicoumarol by rats. Mogenson, Fisher & Jaques (1958) and Boyd & Warner (1948) concluded that rats developed a tolerance after 1 and 2 weeks dicoumarol treatment respectively, whereas Slätis (1958) found no evidence that rats became tolerant to this anticoagulant following its administration for 5 weeks. Chandrasekhar *et al.* (1965) observed no development of tolerance following short-term administration of dicoumarol but found that tolerance developed during longer term administration. A similar conclusion with regard to warfarin is drawn from the present studies. The clotting times of N mice given two identical doses at an interval of 3 weeks were found to be similar. Most N individuals given 1 mg./kg. doses of warfarin daily for 10 days, however, appeared to develop a tolerance to the drug (Table 5). Not unexpectedly, the development of tolerance appeared to be dependent on the dose level of warfarin administered, the proportion of mice showing tolerance decreasing with increasing dosage. Home-bred resistant mice were able to develop tolerance to daily doses of warfarin at higher dosage levels than N mice. No male or female HBR mice died after receiving daily doses of either 1 or 5 mg. of warfarin/kg. body weight for 10 days and clotting times tended to decline from peak values during the test periods. At 50 mg./kg. some HBR males died but no females.

Differences in the response to warfarin of N, HBR and FR mice as judged by the relative amounts of poison bait consumed, clotting time determinations and overall mortality were clearly evident in the comparative feeding experiments (Table 6). Of twenty-seven N mice fed 0·025 % warfarin in oatmeal only eight were well enough to feed during the second week. Examination of blood samples showed that each animal had uncoagulable plasma by the third day and all the mice were dead after 12 days. In the similar study with sixty-two HBR mice, however, forty-seven animals continued to feed in the second week and twenty-eight in the third week. Of the twenty-two survivors, seventeen fed fairly evenly throughout the 3-week period; the bait consumption of the other five declined markedly during the second week but increased in the third week. Although some individuals had uncoagulable plasma after 3 days feeding, the plasma of all but three of the twenty-two surviving mice (mortality 64·5 %) was still coagulable on day 21, the last of the test period. In the study of FR mice, forty of the forty-four animals fed during the second week and thirty-four continued to feed during the third week and survived the test period. All but five of the thirty-four survivors (mortality 22·7 %) fed adequately throughout the test periods. On day 21 the clotting time of all but one of the survivors was near the pre-treatment value. In the above tests with HBR and FR mice relatively more males died (35/59) than females (15/47) the difference being highly significant [$\chi^2 = 6{\cdot}8$; $P = 0{\cdot}01$–$0{\cdot}001$]. This result is in agreement with our earlier findings that a sex difference in susceptibility to warfarin exists in mice (Rowe & Redfern, 1964, 1965, 1967). A similar conclusion was reached by Roll (1966).

The coagulability of the plasma of N, HBR and FR mice was impaired comparatively earlier when they fed on 0·025 % warfarin in the oily bait-base POFOSO instead of in plain oatmeal and mortality was also higher (Table 6). Markedly

prolonged clotting times were also observed within 2 days when seven HBR mice were fed on warfarin in POFOSO after having fed on warfarin in plain oatmeal for 12 weeks (Table 7). Neither an increase in the concentration of warfarin to 0·1 % nor the inclusion of sulphaquinoxaline or corn oil in the latter bait was as effective as 0·025 % warfarin in POFOSO in decreasing the coagulability of the plasma of these individuals. Although no marked increases in plasma clotting times were observed in other tests when mice were fed plain POFOSO, it is possible that as with rats (Drummond, 1967), mineral oil produces a situation favourable to the action of warfarin by interfering with the absorption of vitamin K from the gastro-intestinal tract.

At the present time mice are screened for resistance to warfarin in this laboratory on the basis of their ability to withstand a standard 21-day feeding test on 0·025 % warfarin in plain medium oatmeal. Clearly it would be advantageous to assess levels of resistance by a less laborious and time consuming method such as the change in clotting time following the injection of a single dose of warfarin. The results of injection and feeding tests on mice in the present study are sufficiently encouraging in the case of female animals to warrant further investigation of this aspect.

The data presented here support our previous conclusion that wild mice differ considerably in their susceptibility to warfarin. The abnormal level of resistance to warfarin shown by HBR mice also supports the earlier conclusion that warfarin resistance in wild mice is inherited. A similar conclusion may be drawn from the differences in response to warfarin shown by different laboratory strains of mice (Rowe & Redfern, unpublished) and from the increased level of resistance found by Roll (1966) when two strains of laboratory mice were exposed to warfarin in breeding and selection experiments conducted over several generations. Selection for resistant individuals is also likely to occur when free-living wild house mouse populations are continually exposed to warfarin during poison treatments and, as a result, populations having an order of resistance shown by the FR mice examined in this study may arise. The physiological mechanisms involved in warfarin resistance in mice are unknown, but the present experiments clearly show that some individuals that survived prolonged feeding on 0·025 % warfarin in oatmeal developed a tolerance to the drug. The combined level of factors II, VII and X in their blood diminished initially but then tended to recover. It is suggested that the development of tolerance is most likely an outcome of an increased ability to metabolize warfarin and that detoxification results from increased enzyme activity (Brodie, Maickel & Jondorf, 1958).

Although the response of mice to warfarin was found to increase in the presence of mineral oil it was also found that a few individuals could survive 21 days feeding on 0·025 % warfarin in bait containing mineral oil. These animals showed no haemorrhagic tendencies although their blood was probably uncoagulable for most of that time. In these very resistant individuals it may be further necessary therefore to interfere with other haemostatic mechanisms such as the adhesiveness of the blood platelets or vascular integrity in order to bring about haemorrhage and death (Jaques, 1959).

SUMMARY

The coagulability of the plasma of blood taken from the retro-orbital sinus of untreated and warfarin-treated wild house mice (*Mus musculus* L.) was determined. Individual differences were observed in the resting clotting times of animals drawn from three different sources, so-called 'normal' (N) mice, home-bred warfarin resistant (HBR) mice and field-caught suspected warfarin resistant (FR) mice. Mice from the three sources also showed wide individual variation in response to injected doses of 1, 5 and 50 mg. of warfarin/kg. body weight. The overall response shown by HBR and FR mice, particularly females, was less than that shown by N animals. Some FR females failed to respond to a dose of 100 mg. of warfarin/kg. body weight.

Normal mice showed a similar response to two identical doses of warfarin (1, 5 or 50 mg./kg. body weight) given at an interval of 3 weeks. They developed a tolerance to repeated daily injected doses of 1 mg. of warfarin/kg. body weight but not to the higher doses (5 and 50 mg./kg.) to which some HBR animals became tolerant.

In comparative 21-day feeding tests with 0·025% warfarin in medium oatmeal, the mortality in N, HBR and FR mice was 100% (27), 64·5% (40/62) and 22·7% (10/44) respectively. Whereas the plasma of all N mice was rendered uncoagulable after 3 days and the animals died within 12 days, the clotting times of the surviving HBR and FR mice (24/59 males and 32/47 females) either were not at any stage appreciably increased or had declined to near normal values by the end of the test period.

In similar tests with 0·025% warfarin in oatmeal bait containing 5% mineral oil, it was found that the clotting ability of the plasma of N, HBR and FR mice was impaired earlier and that mortality in HBR and FR animals was significantly higher (62/67, 92·5% and 7/8, 87·5% respectively).

Three of seven HBR mice died after prolonged feeding on various warfarin baits but only after 186, 197 and 209 days respectively. The inclusion of mineral oil in bait containing 0·025% warfarin was more effective in decreasing the coagulability of the blood of these mice than either increasing the concentration of warfarin (to 0·1 and 0·2%) or including sulphaquinoxaline or corn oil.

Studies on mice given warfarin by injection and in feed indicated that in females the level of resistance to warfarin may be assessed on the basis of their response to a single injected dose of warfarin.

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