Vol. 69, No. 3 September 1971

THE JOURNAL OF HYGIENE

(Founded in 1901 by G. H. F. Nuttall, F.R.S.)

EDITED BY

R. M. FRY, M.A., M.R.C.S.

IN CONJUNCTION WITH

P. ARMITAGE, M.A., PH.D.

R. D. BARRY, M.A., B.V.Sc., PH.D.

W. I. B. BEVERIDGE, D.V.Sc., M.A.

A. W. DOWNIE, M.D., F.R.S.

R. I. N. GREAVES, M.D., F.R.C.P.

E. T. C. SPOONER, C.M.G., M.D., F.R.C.P.

Sir GRAHAM WILSON, M.D., LL.D., F.R.C.P., D.P.H.

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W.1

American Branch: 32 East 57th Street, New York, N.Y. 10022

Subscription per volume £7 net ($23.00) (4 parts)

Single parts 40*s. net* ($6.50)

The Journal of Hygiene

Papers for publication should be sent to Dr R. M. Fry, Department of Pathology, Tennis Court Road, Cambridge CB2 1QP. Papers forwarded to the Editor for publication are understood to be offered to *The Journal of Hygiene* alone, unless the contrary is stated.

Contributors receive twenty-five copies of their paper free. Additional copies may be purchased; these should be ordered when the final proof is returned.

SUBSCRIPTIONS

The Journal of Hygiene is published quarterly.

Single parts £2.00 net ($6.50 in the U.S.A.) plus postage.

Four parts form a volume. The subscription price of a volume (which includes postage) is £7.00 net ($23.00 in the U.S.A.).

Second class postage paid at New York, N.Y.

Quotations for back volumes may be obtained from the publisher.

Claims for missing issues cannot be entertained unless received within three months after the date of publication.

Orders should be sent to a bookseller or to the publisher:

Cambridge University Press

Bentley House, 200 Euston Road, London NW1 2DB

32 East 57th Street, New York, N.Y. 10022

J. Hyg., Camb. (1971), **69**, 323
Printed in Great Britain

Strontium chloride B and E.E. enrichment broth media for the isolation of *Edwardsiella*, *Salmonella* and *Arizona* species from tiger snakes

BY J. B. IVESON

Salmonella Reference Laboratory, State Health Laboratory Service, Perth, Western Australia

(*Received* 17 *September* 1970)

SUMMARY

Strontium chloride B medium and E.E. broth have been found effective in the recovery of *Edwardsiella*, *Salmonella* and *Arizona* species from the cloacal contents of tiger snakes (*Notechis scutatus*). Strontium chloride B medium was superior to E.E. broth.

At least one bacterial species was detected in each of the 60 reptiles examined, and all three organisms were recovered from each of 29 snakes on a single examination.

Strontium chloride M, strontium selenite and Rappaport enrichment media and bismuth sulphite agar, although satisfactory for the isolation of *Salmonella* and *Arizona* species, were found unsuitable for *Edwardsiella tarda*.

INTRODUCTION

A new genus of Enterobacteriaceae, embracing a single species, was described as *Edwardsiella tarda* by Ewing, McWhorter, Escobar & Lubin (1965). The species has been isolated from man (Sonnenwirth & Kallus, 1968; Okubadejo & Alausa, 1968; Jordan & Hadley, 1969) and an increasing range of animals, particularly reptiles. In contrast to the four *Salmonella* subgenera, few details of culture procedures have been published. However, in India, Bhat, Myers & Carpenter (1967) reported four *Edwardsiella* isolations from children and commented that colonies on MacConkey and deoxycholate agar resembled *Salmonella* species. The organisms grew on bismuth sulphite agar and survived culture in selenite F broth. It was further reported by Sakazaki (1967) that strains isolated from Japanese reptiles grew on brilliant green agar. Satisfactory growth of laboratory strains on xylose lysine agar was also noted by Taylor (1965).

In Western Australia the first isolation of *Edwardsiella tarda* was made from the faeces of an infant with acute gastro-enteritis. Subsequently, the organisms were isolated from the cloacal contents of three tortoises, three crocodiles, a sewage effluent swab and the litter from eight tiger snakes. It was observed that *E. tarda* occasionally survived, but did not multiply, in selenite F, Rappaport or strontium selenite enrichment media. It was also noted that the organisms usually failed to produce colonies on bismuth sulphite (B.S. agar).

0 C O 2514

Further investigations showed that *Edwardsiella* colonies, although somewhat smaller than those of *Salmonella* species, developed satisfactorily on deoxycholate-citrate-agar and S.S. agar. The species also multiplied in the Enterobacteriaceae enrichment broth (E.E. broth) of Mossell, Visser & Cornelissen (1963), and in the strontium chloride medium of Iveson & Mackay-Scollay (1969) when the malachite green was omitted (strontium chloride B medium).

Following favourable preliminary tests comparative trials were undertaken to evaluate the efficiency of E.E. broth (Oxoid) and strontium chloride B medium, for the isolation of *Edwardsiella*, *Salmonella* and *Arizona* species from reptilian materials. The technique involved and the results obtained are presented in this report.

MATERIALS AND METHODS

Specimens

A total of 60 tiger snakes (*Notechis scutatus*), together with 15 contaminated sawdust-litter samples from 39 of the snakes, were examined. The reptiles were active, healthy adults, captured in a remote swampy area near the southern coast of Western Australia. Cloacal swabs were taken on five separate occasions within a few days of capture.

Swabs were placed in 1–2 ml. volumes of Sachs (1939) buffered glycerol saline at the time of sampling, and approximately 2 g. of sawdust litter were sown in 10 ml. volumes of the transport medium. All samples were well mixed before processing the same day.

Plating media

S.S. agar (Oxoid) and modified bismuth sulphite agar (B.S.A.), were used for direct plating of reptile samples and subculturing from enrichment media. The B.S.A. medium was prepared by reconstituting dehydrated bismuth sulphite agar (Oxoid), as recommended by the manufacturer, but immediately before pouring, 10 ml. of 1 % ferrous sulphate and 3·0 ml. 10 % ferric citrate were added to 1 l. of the molten medium. Plates were dried for approximately 30 min. with the lids removed, and were stored at 4° C. overnight, or up to 4 days, before use. The additive solutions were sterilized by heating at 60° C. for 1 hr. and were stored for use at 4° C.

Enrichment media (strontium chloride B enrichment broth)

Bacto tryptone (Difco)	0·5 g.
Sodium chloride	0·8 g.
Potassium dihydrogen phosphate	0·1 g.
60 % strontium chloride	6·0 ml.
Distilled water	100 ml.

The medium was distributed in 10 ml. volumes and sterilized by steaming for 30 min. The final concentration of strontium chloride was 3·4 % and the pH was 5·0–5·5.

Oxoid E.E. broth

The dehydrated medium was reconstituted as recommended by the manufacturers, distributed in 10 ml. volumes and sterilized by steaming for 30 min.

Other enrichment broth media

The strontium selenite, strontium chloride M, Rappaport, Selenite F. and tetrathionate enrichment media used, were prepared as reported by Iveson & Mackay-Scollay* (1969).

Cloacal swabs and litter samples were first inoculated on the solid plating media, and the swabs, together with approximately 0·5 ml. of each sample, were added to 10 ml. volumes of the particular enrichment medium used, in a series of five separate studies.

Subcultures were made from enrichment media after 24 hr. incubation at 37° C. Non-lactose-fermenting colonies on S.S. agar, and colonies resembling *Salmonella* and *Arizona* species on the modified B.S.A. medium, were examined biochemically and serologically.

Approximately 20 colonies were examined from each specimen. During the investigation up to 50 colonies were identified from selected specimens as a control check on routine recoveries.

RESULTS

A total of 241 isolations comprising 109 *Arizona*, 82 *Salmonella* and 50 *Edwardsiella* species were made from 100 cloacal swabs and 15 snake-litter samples. While 117 (49 %) isolations were made by direct culture, 235 (98 %) isolations were made by enrichment culture.

Table 1. *Distribution frequency of* Salmonella, Arizona *and* Edwardsiella *species in* 100 *cloacal samples from* 60 *tiger snakes and* 15 *snake-litter samples*

Species isolations			Specimens		
Salmonella	*Arizona*	*Edwardsiella*	Cloacal swabs	Litter	Totals
+	+	+	34	0	34
+	+	−	32	13	45
+	−	+	1	0	1
−	+	+	12	1	13
+	−	−	2	0	2
−	+	−	16	1	17
−	−	+	2	0	2
	Total positive		99	15	114

Direct plating yielded 83 (76 %) *Arizona*, 24 (29 %) *Salmonella*, and 10 (20 %) *Edwardsiella* isolations, whereas by enrichment methods 105 (96 %) *Arizona*, 82 (100 %) *Salmonella*, and 48 (96 %) *Edwardsiella* isolations were obtained.

* In this paper (*Journal of Hygiene*, 1969, **67**, 457–64) there are three errors on page 459. Line 10: *for* Distilled water 100 ml. *read* Distilled water to 100 ml. Line 14: *for* 10 ml. solution B *read* 6 ml. solution B. Line 36: *for* pH 7·8 *read* pH 6·8.

Table 2. *Relative efficiency of direct and enrichment culture methods in five experiments in isolating* Salmonella, Arizona *and* Edwardsiella *species from* 100 *cloacal swabs of* 60 *tiger snakes and* 15 *snake-litter samples*

			Salmonella			*Arizona*			*Edwardsiella*		
Expt.	Samples	Culture method	Positive		efficiency (%)	Positive		efficiency (%)	Positive		efficiency (%)
I	23	Direct	6	(17)	35·3	18	(23)	78·3	1	(1)	100
		Strontium selenite	11		64·7	21		91·3	0		0·0
		Strontium chloride M	16		94·1	17		73·9	0		0·0
		Rappaport	12		70·6	15		65·2	0		0·0
II	32	Direct	5	(23)	21·7	26	(32)	81·3	3	(12)	25·0
		Strontium selenite	17		73·9	28		87·5	0		0·0
		E.E	15		65·2	24		75·0	11		91·7
III	19	Direct	4	(15)	26·7	12	(18)	66·7	1	(9)	11·1
		Strontium chloride M	13		86·7	17		94·5	0		0·0
		E.E	9		60·0	9		50·0	9		100
IV	20	Direct	1	(11)	9·1	9	(16)	56·3	0	(9)	0·0
		E.E	11		100	14		87·5	9		100
V	21	Direct	8	(16)	50·0	18	(20)	90·0	5	(19)	26·3
		Strontium chloride B	13		81·3	19		95·0	16		84·2
		E.E	11		68·8	11		55·0	10		52·6
Totals	115			(82)			(109)			(50)	

Figures in parentheses indicate samples yielding positive isolations.

* The samples in Experiment I comprised 8 cloacal swabs and 15 snake-litter samples.

At least one bacterial species was recovered from 114 (99 %) of the test samples. Of these (see Table 1), 34 (30 %) samples yielded all three species together, 59 (52 %) two species, and 21 (18 %) a single species isolation.

Table 2 (Expt. I) shows the relative efficiency of culture methods before the introduction of E.E. broth and strontium chloride B medium. The Rappaport, strontium chloride M and strontium selenite media combination produced 23 *Arizona* and 17 *Salmonella* isolations from the 23 test samples. The single *Edwardsiella* isolation was recovered by direct plating on S.S. agar, and not by enrichment methods.

The relative efficiency of direct and enrichment culture methods in the recovery of *Salmonella*, *Arizona* and *Edwardsiella* species in the four subsequent experiments are also illustrated in Table 2. In Expt II strontium selenite was slightly superior to E.E. broth in the isolation of *Salmonella* and *Arizona* spp. In Expt. III strontium chloride M was considerably better than E.E. broth for the same two organisms, and in Expt. V a similar difference was seen between strontium chloride B and E.E. broth. In all these experiments and in Expt. IV, where only direct plating and E.E. broth were compared, direct plating was always inferior to the enrichment methods used.

Enrichment in E.E. broth was always superior to direct culture for the isolation of *Edwardsiella* spp. However, in Expt. V strontium chloride B medium was superior to both these procedures in the recovery of the same organism. Edwardsiella organisms were not recovered by strontium selenite or strontium chloride M enrichment media.

The *Salmonella* and *Arizona* serotypes recovered are as follows (the figures in parenthesis indicate the frequency of serotype isolations): *S. adelaide* (3), *S. fremantle* (49), *S. houten* (2), *S. onderstepoort* (7), *S. potsdam* (1), *S. ramat gan* (43), *S. slatograd* (1), *S. wandsbek* (5), *S. species* (1), *Arizona* 5: 29–30 (3), *Arizona* 16: 23–25 (29), *Arizona* 20: 29–25 (46), *Arizona* species (37).

A single *Salmonella* serotype was recovered from 54 samples, 25 samples yielded 2 serotypes, and 3 samples yielded 3 *Salmonella* serotypes. The serotyping of *Arizona* strains has yet to be completed.

DISCUSSION

Using strontium chloride B enrichment medium and E.E. broth it has been demonstrated that tiger snakes in a remote habitat are naturally infected simultaneously with *Salmonella*, *Arizona* and *Edwardsiella* species. The reluctance of the latter species to grow in the widely used isolation media, together with unusual biochemical characteristics, may account in part for its infrequent isolation and tardy recognition by bacteriologists.

The occurrence of *E. tarda* in animals and its pathogenic role in man requires further investigation. The species referred to as 'Serotype 1483–59' was first investigated by Ewing in America, and later designated *Edwardsiella tarda* by Ewing *et al.* (1965). It is the same type organism as the Bartholomew group of King & Adler (1964), the Asakusa group of Sakazaki & Murata (1962) and Sakazaki (1965, 1967).

Human isolations reported in America by Ewing *et al.* (1965), Jordan & Hadley (1969) and from India by Bhat *et al.* (1967) indicate that the organism may be present both with and without symptoms in juveniles and adults. The species has also been recovered in America from a fatal adult meningitis case by Sonnenwirth & Kallus (1968) and from a neonatal meningitis, also fatal, reported from Nigeria by Okubadejo & Alausa (1968). Further isolations, predominantly from snakes but including five from cases of human enteritis and two from seals, were reported in Japan by Sakazaki (1965). The organisms have also been recovered from a sea-lion and two alligators in a Florida zoo by Wallace, White & Gore (1966), and from three samples of pig bile in a Phillipine abattoir by Arambulo, Westerlund & Sarmiento (1968).

The widespread distribution of *Edwardsiella* species is further illustrated in a report by d'Empaire (1969), who investigated the growth requirements of strains isolated in France, Tahiti, Tchad and Vietnam. The study included cultures recovered from humans, cattle, reptiles, a pig and a panther.

In an ecological survey of reptiles in Western Australia conducted before the introduction of E.E. broth and strontium chloride B medium, Iveson, Mackay-Scollay & Bamford (1969) were unable to recover *E. tarda* in 116 reptiles examined, although *Salmonella* and *Arizona* serotypes were detected in 83·6 %. However, *E. tarda* was later isolated, using direct culture on S.S. agar, from human faeces, and the cloacal contents of three reptiles, all originating in geographically remote areas. The later recovery of *E. tarda*, again by direct culture from the samples illustrated in Table 1, indicated that routine enrichment methods might be too inhibitory, a factor influencing the selection and trial of the less inhibitory E.E. broth and strontium chloride B enrichment.

E.E. broth was recommended as an enrichment medium for Enterobacteriaceae in the bacteriological examination of foods by Mossell *et al.* (1963), and animal feedingstuffs by Schothorst, Mossell, Kampelmacher & Drion (1966). Additionally, it was suggested that it might be of value as a pre-enrichment medium for labile salmonellas before exposing cells to the more toxic selenite or tetrathionate enrichment broths. The medium was, however, more inhibitory to non-Enterobacteriaceae than the mannite broth of Taylor (1961) and the lactose broth used by North (1961).

The ability of *Escherichia coli*, as well as other non-pathogenic Enterobacteriaceae, to multiply in E.E. broth, severely limits the performance of the medium in the isolation of *Salmonella* and *Edwardsiella* species from human and domestic animal faeces. However, by contrast, the more selective strontium chloride B medium, has shown a marked ability to inhibit other Enterobacteriaceae and to grow *Salmonella*, *Arizona* and *Edwardsiella* species from human and animal excreta when tested at 37° and 43° C., in parallel with our routine isolation procedures.

Salmonella, *Arizona* and *Edwardsiella* species from single cloacal specimens were only recovered together from strontium chloride B or E.E. broth enrichment, subcultured to S.S. agar. The modified B.S. agar in routine use in the laboratory, while it provided ready differentiation of *Salmonella* and *Arizona* colonies, was unsatisfactory for the recognition or recovery of *Edwardsiella*, and these organisms were

identified exclusively on S.S. agar. It was found essential to use the S.S. and B.S. media with their different inhibitory and colony indicator systems, in order to recover the three species together from single samples.

Few workers have reported on the colony morphology of *Arizona* and *Edwardsiella*, or on the suitability of routine enrichment procedures for their simultaneous growth. However, in a report on the occurrence of the *Arizona* group in animals and man, Edwards, McWhorter & Fife (1956) drew attention to the frequent occurrence of lactose-fermenting *Arizona* serotypes, and, as a consequence, isolation difficulties, when only S.S. or D.C. agar were used. Harvey, Price & Dixon (1966) reported that Wilson and Blair medium was more suitable for the easy recognition of *Arizona* spp. In the present study it was noted that, in addition to the familiar black colonies on the modified B.S. agar, *Arizona* species also developed as dark-green to black centred colonies with a pale-green periphery and without a metallic sheen. On S.S. agar *Arizona* colonies often resembled typical lactose-fermenting *Esch. coli*.

The modified B.S. agar was found too inhibitory for *Edwardsiella*. The few laboratory strains which grew on the surface of the medium provided minute, pale-green colonies which failed to develop on continued incubation. On S.S. agar, after incubation for 18–24 hr., *E. tarda* yielded small non-lactose fermenting colonies, frequently resembling *S. pullorum* colonies. With further incubation they became larger and occasionally developed dark centres somewhat resembling *Proteus* colonies.

In separate experiments, using ten pure cultures of *E. tarda* previously isolated in the laboratory, it was found that the species failed to multiply at 37° C. in selenite F, tetrathionate, Rappaport, strontium chloride M or strontium selenite enrichment broths. The sensitivity of *E. tarda* to selenite salt was also observed by Lapage & Bascomb (1968) during selenite reduction tests. All ten strains multiplied rapidly in 1 % peptone water, meat infusion and E.E. broth. The organisms grew readily on D.C. agar, and usually formed characteristic dark-centred colonies within 24 hr.

My thanks are due to the late Dr K. Patricia Carpenter for confirming the identity of *Edwardsiella* strains, to Dr Joan Taylor for confirming the *Salmonella* serotypes and Dr R. Rohde for serotyping the *Arizona* strains; to Mr M. H. Lello for preparing the strontium hydrogen selenite salt used in the investigation; to my colleagues, Mr V. Bamford and Mrs Joy Carter; to Mr Softly and Mr J. Stewart for procuring and handling the venomous tiger snakes; and to Dr W. S. Davidson, Commissioner of Public Health, Western Australia, for permission to publish.

REFERENCES

Arambulo, P. V., Westerlund, N. C. & Sarmiento, R. V. (1968). On the isolation of human enteric organisms from the bile of pigs and cattle. *Acta medica phillippina* **5**, no. 2, 84.

Bhat, P., Myers, R. M. & Carpenter, K. P. (1967). *Edwardsiella tarda* in a study of juvenile diarrhoea. *Journal of Hygiene* **65**, 293.

d'Empaire, M. (1969). Les facteurs de croissance des *Edwardsiella tarda*. *Annales de l'Institut Pasteur, Paris* **116**, 63.

EDWARDS, P. R., MCWHORTER, A. C. & FIFE, M. A. (1956). The *Arizona* group of Enterobacteriaceae in animals and man. *Bulletin of the World Health Organization* **14**, 511.

EWING, W. H., MCWHORTER, A. C., ESCOBAR, M. R. & LUBIN, A. H. (1965). *Edwardsiella* – a new genus of Enterobacteriaceae based on a new species – *Edwardsiella tarda*. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **15**, 33.

HARVEY, R. W. S., PRICE, T. H. & DIXON, J. M. S. (1966). Salmonellas of subgenus III (Arizona) isolated from abattoirs in England and Wales. *Journal of Hygiene* **64**, 271.

IVESON, J. B. & MACKAY-SCOLLAY, E. M. (1969). Strontium chloride and strontium selenite enrichment broth media for the isolation of *Salmonella*. *Journal of Hygiene* **67**, 457.

IVESON, J. B., MACKAY-SCOLLAY, E. M. & BAMFORD, V. W. (1969). Salmonella and Arizona in reptiles and man in Western Australia. *Journal of Hygiene* **67**, 507.

JORDAN, G. W. & HADLEY, W. K. (1969). Human infection with *Edwardsiella tarda*. *Annals of Internal Medicine* **70**, 283.

KING, M. B. & ADLER, D. L. (1964). A previously undescribed group of Enterobacteriaceae. *American Journal of Clinical Pathology* **41**, 230.

LAPAGE, S. P. & BASCOMB, S. (1968). Use of selenite reduction in bacterial classification. *Journal of Applied Bacteriology* **34**, 568.

MOSSELL, D. A. A., VISSER, M. & CORNELISSEN, A. M. R. (1963). The examination of foods for Enterobacteriaceae using a test of the type generally adopted for the detection of Salmonellae. *Journal of Applied Bacteriology* **26**, 444.

NORTH, W. R. (1961). Lactose pre-enrichment for the isolation of Salmonellae from dried egg albumen. Its use in a survey of commercially produced albumen. *Applied Microbiology* **9**, 188.

OKUBADEJO, O. A. & ALAUSA, K. O. (1968). Neonatal meningitis caused by *Edwardsiella tarda*. *British Medical Journal* iii, 357.

SACHS, A. (1939). Difficulties associated with the bacteriological diagnosis of bacillary dysentery. *Journal of the Royal Army Medical Corps* **73**, 235.

SAKAZAKI, R. (1965). A proposed group of the family Enterobacteriaceae; the Asakusa group. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **15**, 45.

SAKAZAKI, R. (1967). Studies on the Asakusa group of Enterobacteriaceae (*Edwardsiella tarda*). *Japanese Journal of Medical Science and Biology* **20**, 205.

SAKAZAKI, R. & MURATA, Y. (1962). The new group of Enterobacteriaceae, the Asakusa group. *Japanese Journal of Bacteriology* **17**, 616.

SCHOTHORST, VAN M., MOSSELL, D. A. A., KAMPELMACHER, E. H. & DRION, E. F. (1966). The estimation of the hygienic quality of feed components using an Enterobacteriaceae enrichment test. *Zentralblatt für Veterinärmedizin* B **13**, 273.

SONNENWIRTH, A. C. & KALLUS, B. A. (1968). Meningitis due to *Edwardsiella tarda*. *American Journal of Clinical Pathology* **49**. 92.

TAYLOR, W. I. (1961). Isolation of *Salmonellae* from food samples. V. Determination of the method of choice for enumeration of *Salmonella*. *Applied Microbiology* **9**, 487.

TAYLOR, W. I. (1965). Isolation of Shigellae. I. Xylose lysine agars; new media for isolation of enteric pathogens. *American Journal of Clinical Pathology* **44**, 471.

WALLACE, L. J., WHITE, F. H. & GORE, H. L. (1966). Isolation of *Edwardsiella* from a sea-lion and two alligators. *Journal of the American Veterinary Medical Association* **149**, 881.

J. Hyg., Camb. (1971), **69**, 331
Printed in Great Britain

Myxomatosis: the introduction of the European rabbit flea *Spilopsyllus cuniculi* (Dale) into wild rabbit populations in Australia

BY W. R. SOBEY AND DOROTHY CONOLLY
C.S.I.R.O., Division of Animal Genetics, P.O. Box 90,
Epping, N.S.W., 2121, *Australia*

(*Received* 8 *December* 1970)

SUMMARY

1. The European rabbit flea *Spilopsyllus cuniculi* (Dale) bred successfully in wild rabbits on three properties in New South Wales and, within two breeding seasons, almost every rabbit shot within a quarter of a mile of a release site was infested.

2. It was demonstrated that the flea transmitted myxoma virus in the field.

3. In areas where more than 75% of the rabbits shot at the beginning of the breeding season were flea-infested and myxoma virus was present, populations failed to show the expected summer build-up.

INTRODUCTION

Myxomatosis is still of considerable economic importance in Australia, although the way it kills rabbit populations is less dramatic than it was when myxoma virus was first released in 1950. The transmission of myxomatosis has been mainly by mosquitoes (Fenner & Ratcliffe, 1965). Because they confer a selective advantage on attenuated 'field strains' of virus and because they are most prevalent in the hot months, during which rabbits have the best chance of recovery from myxomatosis (Mykytowycz, 1956; Marshall, 1959), mosquitoes are not ideal vectors of virus for rabbit control. Further, the presence of mosquitoes in numbers large enough to cause intense annual epizootics has a restricted geographical distribution. In many of the more elevated areas – on the northern tablelands of N.S.W., for example – mosquito-induced intense epizootics have been as infrequent as one year in four. There is evidence (R. T. Williams, personal communication) that the mite *Listrophorus gibbus* (Pagen) may be the vector responsible for winter epizootics in the Snowy Plains and Canberra areas. This mite is widespread throughout Australia and is thus probably part of the ecological background to any field studies of the flea.

The reintroduction of virulent virus into the field has been of limited value since it has not been able to maintain itself there (Fenner, Poole, Marshall & Dyce, 1957). To obtain the maximum usefulness from a reintroduction of virulent virus, it has been necessary to time the release of virus to coincide with mosquito activity and to release the virus on a wide scale (G. W. Douglas, personal communication). These procedures are likely to prove beyond the powers of the average landholder.

As early as 1956 the usefulness of inoculation campaigns to reintroduce virulent virus into the field came into question. Although it was conceded that, under favourable conditions, such campaigns could be useful, it was felt that it would be most profitable to allow myxomatosis to run its course and direct available resources into poisoning and other conventional methods of control (Fenner & Ratcliffe, 1965).

There seems every reason to believe that a better vector would improve the usefulness of myxomatosis in rabbit control, particularly if the vector were less limited by seasonal and geographical variation than the mosquito. In Britain the European rabbit flea *Spilopsyllus cuniculi* (Dale) is considered to be the principal vector for myxomatosis (Lockley, 1954; Armour & Thompson, 1955). Fleas are present on rabbits throughout the year (Allan, 1956), with the result that myxomatosis can be active throughout the year. In Kent 1964–7 at least 20% of each quarter year sample had virus (Vaughan & Vaughan, 1968). An understanding of the life-cycle of the flea by Mead-Briggs & Rudge (1960) enabled fleas to be bred in captivity. With the aid of this knowledge, the flea was introduced into Australia in 1966 (Sobey & Menzies, 1969) and released from quarantine restrictions in 1968.

Studies to determine how readily the flea would establish in the field were begun in June 1968. The present paper is an account of observations made during the 2 years following the release of the flea into wild rabbit populations.

MATERIALS AND METHODS

Experimental areas

Fleas were released on three properties in New South Wales:

(1) 'Millambri', a property of 3500 acres about 14 miles east of Canowindra in N.S.W., elevation about 2000 ft. One paddock of 500 acres was used on this property. Intense annual epizootics spread by mosquitoes occur on this property between December and March. When observations were begun in 1967 rabbit infestation was severe.

(2) 'Wing Vee', a property of 8500 acres about 45 miles south west of Mudgee in N.S.W. Several paddocks with a total area of 1700 acres were used; these are decribed more fully in the text. The elevation of the experimental area is about 2000 ft. Myxomatosis on this property has been sporadic and isolated and appears to have contributed little to rabbit control. During 1965–6 an extensive programme of poisoning by sodium fluoroacetate (1080) (Lazarus, 1956; Rowley, 1968) reduced the rabbits to very low numbers. By 1968, when experimental work was begun, rabbit numbers were increasing and during 1968 reached a high level.

(3) 'Longford', a property of 2500 acres about 25 miles west of Armidale. The elevation is 3400–3800 ft. Prior to experimental work myxomatosis was rarely reported on this property; there had been no explosive epizootic for the past 5 years.

Population density

On 'Millambri' a standard walk count (Myers, 1954; Rowley, 1968) was used as an estimate of population density. The same walk of about 1 mile, covered between 4.30 and 5.00 p.m., was made on each occasion. On 'Wing Vee' and 'Longford' counts were made by spotlight at night from a vehicle at a speed not exceeding 7 m.p.h. and expressed as rabbits per spotlight mile. Spotlight counts are much affected by the abundance of vegetation (B. Cooke, personal communication). Seasonal variation in vegetation cover can account for a two- or threefold variation in the number of rabbits counted.

Viruses

Two strains of virulent virus cloned in our laboratory were used: Glenfield (G.V.) strain no. 5 and Lausanne (Lu) strain no. 7: strain numbers according to Fenner & Marshall (1957). Virus samples collected from the field were passaged once and classified as 'virulent' or 'field strain' according to the type of lesion resulting from their subsequent intradermal inoculation into the shaved back of an unselected laboratory rabbit. Occasional checks on the classification of viruses as virulent, were made by noting survival time (S.T.); in no case did survival time contradict lesion-type assessment. Since virulent virus is seldom recovered from the field (Fenner & Chappel, 1965), any virulent virus recovered during the present study was regarded as having originated from releases made during the study.

After 1967 virus was disseminated on 'Millambri' and 'Wing Vee' by releasing infected fleas and on 'Longford' by inoculation via the eye of trapped rabbits that were then released (Sobey, Conolly & Adams, 1967). Fleas were infected by allowing them to probe skin, rich in virus, which had been prepared by scarifying as described by Rivers & Ward (1937). The skin was removed from the back of a rabbit 7 days after infection, stored at $-60°$ C. and thawed and warmed to 37° C. before feeding the fleas. Random samples from the infected fleas were tested for infectivity by feeding each flea on a marked area of the shaved back of an unselected domestic rabbit and noting the number which initiated lesions. Batch infectivity varied between 20% and 80% with a mean of 45%.

Rabbits were caught for infection via the eye by two methods:

(1) *Trapping.* Rabbits were trapped in gin-traps and the least damaged rabbits infected and released. Mature female rabbits were destroyed. On average, about 70% of the rabbits caught were inoculated.

(2) *Spotlight.* Rabbits caught in the beam of a spotlight mounted on the head of the operator could be immobilized by firing a shot from a 0·22 rifle an inch or two above the rabbit's head; the operator keeping the beam on the rabbit could then walk up to the rabbit and capture it.

Shot sample

Periodically, a sample, shot during the day or at night by spotlight, was taken from each property. A shot rabbit was collected immediately and combed for fleas. Only the head and ears were combed and only for 1 min. In Britain fleas were

found in numbers on the body for only about a fortnight during the whole year (Allan, 1956). Allan found most of the fleas on the ears (80 %) whereas in the present study fleas were found mainly on the head. A blood sample was taken from each rabbit (Sobey, Conolly & Adams, 1966). Sex was recorded and the abdominal cavity of all female rabbits was opened and the uterus exposed; where foetuses were present their age was estimated. Lactation (L) was also recorded. The testes of males were scored as exposed or withdrawn. Where present, virus was noted and sampled by taking an eyelid. Age was initially scored as adult or sub-adult on the basis of size. This classification was too coarse to give information about population age distribution and in the samples taken in the later part of the study age was determined by eye-lens weight (Lord, 1959; Myers & Gilbert, 1968).

Fleas

Fleas were bred in a small animal-house at a temperature of 72 ± 5° F. with a minimum relative humidity of 55 %. One week before parturition a doe had 200 fleas placed on her head. She was supplied with a littering box with a removable bottom. Twelve to fourteen days after the litter was born, the litter and the doe were combed free of fleas and the nest in the cage bottom was removed and emptied into a large plastic bag which was hung from the ceiling of the animal house. From the time nests reached 20 days of age, measured from the time of parturition (i.e. 20 days after the litter was born), they were examined two to three times a week by being emptied into a large enamelled tray; any fleas were aspirated into 1 oz. McCartney bottles containing a strip of filter paper. Fleas were stored at +2° C.

The introduction of the flea into the field in the early stages of the work was done by simply dropping fleas into an active warren opening in lots of 100 or 200. Infected fleas (IF) were spread similarly but in lots of 10–20 fleas.

RESULTS

Millambri

In each of the years 1966 and 1967, during the months October, November and December, about 500 rabbits were trapped across the whole property, inoculated via the eye with G.V. virus and released. Severe mosquito-borne epizootics occurred in December/January of both years, reducing the rabbit population in each case by an estimated 80–90 %. However, no G.V. virus was recovered during the course of either epizootic, thus confirming the findings of Fenner *et al.* (1957) that mosquitoes as vectors confer an overwhelming selective advantage on moderately attenuated strains of virus. In spite of the quite spectacular kills, sufficient survivors remained to ensure a resurgence of rabbits in the spring of 1967 and 1968.

Observations on fleas were made in a single paddock, 'Oak Hill', of about 500 acres which was inaccessible to vehicles and free from stock during the period of observation. No poisoning or other conventional forms of rabbit control were undertaken during the period of observation. The paddock is intersected by

numerous steep gullies and has a large, very steep, rocky outcrop at one end, from which it derives its name of 'Oak Hill'. From the base of the hill the paddock slopes up to a wooded rocky escarpment. The soil is shaley and not well grassed. There were numerous extensive rabbit warrens, particularly near the centre of the paddock in the region of two earth dams. The walk over which the count was made extended around the rocky outcrop to and from the dams.

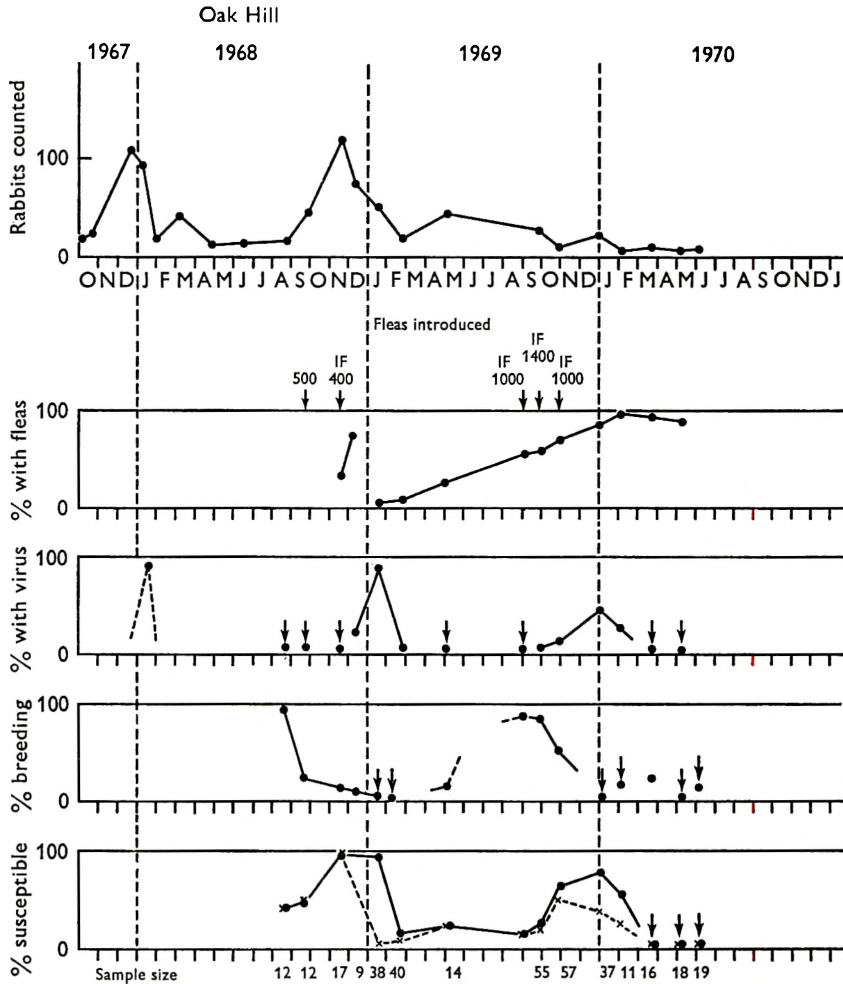


Fig. 1. Walk counts made in the paddock Oak Hill and the number of fleas introduced, together with data from shot samples showing the percentage of animals with fleas, with virus, breeding and susceptible. [× --- ×, including those with active virus; ●—●, excluding those with active virus, of the % susceptible.]

Fleas were introduced into Oak Hill in September 1968 towards the end of the breeding season, as shown in Fig. 1. One hundred fleas were introduced into each of five major warrens in and around the area of the dams near the centre of the paddock. Fleas were found on rabbits from a shot sample in November, at which time Lu virus was released by the introduction of 400 infected fleas. Of nine rabbits shot in December seven had fleas and two of those with fleas were infected with Lu virus.

During January 1969 there was an epizootic probably involving mosquitoes from which only attenuated field strains were recovered and the proportion of rabbits with fleas fell to two out of thirty-eight sampled. In February 1969 the proportion of rabbits with fleas was still low, but subsequently, and coinciding with the 1969 breeding season, the proportion increased linearly with time until February 1970, when almost every rabbit shot had fleas. Infective fleas were distributed in Oak Hill in September and October 1969. The eight virus samples collected in September and October were all Lu virus. In January 1970, at the height of the epizootic, Lu virus was recovered from some of the infected animals, but in February all the virus recovered was attenuated field-strain.

Throughout the 1969 breeding season the shot samples contained fewer susceptible animals than from the previous year and this could be attributed to the fleas transmitting the virus to the young animals before they entered the count. In October 1968 58% of the shot sample was adult and in October 1969 84% was adult.

The salient features of the data presented in Fig. 1 are (1) the linear increase in the population of rabbits with fleas from about 5% to about 100% during the 1969 breeding season, (2) the absence of a population build-up in the spring of 1969 when compared with 1967 and 1968 as judged by the walk count, (3) the extended and less intense 1969 epizootic compared with the shorter and more dramatic 1968 epizootic, and (4) a reduced build-up of susceptible animals in the population of 1969/70 when compared with that of 1968/69.

Wing Vee

The counts on this property were made by the two owners of the property, one driving and one counting. This arrangement enabled counts to be made by a single operator using the same spotlight and vehicle throughout. The same fixed transect was driven on each occasion and care was taken to select dark nights, avoiding rainy or windy conditions. Counts using a tallycounter were made while driving at speeds not exceeding 7 m.p.h.

Fleas were first released in June 1968 into a paddock of 350 acres called Cattle Station. Nine hundred fleas were released into four openings in a major heavily populated warren. The first flea to be recovered from the field was found on a rabbit shot in this paddock in August of 1968. By the end of October 1968, 70% of the shot sample had fleas, these rabbits being shot over the whole paddock and not just in the vicinity of the release site. As shown in Fig. 2, the percentage of rabbits with fleas fell to just below 50% during March and April 1969 but then steadily rose during the breeding season to 100% in July 1969. The percentage of rabbits with fleas remained high thereafter, with a fall to 80% during March/April and a subsequent rise to 100% in May 1970.

There was an increase in the rabbit count during 1968/9 but, as the percentage of rabbits with fleas rose above 75%, the count fell and remained low without any summer build-up in 1969/70 in spite of almost continuous breeding. Counts recorded in Braziers, a paddock of 435 acres adjoining Cattle Station, are given in Fig. 2. In contrast with the population in Cattle Station, the population in Braziers rose steeply in the breeding season of 1968/9 when there were few fleas

in Braziers. There were two transects through Braziers, No. 1 running the length of the western boundary and No. 2 circuiting the area adjoining Cattle Station. Although the shot sample from Braziers was not separated in relation to these two transects, it was observed that the percentage of rabbits with fleas increased more rapidly in the area of Route 2 than Route 1. Running parallel to a non-rabbit

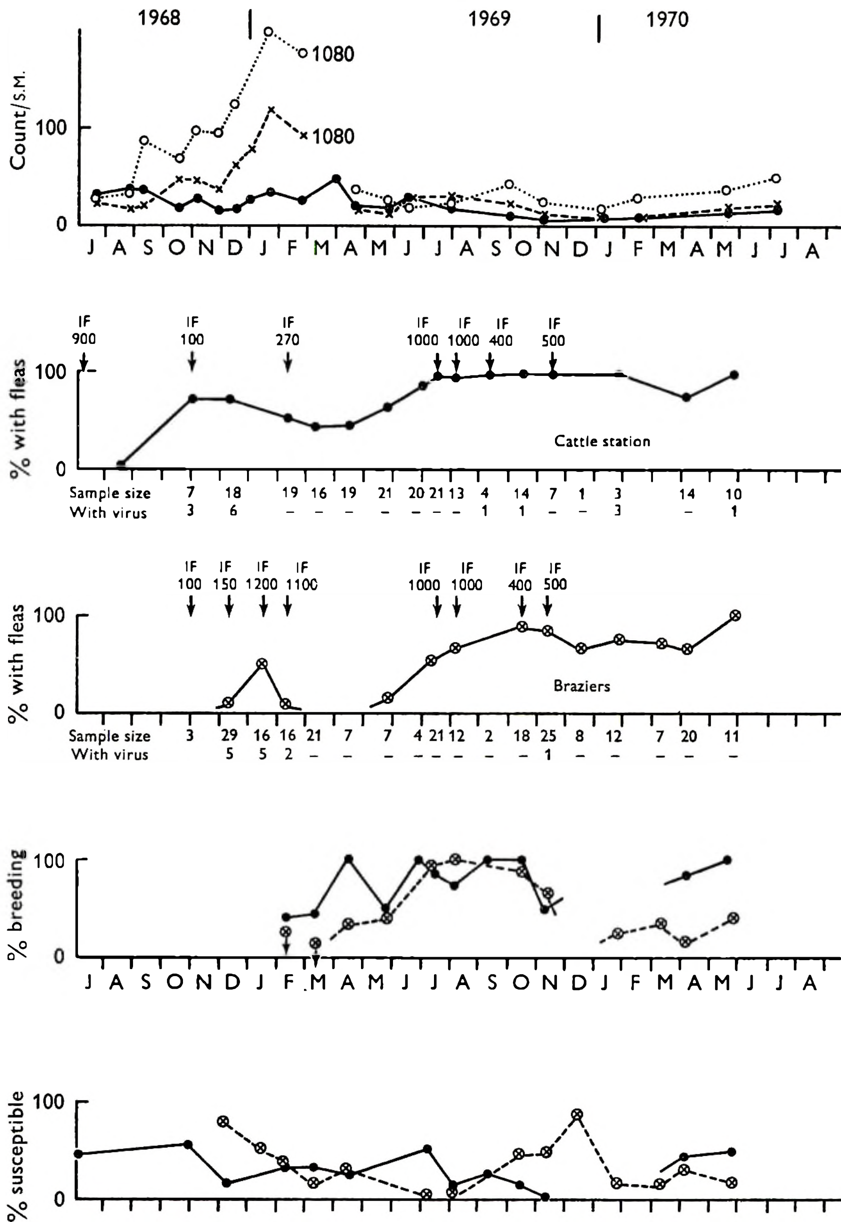


Fig. 2. Spotlight counts in two routes in Braziers prior to and after 1080-poisoning and in Cattle Station. Fleas added to and the build-up of fleas in these two paddocks is shown together with virus found, percentage breeding and percentage susceptible. ● — ●, Cattle Station; ○ · · · · ○, Braziers route 1; x – – – x, Braziers route 2; ⊗ – – – ⊗ or ⊗———⊗, Brazier's route (1 and 2).

netting fence, Route 1 was more prone to changes due to the movement of rabbits from untreated areas. Infected fleas were introduced into Braziers towards the end of 1968 and fleas were recovered in January 1969. During February, March and April 1969, when rabbit numbers were very high, fleas were not recovered. Subsequent to 1080-poisoning in April 1969, fleas were recovered in a progressively increasing proportion of rabbits during the 1969 breeding season, reaching 90% of rabbits in October of that year. The proportion of rabbits with fleas remained high but did not approach 100% until June 1970. There was no marked build-up in rabbit numbers after the 1968/9 breeding season.

Fleas were released in a number of paddocks along the transect route late in the 1968 breeding season, and before the middle of the 1969 breeding season almost every rabbit shot in these areas had fleas. In three areas, Davis A, Davis B and Hoppin' Charlie, which had a different history of flea release, the build-up of fleas was slower and it was not until the end of the 1969 breeding season that flea infestation reached very high levels. Changes in the rabbit spotlight count, expressed as a fraction of the June count in relation to the build-up in rabbits infested with fleas, are illustrated in Table 1.

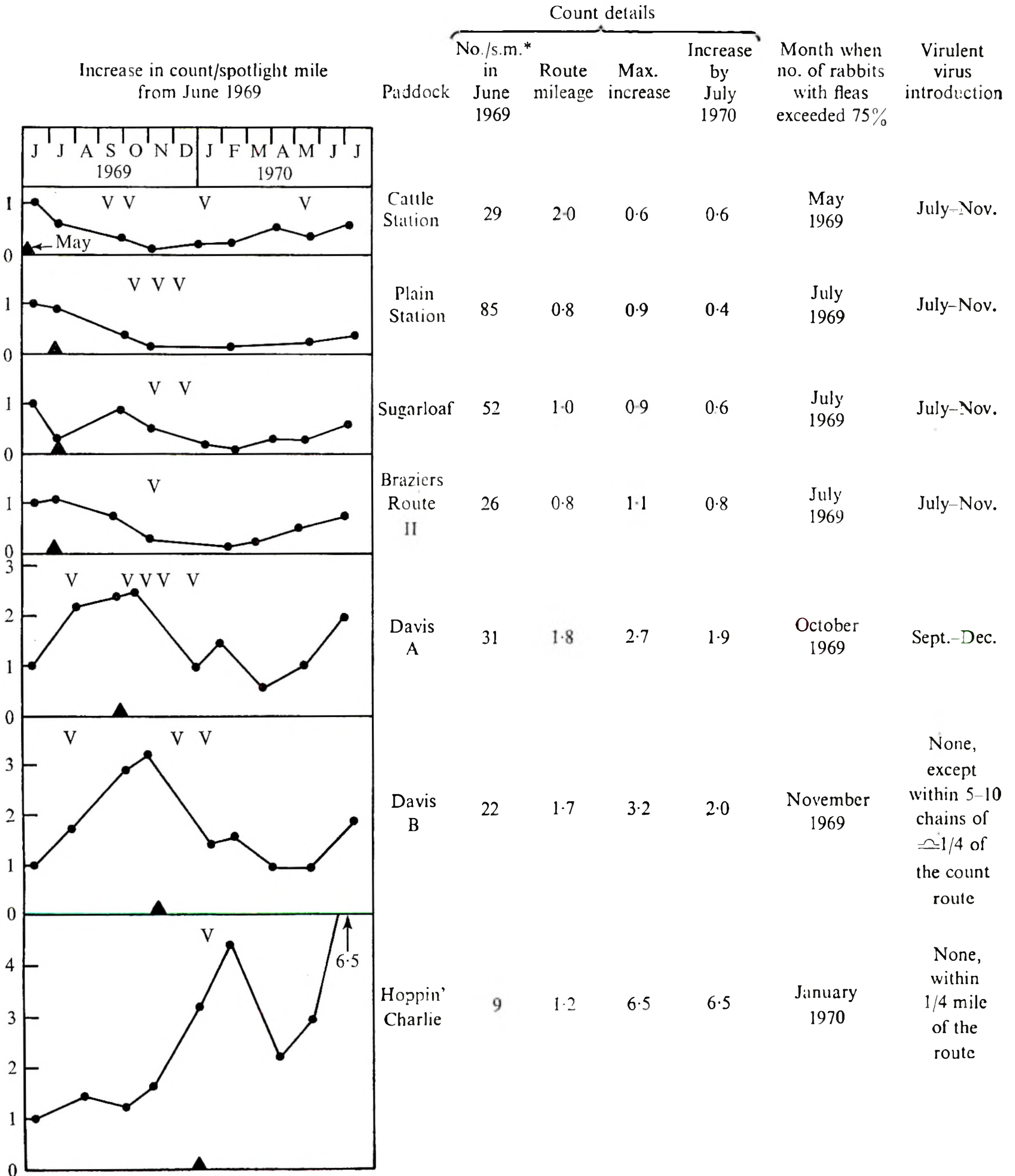
Cattle Station, Plain Station, Sugarloaf and Braziers all reached a high percentage of rabbits with fleas (75% or more) by July 1969 and each showed a decline in the rabbit population by July 1970, with an absence of any summer build-up in rabbit numbers in spite of a normal breeding season. By October 1969 none of the shot samples from these areas contained more than 20% young animals. No fleas were introduced into Davis A and Davis B during 1968. An introduction of 1500 fleas was made into each of Davis A and Davis B in June 1969 and Davis A was subsequently treated with infected fleas, whereas no further fleas were introduced into Davis B. The build-up of fleas in Davis A was advanced by a month when compared to Davis B, but any difference this might have made to changes in population went unrecorded owing to the absence of counts during November and December 1969. In both areas a build-up of rabbits occurred between June and October 1969 (60% young in the October shot samples), at about which time a high level of flea infestation was reached, and in both areas the count fell steeply. By July 1970 the count was double the June 1969 number in both Davis A and B.

Three hundred fleas were introduced into Hoppin' Charlie in November 1968, i.e. at the end of the breeding season. This paddock was 1080-poisoned in April of 1969 and the build-up of flea infestation was slow, not reaching a high level until January 1970. No virulent virus was introduced into Hoppin' Charlie. By February 1970 a 4·5 times count increase had been observed. There was a fall in the count during March and April 1970, following myxomatosis, but by July a 6·5 times increase was observed.

Spotlight counts can be affected by the density of the pasture; when pasture is dense counts can be low because rabbits are not seen, and when pasture is flattened counts can be high because all rabbits are easily seen. An increase in pasture density from June to December 1969 and thereafter a decrease in the pasture density from March to July 1970 could have affected the data in Table 1; ageing rabbits by the weight of the eye lens suggests that very few (9/146) new rabbits entered the popu-

lation between March and July 1970 and yet the count doubled in most areas. Factors other than pasture cover must also have affected counts. Rabbits counted in June 1969 were clearly born before this date and 80% of the shot sample were immune and thus unlikely to be affected by myxomatosis. By January 1970 the

Table 1. *Counts, expressed as a fraction of the June 1969 count, in different paddocks where the build-up of fleas reached 75% or more of the rabbits at different times*



Paddock	Count details: No./s.m.* in June 1969	Count details: Route mileage	Count details: Max. increase	Count details: Increase by July 1970	Month when no. of rabbits with fleas exceeded 75%	Virulent virus introduction
Cattle Station	29	2·0	0·6	0·6	May 1969	July–Nov.
Plain Station	85	0·8	0·9	0·4	July 1969	July–Nov.
Sugarloaf	52	1·0	0·9	0·6	July 1969	July–Nov.
Braziers Route II	26	0·8	1·1	0·8	July 1969	July–Nov.
Davis A	31	1·8	2·7	1·9	October 1969	Sept.–Dec.
Davis B	22	1·7	3·2	2·0	November 1969	None, except within 5–10 chains of ≃1/4 of the count route
Hoppin' Charlie	9	1·2	6·5	6·5	January 1970	None, within 1/4 mile of the route

* Number per spotlight mile. ▲ Rabbits with fleas exceed 75%. V Virus recovered.

counts had fallen, and age estimates of shot samples by eye-lens weight indicated that only 10 % were born before May 1969. However, by July 1970 the counts had risen and 30–50 % of the shot sample were found to have been born before May

Table 2. *The number of infected fleas per* 10 *acres introduced into different paddocks and the virus recovered* (*only virulent virus was introduced*)

	1969						1970					
Paddock	J	A	S	O	N	D	J	F	M	A	M	J
Cattle Station												
Virus added*	30	30	10	—	15	—	—	—	—	—	—	—
Virus found†	—	—	V	V	—	—	AA	—	—	—	A	—
Plain Station												
Virus added	55	55	—	20	30	—	—	—	—	—	—	—
Virus found	—	—	—	V	V	V	—	—	—	—	—	—
Sugarloaf												
Virus added	25	25	—	10	—	—	—	—	—	—	—	—
Virus found	—	A	—	—	V	VA	—	—	—	—	—	—
Braziers												
Virus added	25	25	—	10	10	—	—	—	—	—	—	—
Virus found	—	—	—	—	V	—	—	—	—	—	—	—
Davis A												
Virus added	—	—	400	150	100	100	—	—	—	—	—	—
Virus found	—	A	—	V	VVA	VVA	VAA	—	A	—	—	—
Davis B												
Virus added	—	—	—	—	—	—	—	—	—	—	—	—
Virus found	—	A	—	—	—	AA	VAA	—	—	—	—	—
Hoppin' Charlie												
Virus added	—	—	—	—	—	—	—	—	—	—	—	—
Virus found	—	—	—	—	—	—	AAAA	—	—	—	—	—

* Expressed in terms of infected fleas per 10 acres.
† V, Virulent virus; A, attenuated virus. The number of A's or V's indicates a quantitative estimate of virus found.

Table 3. *Illustrating the greater proportion of old immune rabbits* (*born before March* 1969) *and the higher proportion of immunity in current season's rabbits* (*born March–December* 1969) *on Wing Vee where fleas were active than on adjoining properties where there were no fleas*

	Wing Vee Cattle Station and Plain Station	Edge Hill and Gundowda	χ^2 (1)	P
No. in sample*	50	60	—	—
Rabbits born before March 1969	13	7	38	< 0·001
No. immune	13	6	—	—
Rabbits born March–Dec. 1969	37	53	—	—
No. immune	17	11	6·5	0·002–0·01

* Rabbits shot March–May 1970

1969. In spite of these general trends, the four paddocks which had a high percentage of rabbits with fleas early completed the year with a decreased count. In the three paddocks where high flea infestation was late the counts increased rapidly at a time when pasture density would have been depressing the count. All three showed a marked fall in count when the percentage of rabbits with fleas became high.

Where virulent virus was introduced into the field via infected fleas it was recovered from infected rabbits shot a month later, as shown in Table 2: thus, the virulent virus was transmitted at least once from rabbits infected in the field. Attenuated strains were active before, during and after the time when virulent virus was recovered.

During March, April and May 1970 a shot sample of rabbits was taken (by spotlight) from two properties each 4–5 miles from Wing Vee. A comparison of these rabbits with rabbits shot in Cattle Station and Plain Station on Wing Vee during the same period is given in Table 3. The Wing Vee shot sample contained a smaller proportion of animals born in the 1969/70 breeding season and of these more were immune than those from the adjoining properties.

Longford

This property is run by C.S.I.R.O. as an experimental sheep-breeding station. Counts begun in March 1968 on Longford were made by the same two C.S.I.R.O. employees stationed on the property, using a fixed transect of 3·8 miles throughout. From October 1967 until fleas were first released in July 1968, between five and fifteen rabbits were caught once or twice a week. The mature females, as judged by appearances, were destroyed and the rest were infected with virulent G.V. virus via the eye. Subsequent to January 1969 rabbits were caught only by spotlight. The monthly totals of rabbits infected are shown in Fig. 3, together with other relevant data.

Virus was recovered every month between October 1967 and July 1968. The susceptibility remained between 50 and 75 %, and as no counts were made until March no assessment of population fluctuations can be made. All of the nine virus samples tested during this period were attenuated strains. The prolonged virus activity suggests the presence of some vector other than mosquito or the flea or of contact transmission.

The population was reduced to a very low level in June 1968 by poisoning. Fleas were introduced in July, September and November of 1968 and, by July 1969, were found on every rabbit shot or captured. From June 1968 to May 1969 the count had increased by a factor of 5 or 6 and was significantly lowered by further poisoning. During the following year there was very little change in the count in spite of continuous breeding; flea infestation remained high. When virulent Lu virus was introduced into the population in January and February 1969, the rabbits were largely susceptible and there was a rapid spread of virus with a consequent fall in number of susceptible animals. Of ten virus samples collected during March, April and May 1969, five were virulent Lu and five attenuated field-strain. With the fall in the number of susceptible animals the reintroduction of

virulent virus became increasingly ineffectual, as may be seen in Fig. 3, from the number of susceptible animals that were infected – an average of only two per month. Virus was recovered during most months of the year and two of those sampled in June 1970 were attenuated field-strains. The rise in the number of susceptible rabbits during December, January 1969/70 and the subsequent fall, without any apparent change in size of the total population, suggests some continuing change in the age structure of the population during this period.

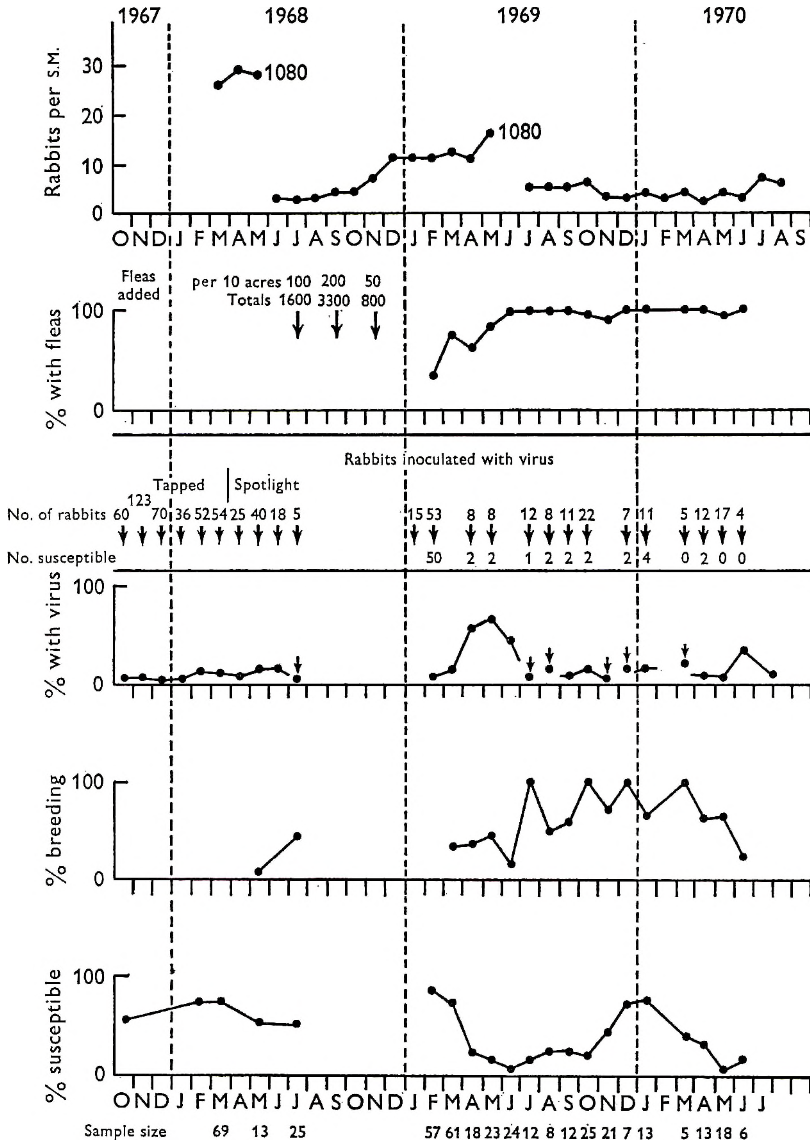


Fig. 3. Spotlight counts on Longford, together with data on fleas added and percentage fleas, virus, breeding and susceptible, in shot samples. Not all rabbits inoculated with virus were susceptible: where blood samples were taken the number of susceptible rabbits is shown.

Fleas and virus transmission

During the period fleas were building up in the field, before all rabbits had fleas, it was possible to compare the proportion of rabbits, with and without fleas, from the same area, that were infected with myxomatosis. Shot samples taken during September, October and November 1969, in areas where Lu virus was being actively disseminated, are shown in Table 4. All of the infected rabbits had fleas. Not all the diseased rabbits were infected with Lu, some were infected with attenuated field strains, suggesting that the fleas were the main vectors and were transmitting whatever virus they came in contact with.

Table 4. *To illustrate the association of virus and fleas in rabbit populations where the proportion of rabbits with fleas was increasing* (*Sept.-Nov.* 1969)

	Rabbits with fleas			Rabbits without fleas		
	No. in sample	No. with virus	% with virus	No. in sample	No. with virus	% with virus
Wing Vee	128	25	20	43	0	< 2
Millambri	35	9	26	14	0	< 7
Total	163	34	21	57	0	< 2

Flea infestation rates

When it became clear that most rabbits in the experimental areas were carrying fleas, an attempt was made to estimate the numbers of fleas on each rabbit. From March 1970 each shot rabbit was scored by inspection on the following scale: +, 1–4; ++, 5–20; +++, 21–100; ++++, 101–500; and +++++, > 500 fleas. The data collected from March to September 1970 on Wing Vee are summarized in Table 5, where the scale has been converted back to flea numbers on the basis + = 3, ++ = 15, +++ = 50, ++++ = 200 and +++++ = 700 fleas. It is probable that the numbers arrived at are underestimates of the actual numbers. Scoring was done by artificial light at night on wild agouti rabbits and the time allowed for scoring was restricted to 1 min. From the September sample, pairs of ears from seven shot rabbits were put into plastic bags, brought back to the laboratory and the fleas in each counted. A +++++ gave 765 fleas and six ++++'s gave 246, 378, 727, 241, 123 and 196 fleas. The complete rabbits would undoubtedly have had additional fleas in each case.

In general, the breeding animals carried more fleas than the non-breeding animals. Females that were lactating only or lactating and less than 10 days pregnant carried fewer fleas than females pregnant and not lactating, or lactating and pregnant more than 10 days. This is simply explained in that most of the fleas these animals were carrying prior to parturition would have been in the nest. The 1970 breeding season commenced about April and as it proceeded to September the mean number of fleas per rabbit increased. Flea infestation in excess of 100 per rabbit was generally restricted to pregnant does or does lactating and pregnant more than 10 days. However, an occasional breeding buck (testes exposed) was

found with more than 100 fleas. The very high numbers, greater than 700 fleas per rabbit, are in excess of the highest numbers, 450 fleas per rabbit, reported by Allan (1956) from rabbits captured in Scotland.

Table 5. *Estimates of the mean number of fleas found on different classes of rabbits at different times between March and September* 1970

	Males		Females				
Date	Testes up	Testes down	Non-breeding	P only	L and P < 10 days	L and P > 10 days	Total
10.iii.70	2 (37)	5 (12)	3 (29)	—	3 (1)	—	3 (79)
6.iv.70	3 (12)	18 (35)	7 (33)	10 (2)	6 (7)	—	11 (89)
26.v.70	19 (3)	13 (43)	8 (10)	25 (3)	18 (7)	—	13 (66)
4.viii.70	8 (5)	8 (44)	22 (8)	70 (13)	11 (24)	50 (11)	22 (105)
15.ix.70	*5 (24)	12 (34)	*5 (9)	125 (2)	25 (19)	140 (16)	34 (104)

P, Pregnant; L, lactating.
The numbers in parenthesis are the numbers of animals on which the estimates are based.
* Kittens present in sample.

DISCUSSION

Within two breeding seasons the fleas released on each of three properties in New South Wales multiplied to a point where almost every rabbit shot within at least a quarter of a mile radius of a release site was infested with fleas. When, within a given area, the number of rabbits infested with fleas reached a high level, it remained high. The number of fleas per rabbit was highest during the breeding season, particularly on the ears and heads of pregnant does. The highest numbers per rabbit counted were in excess of those reported by Allan (1956) in Scotland. During the hot summer months fleas were particularly active and often deserted a shot rabbit within minutes.

There is no doubt that the flea transmits virus in the field. Virulent Lu virus was recovered from the field in areas where it had been introduced only via infected fleas and during the build-up of flea populations there was a very strong association between fleas and the presence of virus of all kinds, no virus being found in 57 rabbits that had no fleas, whereas out of 163 rabbits with fleas 34 had virus. The data presented suggest the long-term usefulness of the flea as an aid in rabbit control. It is interesting that in the areas on all three of the properties where the flea has been established, by the beginning of the breeding season, the rabbit population showed no increase over the period of a year in spite of a normal breeding season, and that whereas before the flea reached high numbers there was a steep increase in the rabbit count during each breeding season, there has been no such rise since.

With the introduction of the flea to assist in rabbit control in Australia it seems that its most immediate use would be in maintaining low rabbit populations following 1080-poisoning as illustrated by the results from Longford. It is not clear from the data whether the reintroduction of virulent viruses into areas where the flea has been established is of value in rabbit control. However, it has been demon-

strated that virulent virus can be reintroduced and maintained for some time in the field, and it is suggested that such reintroduction might be most useful from a control point of view during the breeding season, when both the flea and susceptible rabbits are abundant and the colder weather will favour a high mortality.

We are indebted to W. Menzies for his very able assistance in all aspects of the work and the technical assistance of Miss Elizabeth Emery, Mrs Gail McFarland and Mrs Janet O'Keefe; to Bremner Bros. Pty. Ltd. for access to their property 'Wing Vee' and their assistance with counts, to Canargles Estates Pty. Ltd. for access to 'Millambri', and to Mr Jim Hollands for the counts and samples from 'Longford'.

REFERENCES

ALLAN, R. M. (1956). A study of the populations of the rabbit flea *Spilopsyllus cuniculi* (Dale) on the wild rabbit *Oryctolagus cuniculus* in north-east Scotland. *Proceedings of the Royal Entomological Society of London* **31**, 145.

ARMOUR, C. J. & THOMPSON, H. V. (1955). Spread of myxomatosis in the first outbreak in Great Britain. *Annals of Applied Biology* **43**, 511.

FENNER, F. & MARSHALL, I. D. (1957). A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in Australia, Europe and America. *Journal of Hygiene* **55**, 149.

FENNER, F. & CHAPPLE, P. J. (1965). Evolutionary changes in myxoma virus in Britain. An examination of 222 naturally occurring strains obtained from 80 countries during the period October–November 1962. *Journal of Hygiene* **63**, 175.

FENNER, F., POOLE, W. E., MARSHALL, I. D. & DYCE, A. L. (1957). Studies in the epidemiology of infectious myxomatosis of rabbits. VI. The experimental introduction of the European strain of myxoma virus into Australian wild rabbit populations. *Journal of Hygiene* **55**, 192.

FENNER, F. & RATCLIFFE, F. N. (1965). *Myxomatosis.* Cambridge University Press.

LAZARUS, M. (1956). The toxicity and relative acceptability of some poisons to the wild rabbit *Oryctolagus cuniculus* (L). *C.S.I.R.O. Wildlife Research* **1**, 96.

LOCKLEY, R. M. (1954). The European rabbit flea, *Spilopsyllus cuniculi*, as a vector of myxomatosis in Britain. *Veterinary Record* **66**, 434.

LORD, R. D. (1959). The lens as an indicator of age in Cottontail rabbits. *Journal of Wildlife Management* **23**, 358.

MARSHALL, I. D. (1959). The influence of ambient temperature on the course of myxomatosis in rabbits. *Journal of Hygiene* **57**, 484.

MEAD-BRIGGS, A. R. & RUDGE, A. J. B. (1960). Breeding of the rabbit flea, *Spilopsyllus cuniculi* (Dale): requirement of a 'factor' from a pregnant rabbit for ovarian maturation. *Nature, London* **187**, 1136.

MYERS, K. (1954). Studies in the epidemiology of infectious myxomatosis of rabbits. II. Field experiments August–November 1950 and the first epizootic of myxomatosis in the Riverina Plain of south-eastern Australia. *Journal of Hygiene* **52**, 47.

MYERS, K. & GILBERT, N. (1968). Determination of age of wild rabbits in Australia. *Journal of Wildlife Management* **32**, 841.

MYKYTOWYCZ, R. (1956). The effect of season and mode of transmission on the severity of myxomatosis due to an attenuated strain of the virus. *Australian Journal of Experimental Biology and Medical Science* **34**, 121.

RIVERS, T. M. & WARD, S. M. (1937). Infectious myxomatosis of rabbits. Preparation of elementary bodies and studies of serologically active materials associated with the disease. *Journal of Experimental Medicine* **66**, 1.

ROWLEY, I. (1968). Studies on the resurgence of rabbit populations after poisoning. *C.S.I.R.O. Wildlife Research* **13**, 59.

SOBEY, W. R., CONOLLY, D. & ADAMS, K. M. (1966). Myxomatosis: a simple method of sampling blood and testing for circulating soluble antigens or antibodies to them. *Australian Journal of Science* **28**, 354.

SOBEY, W. R., CONOLLY, D. & ADAMS, K. M. (1967). Myxomatosis: The preparation of myxoma virus for inoculation via the eye. *Australian Journal of Science* **30**, 233.

SOBEY, W. R. & MENZIES, W. (1969). Myxomatosis: The introduction of the European rabbit flea *Spilopsyllus cuniculi* (Dale) into Australia. *Australian Journal of Science* **31**, 404.

VAUGHAN, N. E. M. & VAUGHAN, J. A. (1968). Some aspects of the epizootiology of myxomatosis. *Symposia of the Zoological Society of London* **24**, 289.

J. Hyg., Camb. (1971), **69**, 347
Printed in Great Britain

Beta-haemolytic streptococci in saliva*

BY P. W. ROSS
Department of Bacteriology, Medical School, University of Edinburgh

(*Received* 21 *December* 1970)

SUMMARY

Viable counts of beta-haemolytic streptococci per ml. of saliva were made in the following groups: (1) children with acute streptococcal sore throat, (2) children with acute non-streptococcal sore throat, (3) children who had no sore throat but were streptococcal throat carriers, (4) children who neither had a sore throat nor were streptococcal throat carriers.

The mean counts from cases of streptococcal sore throat and from streptococcal carriers were respectively $1{\cdot}4 \times 10^6$ and $2{\cdot}5 \times 10^5$ per ml.

In a comparison of the efficiency of the throat swab, sublingual swab and specimen of saliva in isolating beta-haemolytic streptococci from the upper respiratory tract, culture of saliva produced the best results.

INTRODUCTION

The literature contains comparatively few references to the presence of beta-haemolytic streptococci in the saliva. Hare (1940) reported their presence, as did Hamburger (1944), who studied the incidence of beta-haemolytic streptococci in the saliva of army recruits who were suffering from scarlet fever, pharyngitis or tonsillitis. He isolated these organisms from 64·6 % of 527 cultures of saliva, and found that the numbers of organisms ranged from 10^2 to 5×10^6 per ml. of saliva; the majority, however, fell within the range of 10^3 to 10^6 per ml. Hamburger also stated that patients with heavily positive throat cultures could yield negative salivary cultures and that those with scanty growth from the throat could have large numbers of beta-haemolytic streptococci in the saliva. Duguid (1946*a*) swabbed 87 people who had beta-haemolytic streptococci in the throat, 50 of whom had scarlet fever and 37 of whom were streptococcal carriers, and isolated these organisms, usually in small numbers, from the mouth secretions of 13. Hamburger & Robertson (1948) reported a very high carrier rate in convalescent and healthy carriers, the average count being in the region of $8{\cdot}0 \times 10^5$ per ml. of saliva. Rubbo & Benjamin (1953) examined saliva from nine streptococcal carriers and reported a wide range of counts. The degree of growth obtained from the throat swabs bore no relation to the concentration of beta-haemolytic streptococci in the saliva.

Holmes & Williams (1958) investigated anterior mouth swabs from children in

* Based on part of a thesis accepted for the degree of M.D., University of Aberdeen.

Dr Barnardo's homes and isolated beta-haemolytic streptococci from 62% of 78 children who had these organisms in their throats. In their studies on cross-infection in isolation hospitals Boissard & Fry (1966) reported that beta-haemolytic streptococci could be isolated from the mouths of almost half the patients who had scarlet fever and concluded that the presence of these organisms in the saliva was a feature of the early stages of tonsillar infection. They recommended the use of a nasal and a salivary swab rather than a nasal and throat swab to detect dispersers.

In this study quantitative counts of beta-haemolytic streptococci from the saliva of four groups of children were performed:

(1) Children with streptococcal sore throat.

(2) Children with non-streptococcal sore throat.

(3) Children who had no sore throat but were throat carriers of beta-haemolytic streptococci.

(4) Children who neither had a sore throat nor were throat carriers of beta-haemolytic streptococci.

The relative merits of throat swabs, sublingual swabs and salivary culture in isolating beta-haemolytic streptococci from the throat were evaluated.

MATERIALS AND METHODS

Selection of children

In the investigations of children with sore throat the family doctors obtained the specimens of saliva when they examined the children. Throat swabs were also taken at that time and the cases were divided into those from whom beta-haemolytic streptococci had been isolated and those from whom these organisms had not been isolated. The streptococcal sore-throat group comprised 47 children and the non-streptococcal sore throat group 52. Children who had no sore throat but were streptococcal throat carriers, and the control group (those who had no sore throat and were not streptococcal throat carriers), were selected from schools in the Pilton–Muirhouse areas in Edinburgh. There were 60 children in each of these two groups, and these were selected for the study whenever the bacteriological results of routine weekly swabbings at these schools became available. The control group was matched as closely as possible with the three other groups in age, sex, season and family size.

A completely separate group of children was investigated in evaluating the throat swab, sublingual swab and salivary culture in the isolation of beta-haemolytic streptococci from the upper respiratory tract. This group comprised 248 children who were found to be symptomless streptococcal throat carriers at routine weekly throat swabbing sessions in schools. No salivary examinations were performed on them at these routine visits; they were included in the study group only because throat-swab culture had produced positive results. These children were revisited at school one or two days after their first throat swab had grown beta-haemolytic streptococci and a further throat swab, as well as a sublingual swab and specimen of saliva, were obtained at this time.

Specimen collection

Specimens of saliva from the acute cases were obtained by the family doctors and from the carriers and controls by the author. A specially prepared kit was used, which consisted of a sterile Pasteur pipette calibrated to deliver 0·02 ml. drops contained in a sterile test-tube. This test-tube had a metal cap, and cotton-wool at the foot to prevent damage to the pipette. The pipette was placed on the floor of the anterior mouth and saliva sucked up by means of a rubber teat. Occasionally it was difficult to get saliva from children, especially from those who were apprehensive. No sialogogues were used but, on the promise of a sweet, saliva was obtained from most of the previously recalcitrant children. The 248 children were swabbed twice, but apart from this group no child was sampled on more than one occasion and a specimen of saliva was taken from each child once only. As well as specimens of saliva, throat and sublingual swabs were taken. 'Exogen' swabs, which had tips of buffered absorbent cotton-wool sterilized by ethylene oxide, were used, since beta-haemolytic streptococci had been found to remain viable on these for up to 24 hr. (Ross, 1970).

Laboratory methods

Saliva and swabs were processed within 2 hr. of being obtained. Estimation of the number of beta-haemolytic streptococci was made by surface-viable counts (Miles & Misra, 1938). Saliva was diluted 1/4 with distilled water and tenfold serial dilutions were made from this. Blood agar plates incubated aerobically and crystal violet blood agar plates incubated anaerobically for 14–18 hr. were used for the streptococcal counts.

Sublingual and throat swabs were plated out on blood agar and crystal violet blood agar and incubated as above. Grouping of the beta-haemolytic streptococci was by the acid extraction method (Lancefield, 1933) and by the use of a bacitracin disk (Maxted, 1953); groups A, B, C and G were tested for. Strains were screened by the bacitracin disk method and if they were sensitive to bacitracin they were taken to be group A; no serological confirmation was obtained. This occurred at a later stage when presumptive group A cultures were typed; if any were untypable they were then tested to ensure that they did in fact belong to group A. All bacitracin-resistant strains, however, were grouped by Lancefield's method. Typing was by slide agglutination (Griffith, 1934) using T-antisera (Williams, 1958).

RESULTS

All 47 children with streptococcal sore throat had beta-haemolytic streptococci in the saliva. Mean counts per ml. of saliva varied from child to child and the range was from $4{\cdot}8 \times 10^5$ to $4{\cdot}4 \times 10^6$, with a mean of approximately $1{\cdot}4 \times 10^6$. The distribution of these counts is seen in Table 1. This table also shows the distribution of counts obtained from the symptomless streptococcal throat carriers. Fifty-five out of sixty (91·7 %) had beta-haemolytic streptococci in the saliva, but mean counts

per ml. were considerably lower than those obtained from the acute cases. They ranged from 4×10^3 to $3 \cdot 2 \times 10^6$, with a mean of approximately $2 \cdot 5 \times 10^5$ per ml.

Of the 102 pairs of beta-haemolytic streptococci isolated from throat and saliva of the cases of streptococcal sore throat and also the symptomless throat carriers, 99 pairs (97 %) belonged to group A; 46 from the 47 cases and 53 from the 55 symptomless carriers produced growth of these organisms. Two of the non-group A pairs belonged to group G, but the other was ungroupable. The streptococcal group in the throat was the same as that in the saliva in each child.

Table 1. *Distribution of salivary counts of beta-haemolytic streptococci in* 47 *cases of acute streptococcal sore throat and in* 55 *streptococcal throat carriers*

Salivary count (millions/ml.)	Cases	Carriers
$< 0 \cdot 1$	0	31
0·1–0·49	5	19
0·5–0·99	15	3
1·0–1·49	13	1
1·5–1·99	2	0
2·0–2·49	5	0
2·5–2·99	1	0
3·0–3·49	1	1
3·5–3·99	3	0
4·0–4·49	2	0

The group A organisms from the cases and carriers were typed; in two of the carriers the organisms from both the throat and saliva were untypable, but the remaining 97 *Strep. pyogenes* from both throat and saliva were typed. Eighty-seven (89 %) of the strains in the throat and saliva were of the same serotype; two cases and eight carriers had different serotypes.

Fifty-two children had sore throats from which no beta-haemolytic streptococci had been isolated, but three had these organisms in the saliva, at counts of 10^5; $2 \cdot 4 \times 10^5$ and $9 \cdot 6 \times 10^5$ per ml. One child had group A organisms and the others group G. Three of the 60 control children, who had no sore throat and from whose throats no beta-haemolytic streptococci had been isolated, had these organisms in the saliva; the counts were $2 \cdot 4 \times 10^5$; 3×10^5 and $3 \cdot 8 \times 10^5$ per ml. One had group A, another group C and the third ungroupable organisms.

Beta-haemolytic streptococci were grouped using antisera to groups A, B, C and G; 210 strains were grouped, comprising 47 pairs from the throat and saliva of the acute streptococcal cases; 55 pairs from both throat and saliva of the symptomless carriers; 3 strains from the saliva of the non-streptococcal cases of sore throat; and 3 strains from the saliva of the controls. Two hundred (95 %) belonged to group A and 6 (2·9 %) to group G; only 1 belonged to group C; 3 were ungroupable. The bacitracin screening test correctly identified 99 % of group A and 88·9 % of non-group-A strains.

Table 2 shows the growths obtained from culture of throat swabs, sublingual swabs and salivary specimens from the separate group of 248 school-children with

symptomless streptococcal throat carriage, and it can be seen that whereas previous throat-swabbing had produced growth of beta-haemolytic streptococci from each child, repeat throat-swabbing produced growth from only 66·9 %.

Table 2. *Growth of beta-haemolytic streptococci from the throat swab, sublingual swab and specimen of saliva from* 248 *school-children*

	No.	%
Growth from throat swab	166	66·9
Growth from sublingual swab	56	22·6
Growth from saliva	236	95·1

DISCUSSION

Beta-haemolytic streptococci were isolated from the saliva of 102 out of 107 (95·3 %) streptococcal sore throat cases and symptomless carriers. This isolation rate compares with that reported by Hamburger & Robertson (1948) but is higher than those reported by Hamburger (1944), Duguid (1946*a*) and Rubbo & Benjamin (1953).

Although the mean count in the cases of sore throat, $1·4 \times 10^6$ per ml., differed considerably from the $2·5 \times 10^5$ per ml. found in the carriers, it is impossible to draw a line of demarcation between counts obtained from cases and carriers. In general, however, those of the sore throat cases were above 5×10^5 per ml., although cases with low counts occurred, and those of the carriers were below this figure. Important findings were that not only in 97 % of children were group A organisms present in both throat and saliva, but that in 89 % the group A serotypes in both situations were identical. Clearly the saliva is a reliable indicator of the presence of beta-haemolytic streptococci in the throat in the majority of cases.

Isolation of streptococci of one group from the throat and of another group from the saliva did not occur in any of the cases or carriers, but in ten children the group A serotypes isolated from the sore throat and saliva were different. This latter finding does not necessarily mean that quite separate serotypes colonized both sites, because it is possible that the colonies of beta-haemolytic streptococci which were typed randomly did not represent the predominant serotype of either site.

The isolation rate of *Strep. pyogenes* was high and that of the other groups very low. It was interesting to note scarcely any difference between the cases of streptococcal sore throat and the symptomless throat carriers in the isolation rate of *Strep. pyogenes*. This could not be explained by the presence of a particularly virulent serotype in the community because the T-agglutination patterns of the group A strains were very varied. The number of ungroupable strains of beta-haemolytic streptococci was insignificant; strains were ungroupable presumably because they belonged to groups other than A, B, C or G.

The bacitracin disk tests proved satisfactory and results correlated well with serological grouping of the strains. The grouping regime used in the present series can be employed with accuracy only if typing is also performed. Because there is a slightly greater chance of group A strains being resistant than non-group A strains

sensitive to bacitracin (Maxted, 1953), and because group A strains are generally far more frequent than non-group A strains in the community, it is economical in time and materials to confine serological grouping initially to bacitracin-resistant strains. There is no need to group bacitracin-sensitive strains serologically if typing is to be performed, because if any strain proves untypable its group can then be checked serologically. Two such strains were detected in the present series.

With regard to the second part of this study, it can be seen in Table 2 that culture of saliva appears to be the most efficient way of recovering beta-haemolytic streptococci from the upper respiratory tract. An isolation rate of 95·1 % is very high and that of 66·9 % from the throat swab surprisingly low. The isolation rate using the sublingual swab (22·6 %) is very low and the discrepancy between the results of this and culture of saliva is considerable. Growth from all three specimens occurred simultaneously in only 17·7 % of the children. Cases or carriers not detected by the sublingual swab were mostly those with very few organisms in the saliva. Duguid (1946*a*) isolated beta-haemolytic streptococci from the saliva of only 13 out of 87 persons who had these organisms in the throat and this poor isolation rate may well have been due to his use of sublingual swabs.

These results clearly demonstrate that saliva from certain cases of sore throat may contain many beta-haemolytic streptococci and that saliva from others may contain few. Duguid (1946*b*) stated that in the production of true airborne infection by droplet spray the content of the saliva was important. He added, however, that the danger was appreciable only when the saliva was heavily infected. These studies show that the saliva can indeed be heavily infected and it may be that these people should be included in the category of 'dangerous carriers' which Hamburger, Green & Hamburger (1945) reserved for those who were nasal carriers of beta-haemolytic streptococci. An important investigation would be to find out which of the following categories is the most important from the point of view of spreading infection: nasal carriage, nasal and salivary carriage, and salivary carriage.

Clearly culture of the saliva is very useful, and combined with the use of a throat swab will provide maximum numbers of isolations of beta-haemolytic streptococci from cases of sore throat. The sublingual swab is not considered to be of value in studies on streptococcal sore throat.

I am indebted to Dr M. T. Parker and Dr W. R. Maxted for providing antisera and for giving invaluable advice on matters pertaining to streptococci. I am deeply grateful to them and to the staff of the Streptococcus Reference Laboratory, Colindale, for solving difficult problems in the typing of the streptococci.

I also acknowledge with thanks the co-operation and assistance of Drs E. V. Kuenssberg, J. D. E. Knox, G. MacNaughtan, A. R. Laurence and A. A. Robertson.

REFERENCES

BOISSARD, J. M. & FRY, R. M. (1966). Streptococcal school outbreaks: a method of investigation and control. *Journal of Hygiene* **64**, 221.

DUGUID, J. P. (1946*a*). Expulsion of pathogenic organisms from the respiratory tract. *British Medical Journal* i, 265.

DUGUID, J. P. (1946*b*). The size and duration of air carriage of respiratory droplets and droplet nuclei. *Journal of Hygiene* **44**, 471.

GRIFFITH, F. (1934). The serological classification of *Streptococcus pyogenes*. *Journal of Hygiene* **34**, 542.

HAMBURGER, M., JR. (1944). Studies on the transmission of hemolytic streptococcus infections. II. Beta-hemolytic streptocci in the saliva of persons with positive throat cultures. *Journal of Infectious Diseases* **75**, 71.

HAMBURGER, M., JR., GREEN, M. J. & HAMBURGER, V. G. (1945). The problem of the 'dangerous carrier' of hemolytic streptococci. I. Number of hemolytic streptococci expelled by carriers with positive and negative throat cultures. *Journal of Infectious Diseases* **77**, 68.

HAMBURGER, M., JR. & ROBERTSON, O. H. (1948). Expulsion of group A hemolytic streptococci in droplets and droplet nuclei by sneezing, coughing and talking. *American Journal of Medicine* **4**, 690.

HARE, R. (1940). The expulsion of hemolytic streptococci by nasopharyngeal carriers. *Canadian Public Health Journal* **31**, 539

HOLMES, M. C. & WILLIAMS, R. E. O. (1958). Streptococcal infections among children in a residential home. I. Introduction and definitions: the incidence of infection. *Journal of Hygiene* **56**, 43.

LANCEFIELD, R. C. (1933). A serological differentiation of human and other groups of hemolytic streptococci. *Journal of Experimental Medicine* **57**, 571.

MAXTED, W. R. (1953). The use of bacitracin for identifying group A haemolytic streptococci. *Journal of Clinical Pathology* **6**, 224.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732.

ROSS, P. W. (1970). M.D. thesis, University of Aberdeen.

RUBBO, S. D. & BENJAMIN, M. (1953). Transmission of haemolytic streptococci. *Journal of Hygiene* **51**, 278.

WILLIAMS, R. E. O. (1958). Laboratory diagnosis of streptococcal infections. *Bulletin of the World Health Organization* **19**, 153.

J. Hyg., Camb. (1971), **69**, 355
Printed in Great Britain

Bacteriological monitoring in penicillin treatment of streptococcal sore throat

By P. W. ROSS
Department of Bacteriology, The Medical School, University of Edinburgh

(*Received* 21 *December* 1970)

SUMMARY

Bacteriological monitoring of penicillin therapy in 30 children with streptococcal sore throats was performed by means of salivary and throat-swab culture, on the first, third, fifth and ninth days after therapy had started. Counts of beta-haemolytic streptococci per ml. of saliva were also performed.

Results showed that salivary culture and estimation of the numbers of beta-haemolytic streptococci in the saliva were much more sensitive indices of the effectiveness of penicillin treatment than throat-swab culture.

INTRODUCTION

Penicillin is universally accepted as being the best drug, in non-hypersensitive patients, for the treatment of streptococcal sore throat. Many investigators have recommended its use, including Hamburger & Lemon (1946), Denny, Wannamaker & Hahn (1953), Brumfitt & Slater (1957), Denny (1957), Breese & Disney (1958), Stillerman *et al.* (1960), Parker, Maxted & Fraser (1962) and Mitchell & Baber (1965).

Hitherto the criterion of successful penicillin therapy in streptococcal sore throat has been the production of a negative throat culture from the patients (Goerner, Massell & Jones, 1947). An element of chance is present in the use of swabs, however, and negative cultures may occur not necessarily because there are no organisms at the site of swabbing but perhaps because swabbing technique is faulty (Cruickshank, 1953; Ross, 1970) or because swabs which are detrimental to bacterial survival are used (Rubbo & Benjamin, 1951; Bartlett & Hughes, 1969). Ross (1971) reported, however, that a large number of isolations of beta-haemolytic streptococci, and in particular *Streptococcus pyogenes*, were obtained from cases of streptococcal sore throat when specimens of saliva were cultured. As it was felt that the use of the throat swab alone was a rather insensitive approach to the isolation of streptococci from the respiratory tract, it was decided to culture salivary specimens and perform salivary counts of beta-haemolytic streptococci in cases treated with penicillin, in order to ascertain whether or not a more accurate picture of the effect of penicillin therapy was produced by these procedures than by culture of throat swabs.

MATERIALS AND METHODS

Selection of children

Thirty-two children under the age of 15 years who presented with sore throats to family doctors in a group practice, and from whom beta-haemolytic streptococci were isolated, were investigated. These children were part of a group of 47 children who were investigated in studies on beta-haemolytic streptococci in the saliva (Ross, 1971). The doctors obtained specimens of saliva as well as throat swabs and completed a form for each child at the initial examination. At this time 30 of the children received 0·5–1 g. of oral penicillin daily, as recommended by the British National Formulary (1966) and the Monthly Index of Medical Specialities (1968). All daily doses were given orally in four equal amounts; the other children, usually those over 10 years of age, were prescribed 1·0 g. daily and the younger children 0·5 g. (see Table 2). Those who received 1·0 g. daily had a course of treatment of 7 days and those who were prescribed 0·5 g. daily had a course of 5, 6 or 7 days, depending on which of the doctors in the group prescribed the treatment. One of the two children who did not receive penicillin was prescribed 1·0 g. of erythromycin daily for 5 days and the other was given only analgesic.

Specimen collection

This is described in the previous paper (Ross, 1971). On the third, fifth and ninth days after the start of treatment the author visited the children's homes and obtained salivary specimens and throat swabs. At these visits a check was made on the number of tablets or amount of suspensions remaining, to ensure that the children had taken the treatment prescribed for them.

Laboratory methods

These are described in the previous paper (Ross, 1971).

RESULTS

Table 1 indicates the number and percentage of salivary and throat-swab cultures which were positive on the first, third, fifth and ninth days after the start of treatment. All salivary and throat-swab cultures were positive on the first day,

Table 1. *Growth of beta-haemolytic streptococci from the saliva and throats of 30 children with acute streptococcal sore throat, on the first, third, fifth and ninth days after penicillin therapy had commenced*

	Growth from saliva		Growth from throat	
	No.	%	No.	%
First day	30	100	30	100
Third day	21	70	10	33·3
Fifth day	7	23·3	1	3·3
Ninth day	4	13·3	1	3·3

but thereafter the causal organisms were harvested much more often from the saliva.

In Table 2 the salivary counts of beta-haemolytic streptococci and the results of throat-swab culture from the 30 children on the various days of investigation can be seen. The highest initial count was $4{\cdot}4 \times 10^6$ per ml. and the lowest $6{\cdot}4 \times 10^5$ per ml. In most cases the numbers of beta-haemolytic streptococci isolated fell as treatment continued, but these organisms could still be cultured from the saliva of cases 8, 14, 15 and 27 on the ninth day. In the child who received erythromycin, salivary and throat cultures were negative by the fifth day, but the child who was

Table 2. *Salivary counts of beta-haemolytic streptococci (thousands/ml.), and growth from the throat swabs, of* 30 *children with acute streptococcal sore throat undergoing penicillin treatment*

		Day of treatment							
		First		Third		Fifth		Ninth	
Case no.	Treatment class*	Salivary count (10^3/ml.)	TS†	Salivary count (10^3/ml.)	TS†	Salivary count (10^3/ml.)	TS†	Salivary count (10^3/ml.)	TS†
1	A	1040	+	72	+	–	–	–	–
2	B	920	+	48	–	–	–	–	–
3	B	1560	+	36	–	–	–	–	–
4	D	880	+	8	–	–	–	–	–
5	D	1480	+	–	–	–	–	–	–
6	A	1200	+	240	–	–	–	–	–
7	A	2400	+	–	–	–	–	–	–
8	B	1280	+	12	+	8	–	8	–
9	B	960	+	4	+	–	–	–	–
10	B	720	+	48	–	–	–	–	–
11	B	640	+	–	–	–	–	–	–
12	B	920	+	–	–	–	–	–	–
13	B	800	+	64	–	–	–	–	–
14	B	3200	+	2400	+	260	+	160	+
15	A	2400	+	1300	–	300	–	24	–
16	D	2000	+	–	–	–	–	–	–
17	B	900	+	–	–	–	–	–	–
18	B	840	+	104	+	–	–	–	–
19	B	2200	+	760	+	128	–	–	–
20	C	1040	+	440	+	164	–	–	–
21	C	840	+	–	–	–	–	–	–
22	A	1000	+	84	–	–	–	–	–
23	C	1600	+	220	–	–	–	–	–
24	A	1800	+	92	–	–	–	–	–
25	B	2600	+	–	–	–	–	–	–
26	B	4400	+	–	–	–	–	–	–
27	B	1240	+	360	+	76	–	8	–
28	B	2360	+	600	+	–	–	–	–
29	A	1480	+	496	–	–	–	–	–
30	A	3720	+	256	+	44	–	–	–

* The penicillin treatment classes are as follows: A, 1·0 g. daily for 7 days; B, 0·5 g. daily for 7 days; C, 0·5 g. daily for 6 days; D, 0·5 g. daily for 5 days.

† TS = throat-swab result.

prescribed analgesic produced positive throat and salivary cultures on the ninth day.

On the first day of examination, when growth was obtained from all specimens of saliva and all throat swabs, the beta-haemolytic streptococci isolated from the saliva and those from the throat of each child were grouped and in every case the organisms from the saliva and the throat belonged to the same group. Twenty-eight of the 30 pairs of beta-haemolytic streptococci were group A and the other two pairs (cases 11 and 21) were group G. Each pair of *Strep. pyogenes* had similar T-agglutination patterns. The organisms isolated from the throat and saliva of each child were similar not only at the first but also at each subsequent isolation.

DISCUSSION

It was surprising to find that as many as four children who had been prescribed penicillin still retained *Strep. pyogenes*, of the serotype originally isolated from the throat, in the saliva on the ninth day. The counts were not high however (see Table 2). The doses of penicillin which were prescribed for these children were those recommended by the manufacturers of the proprietary preparations and also by the British National Formulary (1966) and the Monthly Index of Medical Specialities (1968). In such antibiotic literature, although daily dose schedules are well documented the duration of treatment is not, although in the British National Formulary (1966) it is stated that in most cases penicillin treatment should not be continued for more than 7 days. The impression is given in much of the literature that the main factor in the control of therapy is clinical response. In the present series, although all the children had recovered clinically from their throat infection by the fifth day, 23 % still retained the infecting organism. It is clear that at times clinical and bacteriological response may be quite unrelated, but the aim of treatment for streptococcal sore throat should be eradication of the streptococci.

The two sampling techniques produced conflicting results; those obtained from throat-swab culture indicated a greater degree of treatment success than those from salivary culture. The treatment failure rate was in the region of 13 % using salivary culture as an indicator of therapeutic efficacy, but only in the region of 3 % using conventional throat-swab culture. It was surprising to record such a large number of children with high counts of beta-haemolytic streptococci even after 5 days' treatment. There is no doubt that by using the results of throat-swab culture rather than those of salivary culture a far more optimistic picture of the efficiency of the therapeutic measure was obtained.

Cases 8, 14, 15 and 27 still retained *Strep. pyogenes* in the saliva on the ninth day; the counts per ml. in cases 8, 15 and 27 were very low, but this was not so in case 14, who had a count of $1{\cdot}6 \times 10^5$ per ml. These treatment 'failures' may have been due to failures in absorption of penicillin or simply to the children not taking the prescribed treatment; although at each home visit checks were made on the amount of penicillin remaining, it does not necessarily mean that the penicillin was actually ingested. The most likely explanation, however, is that the duration of treatment was too short; in oral penicillin therapy a 10-day course is necessary if

eradication or near-eradication of the organisms is to be achieved (Wannamaker *et al.* 1953).

Four of the 30 children, cases 5, 12, 21 and 26, had had their tonsils removed, and it is interesting to note that the organisms were completely eradicated from each of these children by the third day, even from the child who produced the highest initial count of the series, $4{\cdot}4 \times 10^6$ per ml. It may be that these children with tonsils present are more obstinate from the point of view of treatment, the beta-haemolytic streptococci presumably obtaining protection from the antibiotic by lodging in tonsillar crypts, which may also contain protective cellular debris. As the streptococci from the sore throat can be isolated from the saliva, however, this protection is presumably only partial.

Growth was never produced from throat-swab culture and not from culture of saliva, but in many cases growth was obtained from culture of saliva but not from throat-swab culture. Culture of a specimen of saliva has been recommended for the isolation of beta-haemolytic streptococci from the upper respiratory tract (Ross, 1971), and it can be seen that to perform viable counts of beta-haemolytic streptococci in the saliva of cases of streptococcal sore throat can be a useful guide in assessing the efficacy of penicillin, or indeed any antibiotic, therapy; these results also show that the throat swab alone is less sensitive, and may produce erroneous results. It should not be used as a sole agent in the bacteriological monitoring of penicillin therapy for streptococcal sore throat.

I wish to acknowledge with thanks the help of Drs J. D. E. Knox, G. MacNaughtan, A. A. Robertson and A. R. Laurence in obtaining specimens of saliva and throat swabs when they examined the cases of sore throat. I am indebted also to Dr M. T. Parker, Dr W. R. Maxted and the staff of the Streptococcus Reference Laboratory, Colindale, for the gifts of sera, assistance in typing several strains of *Streptococcus pyogenes* and for general advice on streptococcal problems.

REFERENCES

Bartlett, D. I. & Hughes, M. H. (1969). Bacteriological swabs. *British Medical Journal* ii, 450.

Breese, B. B. & Disney, F. A. (1958). Penicillin in the treatment of streptococcal infections: a comparison of the effectiveness of five different oral and one parenteral form. *New England Journal of Medicine* **259**, 57.

British National Formulary (1966). Aylesbury: Hazell, Watson and Viney Ltd.

Brumfitt, W. & Slater, J. D. H. (1957). Treatment of acute sore throat with penicillin: a controlled trial in young soldiers. *Lancet* i, 8.

Cruickshank, R. (1953). Clinical pathology in general practice: taking swabs. *British Medical Journal* ii, 1095.

Denny, F. W. (1957). *Current Therapy.* W. B. Saunders Company.

Denny, F. W., Wannamaker, L. W. & Hahn, E. O. (1953). Comparative effect of penicillin, aureomycin and terramycin on streptococcal tonsillitis and pharyngitis. *Pediatrics* **11**, 7.

Goerner, J. R., Massell, B. F. & Jones, T. D. (1947). The use of penicillin in the treatment of carriers of beta-haemolytic streptococci among patients with rheumatic fever. *New England Journal of Medicine* **237**, 576.

Hamburger, M. & Lemon, H. M. (1946). The problem of the 'dangerous carrier' of hemolytic streptococci: chemotherapeutic control of nasal carriers. *Journal of the American Medical Association* **130**, 836.

Mitchell, R. G. & Baber, K. G. (1965). Infections by tetracycline-resistant haemolytic streptococci. *Lancet* i, 25.

Monthly Index of Medical Specialities (1968). London: Haymarket Press Ltd.

Parker, M. T., Maxted, W. R. & Fraser, C. A. M. (1962). Tetracycline-resistant haemolytic streptococci. *British Medical Journal* i, 1550.

Ross, P. W. (1970). M.D. thesis, University of Aberdeen.

Ross, P. W. (1971). Beta-haemolytic streptococci in saliva. *Journal of Hygiene* **69**, 347.

Rubbo, S. D. & Benjamin, M. (1951). Some observations on survival of pathogenic bacteria on cotton wool swabs. *British Medical Journal* i, 983.

Stillerman, M., Bernstein, S. H., Smith, M. L., Gittelson, S. B. & Karelitz, S. (1960). Antibiotics in treatment of beta-haemolytic streptococcal pharyngitis. *Pediatrics* **25**, 27.

Wannamaker, L. W., Denny, F. W., Perry, W. D., Rammelkamp, C. H., Ekhardt, G. C., Houser, H. B. & Hahn, E. O. (1953). The effect of penicillin prophylaxis on streptococcal disease rates and the carrier state. *New England Journal of Medicine* **249**, 1.

J. Hyg., Camb. (1971), **69**, 361
With 2 plates
Printed in Great Britain

Interaction of porcine mycoplasmas with fresh animal serum

By D. H. ROBERTS
Central Veterinary Laboratory, Weybridge, Surrey

(*Received* 18 *January* 1971)

SUMMARY

When fresh animal serum was dropped onto seeded mycoplasma agar plates, inhibition of growth frequently occurred. This effect was dependent on the mycoplasma serotype and on the animal species from which the fresh serum came. This activity of fresh animal serum was heat-labile and would not diffuse through the agar medium. Growth of all the porcine mycoplasma serotypes was inhibited by fresh sheep serum. *M. hyorhinis*, *M. hyopneumoniae*, B 3 and the P 45 strains were insensitive to fresh horse serum. The addition of fresh horse serum to specific *M. hyorhinis* rabbit antiserum-impregnated disks appeared to have a synergistic effect and the combination of *M. hyorhinis* antiserum-impregnated disk and fresh horse serum always inhibited the growth of *M. hyorhinis* strains.

INTRODUCTION

The disk growth-inhibition test (Clyde, 1964) has proved to be unsatisfactory for the identification of *Mycoplasma hyorhinis* (Dinter, Danielsson & Bakos, 1965; Dinter & Taylor-Robinson, 1969). *M. hyorhinis* strains when grown on solid medium have been subdivided into antiserum-resistant and antiserum-sensitive strains, and it has been suggested that the difference between sensitive and resistant strains is due to changes in the composition of the mycoplasma membranes (Dinter & Taylor-Robinson, 1969).

The effect of fresh animal serum on the disk growth-inhibition test was investigated with particular reference to *M. hyorhinis.* As normal serum is an essential component in the growth medium for many mycoplasma species, it has been difficult to evaluate the role of normal serum factors in the growth inhibition phenomenon.

The blood of animals which have recovered from contagious bovine pleuropneumonia is highly bactericidal for *M. mycoides*. The bactericidal action is dependent upon the presence of antibody and complement (Priestley, 1952). A heat-labile factor in rabbit serum is essential to neutralize *M. pneumoniae* infectivity for cell cultures (Eaton, Farnham, Levinthal & Scala, 1962). In the metabolic inhibition test, antiserum does not inhibit the growth of *M. pneumoniae* in broth if the horse serum in the growth medium has been heated; unheated guinea-pig serum is also essential for demonstrating the growth-inhibiting effect of specific antiserum against the Negroni strain of *M. pulmonis* (Taylor-Robinson, Purcell, Wong & Chanock, 1966). Complement-like substances play an important role in immune inactivation of *M. gallisepticum* (Barker & Patt, 1967).

Mycoplasma species of porcine origin have been classified into seven groups or serotypes (Dinter *et al.* 1965; Goodwin, Pomeroy & Whittlestone, 1967; Taylor-Robinson & Dinter, 1968). These groups include glucose fermenters and arginine-metabolizing strains of mycoplasma. *M. hyopneumoniae* (*suipneumoniae*), *M. hyorhinis*, *M. granularum*, *M. laidlawii* (B 4) and the B 3 strain are glucose fermenters while *M. gallinarum* (B 2) and *M. iners* (B 6) are arginine-metabolizing strains. Roberts & Gois (1970) reported the isolation from the porcine respiratory tract of mycoplasmas which were arginine-metabolizing and antigenically distinct from the other seven serotypes; these mycoplasmas are represented in this study by the P 45 strain. Using the disk growth-inhibition test, the P 45 strain has been found antigenically similar to *M. hyosynoviae* (Ross & Karmon, 1970) and *M. suidaniae* (Friis, 1970).

The experiments reported here were designed to study the effect of normal serum factors on the porcine mycoplasma serotypes.

MATERIALS AND METHODS

Mycoplasma strains

M. hyorhinis, strain 7, *M. granularum* strain 39 and *M. hyopneumoniae* strain 11 were obtained from Dr W. P. Switzer, Iowa State University, U.S.A. The GDL strain of *M. hyorhinis* was obtained from Dr D. Taylor-Robinson, Salisbury. The B 2, B 3, B 4, B 6 strains and the F strain of *M. hyorhinis* were obtained from Professor Z. Dinter, Uppsala, Sweden. The M 244 strain of *M. hyopneumoniae* was obtained from the Mycoplasma Reference Laboratory, Colindale. The EP 33 strain of *M. hyopneumoniae* was obtained from Dr C. L'Ecuyer, Hull, Quebec. Other strains used in the study were isolated at the Central Veterinary Laboratory, Weybridge; these included the P 19, P 22, S 145 and S 150 strains of *M. hyorhinis*. These strains were typed using the gel diffusion method (Dinter *et al.* 1965) and the metabolic inhibition test (Taylor-Robinson *et al.* 1966). The P 45 strain has been described previously (Roberts & Gois, 1970).

Mycoplasma media

The following three media were used.

(1) Difco PPLO broth plus 20% unheated horse serum, dextrose 0·1% and yeast extract 10% prepared by the method of Marshall & Kelsey (1960). Penicillin G (500 i.u./ml) was added and the pH adjusted to 7·6. Agar plates were prepared using 1% ionagar (Oxoid). This medium was primarily used for growing *M. hyorhinis* and P 45 strains.

(2) Difco PPLO broth plus inactivated horse serum 10%, yeast autolysate (Albimi) 1%, dextrose 0·1%, penicillin G(1000 i.u./ml) and the pH adjusted to 7·8. Agar plates were prepared using 1% ionagar (Oxoid). This medium was used for growing *M. granularum*, B 2, B 3, B 4 and B 6 strains.

(3) Acellular medium described by Goodwin & Whittlestone (1966). Agar plates were prepared using 1% agarose (BDH). This medium was used for growing *M. hyopneumoniae* strains only.

Antiserum production

Specific antisera were prepared in rabbits against the mycoplasma strains by the method described by Roberts (1968). Antiserum to *M. hyorhinis* strain 7 was also prepared in pigs. All sera were Seitz filtered and stored at $-20°$ C.

Cultivation

All cultures were incubated at 37° C. *M. hyopneumoniae* agar cultures were placed in sealed plastic bags and other mycoplasma agar cultures were placed in candle jar containers.

Fresh animal serum and disk growth inhibition

The disk growth inhibition technique was that of Hayflick & Stanbridge (1967). The dried antiserum-impregnated disks were stored at $-10°$ C.

Fresh animal serum was obtained from a rabbit, horse, sheep and guinea-pig. Each serum sample was filtered through a 0·8 μ Millipore filter and stored at $-20°$ C. until ready for use. Preserved guinea-pig serum (Wellcome) was included in the investigation.

Agar plates were prepared, and some were seeded with mycoplasma cultures. On these agar plates, both seeded and unseeded, were placed filter-paper disks (6 mm diameter), each soaked with 0·025 ml. of one of the fresh animal sera or of preserved guinea-pig serum. On similar agar plates dry filter-paper disks, and dried antiserum-impregnated disks for the disk growth-inhibition test, were placed, and 0·025 ml. of fresh animal serum or preserved guinea-pig serum was dropped on each of these disks. When the serum was dropped in this way it spread over the surface of the medium in the area surrounding the disks. A similar series of plates was set up in exactly the same way except that the fresh animal sera and the preserved guinea-pig serum had been heated at 60° C. for 30 min.

RESULTS

Inactivation of porcine mycoplasma by fresh animal serum

When fresh animal serum was added to the filter-paper disks and placed on mycoplasma-seeded agar plates inhibition of growth in the region of the disks was not observed. When, however, the filter-paper disks were placed on the seeded agar plates, and fresh animal serum was dropped onto the disk, so that the serum spread over the surface of the agar in the region of the disk, growth of mycoplasma in the area of the agar covered by fresh animal serum was often not observed. This inhibition of growth did not occur when the fresh serum was heated at 60° C. for 30 min. The results of adding fresh animal serum to the different porcine mycoplasma serotypes are recorded in Table 1. The absence of growth is demonstrated in Pl. 1, Fig. 1 with *M. granularum* and *M. laidlawii* (B 4). The effect of fresh animal serum on *M. hyopneumoniae* was difficult to assess. Owing to its slow rate of growth, the plates were incubated for 2 weeks and then examined, but even after this length of time, growth on agar is relatively sparse and it was not possible

by gross examination to assess any increase or decrease in growth. Even on microscopical examination it was difficult to assess, as colonies were found in the region of the disk. There were, however, differences between the various animal sera; sheep and guinea-pig serum appeared to inhibit growth to a greater extent than rabbit and horse serum.

Table 1. *Inhibition of growth of porcine mycoplasmas by animal sera*

Mycoplasma species	Animal sera				
	Rabbit	Sheep	Horse	Guinea-pig	Preserved guinea-pig
B 2	+ +	+ + +	+ +	−	+
B 3	+ +	+ +	−	−	−
B 4	−	+ + +	+ +	+ + +	+ +
B 6	+ +	+ +	+ +	−	±
M. granularum	+ +	+ + +	+ +	+ + +	+ + +
P 45	+ +	+ + +	−	+ + +	+ + +
M. hyopneumoniae					
Strain 11	−*	±*	−*	±*	±*
M. hyorhinis					
Strain GDL	+	+ + +	−	+ +	+ + +
Strain 7	+	+ + +	−	+ +	+ + +

+ + + Inhibition zone round disk > 3 mm.
+ + Inhibition zone round disk > 2 mm. and < 3 mm.
+ Inhibition zone round disk > 1 mm. and < 2 mm.
± Inhibition zone round disk < 1 mm.
− No inhibition.
* Microscopic assessment only

M. hyorhinis strains were insensitive to fresh horse serum. With rabbit antiserum-impregnated disks it is often difficult to type *M. hyorhinis* strains; the growth of mycoplasma in the region of the disk on solid medium is often not inhibited (see Pls. 1, 2, Figs. 2 and 3), although the gel diffusion and metabolic inhibition tests indicate that the strains are similar. The addition of fresh horse serum, dropped onto these rabbit antiserum-impregnated disks appeared to have a synergistic effect and the combination of *M. hyorhinis* antiserum-impregnated disk and fresh horse serum always inhibited *M. hyorhinis* strains. The results are shown in Table 2. Plate 1, fig. 2 and Pl. 2, fig. 3 show the synergistic effect of fresh horse serum and rabbit antiserum-impregnated disks on *M. hyorhinis* strains F and 7.

Precipitation rings were often observed in the agar medium around the disks containing rabbit antisera, similar to those described by Dinter & Taylor-Robinson, (1969). This occurred with both *M. hyorhinis* and *M. hyopneumoniae* strains. A precipitate or halo was often seen in the disk growth-inhibition test with *M. hyorhinis* strains and *M. hyopneumoniae* antiserum-impregnated disks onto which fresh horse serum had been dropped, as demonstrated in Figs. 2 and 3. Occasionally in the disk growth-inhibition test with *M. hyorhinis* strains and *M. hyorhinis* antiserum-impregnated disks, a zone of mycoplasma growth occurred between the disk and the zone of inhibition. This zone of mycoplasma growth was

Table 2. *Disk growth-inhibition tests with* Mycoplasma hyorhinis *strains: antiserum-impregnated disks with and without fresh horse serum*

	Antiserum-impregnated disks																	
	Without horse serum					With horse serum												
M. hyorhinis strain	F	7	S 150	P 22	P 19	F	7	S 150	P 22	P 19	B 2	B 3	B 4	B 6	P45	MG	HP	C
F	+ + + P	+	+ P	+ P	+ P	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–
7	–	+ + +	–	–	–	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–
GDL	+ + + G	+ + G	+ G	+ + G	+ + G	+ + + G	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–
S 150	+ P	–	+ +	–	–	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–
P 22	+ + + P	+ + +	+ + P	+ + P	+ + P	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–
P 19	–	–	–	–	+ + P	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–	–	–	–	–	–

C = Fresh horse serum control.
HP = *M. hyopneumoniae* Str. 11.
MG = *M. granularum*.
P = Precipitation rings were noted.
G = Growth occurring in region of disk with inhibition of growth in peripheral area. See Table 1 for key.

very extensive with disks prepared from pig antiserum to *M. hyorhinis* strain 7, placed on *M. hyorhinis* seeded agar plates, followed by dropping fresh sheep serum onto the disk. The mycoplasma growth zone was then often 3 mm or more, at the periphery of which a zone of inhibition occurred. This phenomenon is demonstrated in Fig. 3.

A feature of the dropping of either fresh or heated animal serum onto filter-paper disks or antiserum-impregnated disks was the constant appearance of pseudocolonies (Hayflick, 1965; Brown, Swift & Watson, 1940) on solid medium 3. These agar plates were incubated for 2 weeks and the pseudocolonies appeared in the region of the disk where the animal serum had come in contact with the solid agar. These pseudocolonies developed on both seeded and unseeded agar plates. Pseudocolonies and mycoplasma colonies were not found together in the same region of the agar plate.

DISCUSSION

It was not established what effect fresh animal serum had on seeded mycoplasma agar plates, whether it was inactivation, inhibition of growth or lysis. Heat-labile accessory factors in fresh serum may either potentiate growth-inhibiting or metabolic inhibiting antibody titres or stabilize the titres so that they remain at their original level on continued incubation (Taylor-Robinson 1968). In the experiments reported here, fresh animal serum appears to have a far greater effect; this effect was dependent on the mycoplasma serotype and on the animal species from which the fresh serum came. This activity of fresh animal serum was heat-labile and would not diffuse through the agar medium; the factor involved could possibly be complement, for agar is known to be strongly anti-complementary (Jerne, Nordin & Henry, 1963).

The disk growth-inhibition test is not very satisfactory for the typing of *M. hyorhinis*. The addition of fresh horse serum to the rabbit antiserum-impregnated disks aided in the typing of *M. hyorhinis*. There was certainly a synergistic effect observed and, on the limited observations carried out, it seems possible that all *M. hyorhinis* cultures could be typed in this way. Gois, Cerny & Veznikova (1970) showed that *M. hyorhinis* produced colonies in liquid media in the presence of specific rabbit antiserum and of pig serum obtained from *M. hyorhinis*-infected herds. A similar phenomenon was seen in these investigations, but it occurred on solid medium, as demonstrated in Fig. 3. This phenomenon was clearly seen when fresh sheep serum was dropped onto pig antiserum-impregnated disks. Growth of mycoplasmas occurred immediately next to the disk and at the periphery of the growth zone an area of inhibition occurred. An explanation of this phenomenon is not apparent, but the ability of *M. hyorhinis* to grow in the presence of antibodies might explain why *M. hyorhinis* cannot be typed satisfactorily using the disk growth-inhibition test. The interaction of fresh animal serum and the various porcine mycoplasma serotypes could also be used as an aid in their classification. For example, *M. hyorhinis* and the P 45 serotype are sensitive to rabbit, sheep and guinea-pig, and insensitive to horse serum, of these two serotypes only *M. hyorhinis* ferments glucose. Differences between other serotypes can be seen in Table 1.

The growth-inhibition, lysis or inactivation effect of fresh animal serum on porcine mycoplasma in the absence of antiserum cannot be adequately explained. It is hardly likely that heat-labile natural antibodies are to be found in sheep to all the porcine mycoplasma serotypes. Non-specific attachment of serum proteins from the growth medium onto the mycoplasma organism may sensitize the organism to complement. Adsorption of medium components onto *M. hyorhinis* and *M. hyopneumoniae* cells have been shown to occur (Roberts & Little, 1970). This does not, however, explain why all porcine mycoplasmas are sensitive to fresh sheep serum whereas only four serotypes are sensitive to fresh horse serum. A bacteraemia rarely occurs in animal mycoplasmosis. When it does occur, it is associated with stress and sometimes it results in arthritis (Switzer, 1964; Roberts & McDaniel, 1967). Priestley (1952) demonstrated that the blood of cattle, which had recovered from contagious bovine pleuropneumonia, was highly bactericidal for *M. mycoides* and that the action was dependent upon the presence of antibody and complement. The blood of animals dying of contagious bovine pleuropneumonia was not bactericidal, and this was due to a deficiency of complement and not to any deficiency of antibody. It would be interesting to see if the blood of animals under stress is deficient in complement.

The addition of fresh and heated animal serum to medium 3 invariably produced pseudocolonies in the absence of mycoplasma colonies. These colonies form on agar with a high serum content and are composed of calcium and magnesium soaps which form crystalline structures on the agar surface (Hayflick, 1965; Brown *et al.* 1940).

The author would like to thank Mr R. Sayer of the photographic section, Mrs J. Jackman and Mr R. Metcalfe for technical assistance.

REFERENCES

Barker, L. F. & Patt, J. K. (1967). Role of complement in immune inactivation of *Mycoplasma gallisepticum*. *Journal of Bacteriology* **94**, 403.

Brown, T. M., Swift, H. F. & Watson, R. F. (1940). Pseudocolonies simulating those of pleuropneumonia-like micro-organisms. *Journal of Bacteriology* **40**, 857.

Clyde, W. A. (1964). *Mycoplasma* species identification based upon growth inhibition by specific antisera. *Journal of Immunology* **92**, 958.

Dinter, Z., Danielsson, D. & Bakos, K. (1965). Differentiation of porcine mycoplasma strains. *Journal of General Microbiology* **41**, 77.

Dinter, Z. & Taylor-Robinson, D. (1969). Susceptibility and resistance of various strains of *Mycoplasma hyorhinis* to antisera, polymyxins and low pH values. *Journal of General Microbiology* **57**, 263.

Eaton, M. D., Farnham, A. E., Levinthal, J. D. & Scala, A. R. (1962). Cytopathic effect of the atypical pneumonia organism in cultures of human tissue. *Journal of Bacteriology* **84**, 1330.

Friis, N. F. (1970). A new porcine mycoplasma species: *Mycoplasma suidaniae*. *Acta veterinaria scandinavica* **11**, 487.

Gois, M., Cerny, M. & Veznikova, D. (1970). Relationship between metabolism-inhibiting antibodies and colonial growth of *Mycoplasma hyorhinis* and other porcine mycoplasma in the presence of rabbit and pig sera. *Research in Veterinary Science* **11**, 161.

Goodwin, R. F. W. & Whittlestone, P. (1966). Enzootic pneumonia of pigs. Growth and behaviour of the causal mycoplasma in liquid media. *British Journal of Experimental Pathology* **47**, 518.

GOODWIN, R. F. W., POMEROY, A. P. & WHITTLESTONE, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *Journal of Hygiene* **65**, 85.

HAYFLICK, L. (1965). Tissue cultures and mycoplasma. *Texas Reports on Biology and Medicine* **23**, 285.

HAYFLICK, L. & STANBRIDGE, E. (1967). Isolation and identification of mycoplasma from human clinical materials. *Annals of the New York Academy of Sciences* **143**, 608.

JERNE, N. K., NORDIN, A. A. & HENRY, C. (1963). In *Cell-bound Antibodies*, p. 109. Ed. H. Amos and H. Koprowski. Philadelphia, U.S.A.: Wistar Institute Press.

MARSHALL, J. H. & KELSEY, J. C. (1960). A standard culture medium for general bacteriology. *Journal of Hygiene* **58**, 367.

PRIESTLEY, F. W. (1952). Observations on immunity to contagious bovine pleuropneumonia, with special reference to the bactericidal action of blood. *British Veterinary Journal* **108**, 153.

ROBERTS, D. H. (1968). Immunological response in cows to *Mycoplasma bovigenitalium*, strain 1836. *Journal of Hygiene* **66**, 585.

ROBERTS, D. H. & MCDANIEL, J. W. (1967). Mechanism of egg transmission of *Mycoplasma gallisepticum*. *Journal of Comparative Medicine* **77**, 439.

ROBERTS, D. H. & LITTLE, T. W. A. (1970). Serological studies in pigs with *Mycoplasma hyopneumoniae*. *Journal of Comparative Medicine* **80**, 211.

ROBERTS, D. H. & GOIS, M. (1970). A previously unreported serotype of porcine my 'oplasma. *Veterinary Record* **87**, 214.

ROSS, R. F. & KARMON, J. A. (1970). Heterogeneity among strains of *Mycoplasma granularum* and identification of *Mycoplasma hyosynoviae*, sp.n. *Journal of Bacteriology* **103**, 707.

SWITZER, W. P. (1964). In *Diseases of Swine*, p. 498. Ed. H. W. Dunn. Iowa State University Press, U.S.A.

TAYLOR-ROBINSON, D. (1968). The biology of mycoplasma. *Journal of Clinical Pathology* **21** (suppl. no. 2), 38.

TAYLOR-ROBINSON, D. & DINTER, Z. (1968). Unexpected serotypes of mycoplasma isolated from pigs. *Journal of General Microbiology* **53**, 221.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *Journal of Hygiene* **64**, 91.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Inactivation of *M. laidlawii* (B 4) and *M. granularum* by fresh animal serum.

Fig. 2. Disk growth-inhibition tests with the F strain of *M. hyorhinis*, rabbit antiserum-impregnated disks with and without fresh horse serum. MHr.F = F strain of *M. hyorhinis*. MHr. 7 = 7 strain of *M. hyorhinis*. M.Hp. 11 = 11 strain of *M. hyopneumoniae*. MG = *M. granularum* and C = fresh horse serum control. Other symbols as designated in text.

PLATE 2

Fig. 3. Disk growth-inhibition tests with strain 7 of *M. hyorhinis*, rabbit antiserum-impregnated disks with and without fresh horse serum, and antiserum-impregnated disks with fresh sheep serum. Hr. = *M. hyorhinis*, Hr. 7 R and Hr. 7 P indicate rabbit and pig antiserum respectively; remaining disks are prepared from rabbit antiserum. Other symbols, see fig. 2 and text.

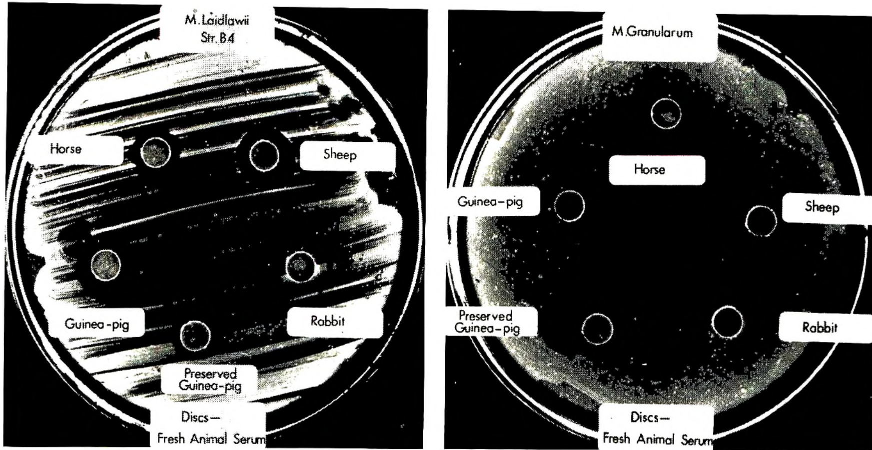


Fig. 1

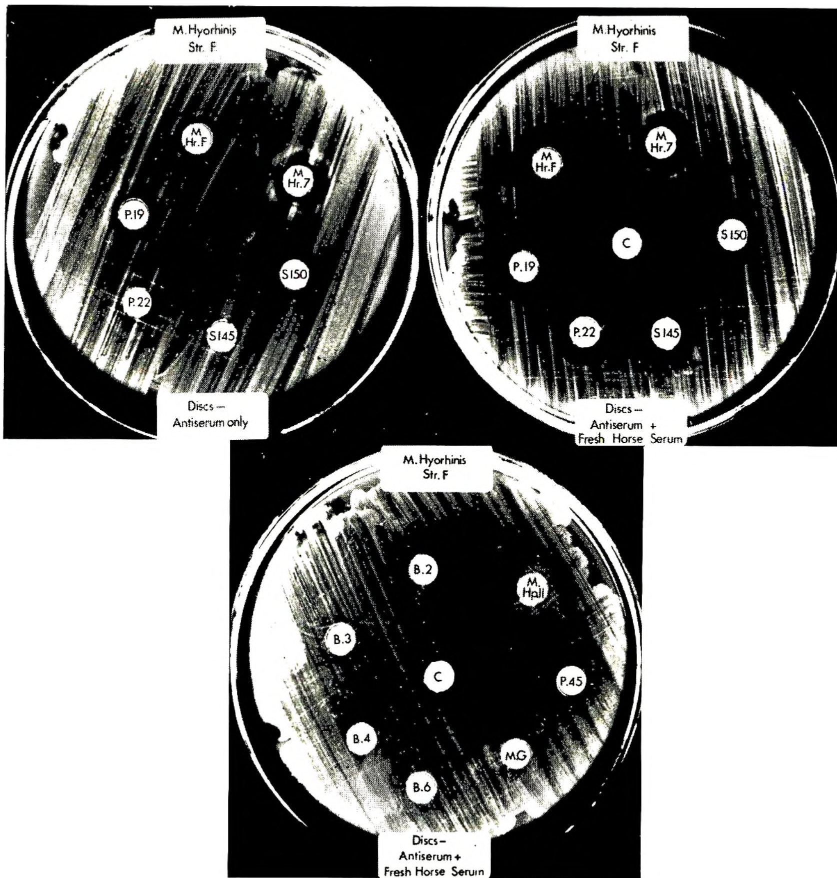


Fig. 2

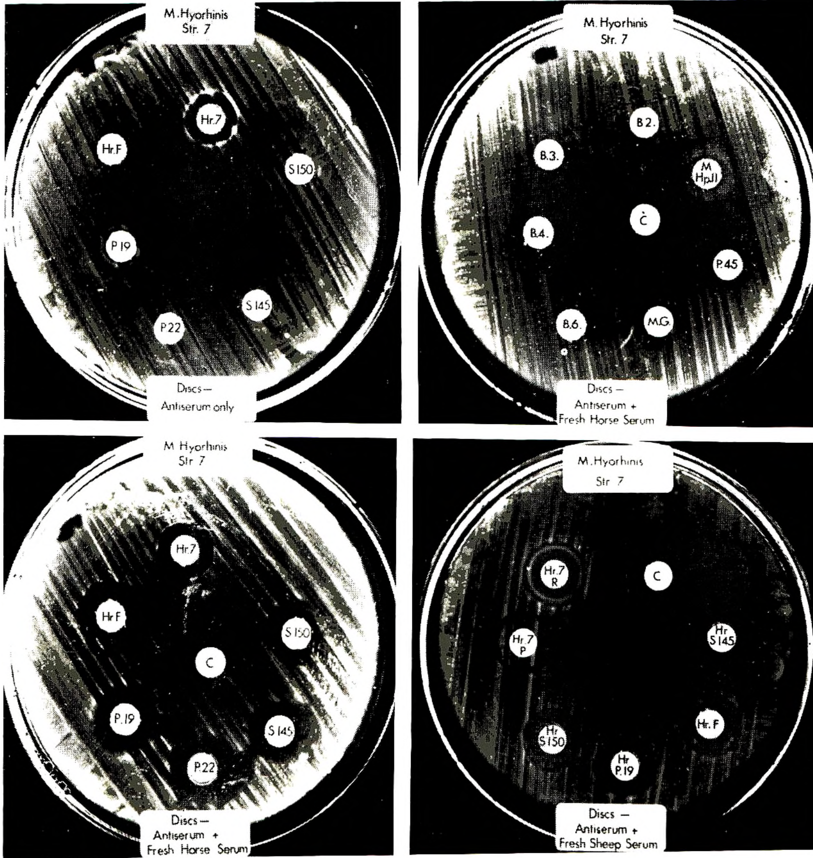


Fig. 3

D. H. ROBERTS

J. Hyg., Camb. (1971), **69**, 369
Printed in Great Britain

Persistence of antibody after subcutaneous vaccination with Wistar RA 27/3 rubella vaccine

BY IRENE B. HILLARY
Department of Medical Microbiology, University College, Dublin

(*Received* 19 *January* 1971)

SUMMARY

After subcutaneous vaccination of rubella sero-negative children with Wistar RA 27/3 rubella vaccine, haemagglutination-inhibiting antibody titres were compared in sera taken 46 days and 2 years after vaccination. Antibody titres were well maintained in the absence of any known exposure to natural infection.

INTRODUCTION

Wistar RA 27/3 rubella vaccine has been licensed recently for general use in the United Kingdom and in the Republic of Ireland. However, since there is no clear evidence that available vaccines are free from the teratogenic and embryotoxic properties of the parent virus, mass vaccination of adult women against rubella is not recommended in any country. There is a risk that vaccine may be given to pregnant women in an adult mass vaccination programme. Thus in some European countries, including the Republic of Ireland and the United Kingdom, the prime group to whom the administration of rubella vaccine is recommended is 11- to 14-year-old girls, with the aim of securing immunity to rubella over their child bearing years. It is essential, therefore, that vaccine-induced immunity is maintained over a prolonged period of time. Booster doses might be indicated if immunity waned. However, even with careful timing some subjects will not be protected for a period before boosting, while others with a minimum level of fading immunity are unlikely to respond to revaccination and will become rubella susceptible later. Furthermore, booster doses may not be free from teratogenic effects. After vaccination with the Cendehill and HPV vaccines serum antibodies have been shown to persist without significant decline for periods up to 2 and 3 years respectively (Meyer *et al.* 1969; Prinzie *et al.* 1969), while Buser, Nicholas & Plotkin (1969) and Plotkin, Farquhar, Katz & Hertz (1969) have described antibody persistence 12 and 14 months after vaccination with Wistar RA 27/3 vaccine. This investigation describes the persistence of haemagglutination-inhibiting (HAI) antibody titres over a two year period in 20 children who took part in a previously reported study of Wistar RA 27/3 rubella vaccine (Hillary *et al.* 1969).

METHOD

In July 1970 samples of venous blood were collected from 20 of the 21 children vaccinated subcutaneously with Wistar RA 27/3 rubella vaccine in July 1968. Blood samples were also obtained from nine unvaccinated rubella seronegative children who served during the first study as sibling contacts for evidence of vaccine virus transmission. In 1968 post-vaccination samples were secured from all children at the 46th day after vaccination.

SEROLOGY

In 1968 rubella HAI antibody titrations were carried out on the 46th day post-vaccination sera by the method described by Stewart *et al.* (1967) which used acid-washed kaolin for the removal of non-specific serum inhibitors. Since then heparin and manganous chloride have replaced kaolin for this purpose (Mann, Rossen, Lehrich & Kasel, 1967; Plotkin, Bechtel & Sedwick, 1968). The original sera held at a temperature of -20° C were therefore retitrated in parallel with the 2-year post-vaccination samples employing the revised techniques.

RESULTS

The modal, median and geometric mean rubella HAI antibody titres of 46-day and 2-year post-vaccination sera are shown in Table 1. Comparisons of the titres for individual sera titrated in parallel after heparin and manganous chloride pre-treatment, 46 days and 2 years after vaccination, are shown in Table 2. No dif-

Table 1. *Rubella haemagglutination-inhibiting antibody titres*

	Time after vaccination		
	46 days		2 years
	Kaolin treated	Heparin–$MnCl_2$ treated	Heparin–$MnCl_2$ treated
Modal titre	160	160	160
Median titre	160	160	80–160
Geometric mean titre	129·9	129·9	113·2

Table 2. *Comparison of haemagglutination-inhibition titres of* 20 *children in sera treated by the heparin–$MnCl_2$ method,* 46 *days and* 2 *years after vaccination.*

Titres at 46 days	Titres at 2 years: 40	80	160	320	640
40	–	–	1	–	–
80	1	5	1	–	–
160	2	2	5	–	1
320	–	–	–	1	–
640	–	–	1	–	–

The stepped lines enclose the area of not more than twofold change of titre.

ference in titre is seen in 11 sera while in 15 the difference in titre was no more than twofold. Fourfold decreases in titre in the 2-year post-vaccination sera were seen in three children and fourfold increases in two children. Statistical comparison of 46-day and 2-year post-vaccination titres shows no significant increase ($P = 0{\cdot}05$). Minor differences are seen between the titres obtained after kaolin treatment and titres obtained after heparin and manganous chloride treatment of 46-day post-vaccination sera (Table 3). However, in no case was the difference between the titres more than twofold. It should be noted that the titrations were carried out almost 2 years apart by different technicians. None of the nine sero-negative unvaccinated sibling contacts had developed rubella HAI antibody in July 1970.

Table 3. *Comparison of haemagglutination-inhibition titres of* 20 *children* 46 *days after vaccination, in sera treated by the kaolin and heparin–$MnCl_2$ methods*

Titres, heparin–$MnCl_2$ method	Titres, kaolin method: 40	80	160	320	640
40	1	–	–	–	–
80	3	–	4	–	–
160	–	2	5	3	–
320	–	–	1	–	–
640	–	–	–	1	–

The stepped lines enclose the area of not more than twofold change of titre.

DISCUSSION

The present study demonstrates that rubella HAI antibody titres produced by subcutaneous administration of Wistar RA 27/3 rubella vaccine in July 1968 were maintained satisfactorily 2 years after vaccination. It is of course conceivable that the immediate post-vaccination sera may have lost some of their HAI antibody during two years storage at $-20°$ C but this seems unlikely, since rubella HAI antibodies are known to be heat-stable (Phillips & O'Brien, 1969). Detailed inquiries failed to produce evidence of any cases of natural rubella in the area concerned since July 1968. This is confirmed to some extent by the finding that nine sibling rubella seronegative contacts had not become seropositive during this period. It is clear therefore that the titres of the vaccinated children are unlikely to have been maintained by subclinical attacks of natural infection.

The relatively high average titres obtained in the first study (Hillary *et al.* 1969) are of some importance, since it has been shown that after vaccination with the Cendehill vaccine, groups with low geometric mean antibody titres are more likely to be reinfected than members of groups with higher titres. In fact, Horstmann *et al.* (1970) showed that reinfection rates were correlated in a general way with HAI antibody titres at the time of exposure, stating that if the titre was $\leqslant 1/64$ the risk of reinfection was considerably greater than if it was $\geqslant 1/128$. However, in the absence of a standard reference serum one cannot really compare antibody titres from different laboratories.

Persistence of antibody 2 years after vaccination is of course of short duration in

comparison with the desirable period of several decades. Nevertheless, assuming that HAI antibody is an indication of immunity to infection, the finding that antibody titres did not significantly decline over the 2-year period indicates that immunity after Wistar RA 27/3 vaccine is likely to be prolonged.

I wish to thank Professor P. N. Meenan for his guidance and to acknowledge the assistance of Dr A. H. Griffith and Dr D. S. Freestone of the Wellcome Foundation in the preparation of the paper. I am also grateful for the co-operation of Dr P. Fox, Dr D. Kelly and Dr D. O'Sullivan, District Medical Officers, in whose areas this work was carried out. I am indebted too to Nurse M. O'Brien for her assistance.

REFERENCES

BUSER, F., NICHOLAS, A. & PLOTKIN, S. A. (1969). Vaccination with the RA 27/3 strain of attenuated rubella virus grown in human diploid cells. International Symposium on Rubella Vaccines, London 1968. *Symposium Series on Immunological Standardisation*, vol. XI, p. 391. Basle: Karger and Karger.

HILLARY, I. B., MEENAN, P. N., GRIFFITH, A. H., DRAPER, C. C. & LAURENCE, G. (1969). Rubella vaccine trial in children. *British Medical Journal* ii, 531.

HORSTMANN, D. M., LIEBHABER, H., LE BOUVIER, G. L., ROSENBERG, D. A. & HALSTEAD, S. B. (1970). Rubella reinfection of vaccinated and naturally immune persons. *The New England Journal of Medicine* **283**, 771.

MANN, J. J., ROSSEN, R. D., LEHRICH, J. R. & KASEL, J. A. (1967). The effect of kaolin on immunoglobulins: an improved technique to remove the non-specific inhibitor of reovirus haemagglutination. *Journal of Immunology* **98**, 1136.

MEYER, H. M., PARKMAN, P. D., HOBBINS, T. E., LARSON, H. E., DAVIS, W. J., SIMSARIAN, J. P. & HOPPS, H. E. (1969). Attenuated rubella viruses. Laboratory and clinical characteristics. *American Journal of Diseases of Children* **118**, 155.

PHILLIPS, C. A. & O'BRIEN, L. (1969). Thermostability of rubella haemagglutination inhibiting antibody. *Applied Microbiology* **17**, 634.

PLOTKIN, S. A., BECHTEL, D. J. & SEDWICK, W. D. (1968). A simple method for removal of rubella haemagglutination inhibitors from serum adaptable to finger tip blood. *American Journal of Epidemiology* **88**, 301.

PLOTKIN, S. A., FARQUHAR, J. D., KATZ, M. & HERTZ, C. (1969). Further studies of an attenuated rubella strain grown in WI-38 cells. *American Journal of Epidemiology* **89**, 232.

PRINZIE, A., HUYGELEN, C., GOLD, J., FARQUHAR, J. & MCKEE, J. (1969). Experimental live attenuated rubella virus vaccine. Clinical evaluation of Cendehill strain. *American Journal of Diseases of Children* **118**, 172.

STEWART, G. L., PARKMAN, P. D., HOPPS, H. E., DOUGLAS, R. D., HAMILTON, J. P. & MEYER, H. M. (1967). Rubella virus haemagglutination – inhibition test. *The New England Journal of Medicine* **276**, 554.

J. Hyg., Camb. (1971), **69**, 373
Printed in Great Britain

Multiple sclerosis distribution in England and Wales and parts of Europe

BY PERCY STOCKS
34 *Brompton Avenue, Colwyn Bay, Denbighshire, Wales*

(*Received* 29 *January* 1971)

SUMMARY

Scotland and Ireland have the highest death rates from multiple sclerosis and high rates are recorded in an area extending south-eastward from Britain through central Europe. The rates tend to diminish with rising latitude and longitude. In England and Wales the county boroughs with notably high rates during 1958–67 were mostly textile towns with cotton and wool mills, situated in the area recording the lowest average levels of sunshine. In the London area mortality from multiple sclerosis was high in those western boroughs and adjacent counties most exposed to the noise of aircraft using the airports of London. The geographical pattern in England suggests that noise and vibration of particular kinds may be a factor in causation along with a climatic factor, but this hypothesis is speculative until further evidence is found to support it.

INTRODUCTION

The causes of multiple sclerosis are shrouded in mystery notwithstanding considerable studies of the geographical incidence of the disease in North America and Australia. Limburg (1950), Kurland (1952) and Westlund & Kurland (1953*a*, *b*) revealed a variation with latitude by surveys of mortality and morbidity rates in Canadian cities of Manitoba and Winnipeg and in New Orleans in the United States. Hypotheses that relations with climate arise from differing intensities of solar radiation or cosmic rays were later discussed by Barlow (1960) and by Acheson, Bachrach & Wright (1960). A similar association with latitude in Australia was found by Sutherland, Tyrer & Eadie (1962) and a hypothesis that poliomyelitis or related virus disease might be connected with appearance of multiple sclerosis in later life was investigated by the same authors (Eadie, Sutherland & Tyrer, 1965).

Kurland & Reed (1964) made a useful summary of the consensus of views regarding the possible causation factors, as did also Miller (1968). A local survey of prevalence has been made in north-east England based on some 50 cases (Poskanzer, Shapira & Miller, 1963), but it is not practicable to make a nation-wide survey of over 1000 cases per annum in order to establish the geographical distribution. For such a purpose statistics provide the only measure of incidence despite their deficiencies for such a chronic disease.

Not much attention has been paid to distribution of death-rates in the British Isles, although, as will be seen, Scotland and Ireland have the highest rates of any

country. In the present paper death-rates from multiple sclerosis in parts of Europe and some other countries during the decade 1951–60 have been calculated from the data published by the World Health Organization, and a detailed study of the deaths during 1958–67 in the towns and counties of England and Wales has been made in the hope of throwing some light on the aetiological factors.

TREND OF MORTALITY IN ENGLAND AND WALES SINCE 1921

It was apparent from the surveys in North America that assessment of rates of prevalence of multiple sclerosis is so difficult that rates of mortality probably provide as good a measure of frequency of the disease. The official records of deaths have been used exclusively in this paper as measures of relative incidence in different places, though a considerable proportion of those affected are recorded as dying of other associated causes. Crude death-rates of males and females have been recorded by the Registrar General of England and Wales for each year since 1921 and the mean annual rates per million living in 5-year periods (or shorter periods when necessary) and in 1966–7 are shown in Table 1, with the ratios between female and male rates.

Table 1. *Mean annual death rates by sex at all ages from multiple sclerosis in England and Wales*, 1921–67

Period	Rates per million		Ratio
	Males	Females	F/M (%)
1921–25	18·6	17·6	95
1926–30	20·2	20·6	112
1931–35	20·0	23·0	115
1936–39	20·0	21·7	108
1940–43	24·7	23·7	91
1944–45	22·5	22·5	100
1946–50	16·2	19·8	122
1951–55	15·2	22·0	145
1956–60	15·2	22·4	147
1961–65	14·4	21·2	147
1966–67	13·5	19·5	144

From 1946 to 1967 the female rate at all ages varied little from about 22 per million. The male rate, after remaining at 20 during 1926–39, rose to 24·7 during the four years 1940–3 owing to the rates being based on the civilian population, only 7 deaths of non-civilians from multiple sclerosis being registered in those years. From the end of the war the rate, based again on the whole population, declined rapidly to 13·5 in 1966–7. The reason for this pronounced fall in male mortality after 1945 must be important from the point of view of aetiology since it had no parallel in the female rate. There was no sex difference of importance before 1946, but since 1950 the crude male mortality level has become established at only two-thirds of that of females.

The improvement shown by males must have been due to removal, or great

diminution, of some factors affecting them such as large changes which took place in industry and conditions of life in factories. For example, heavy work on munitions was replaced by a growth in lighter industries. This will be referred to in the examination of death-rates in large towns.

In order to eliminate the effects on crude rates of the increasing average age of the population and discover at what ages the change in death-rate of males since 1925 has occurred, Table 2 compares the rates per million of each sex at different ages in the recent years 1963–7 with those in 1926–30.

Table 2. *Mean annual death-rates per million by sex and age from multiple sclerosis in* 1963–67 *compared with* 1926–30

Age group	Males		Females		Change of rate % of that in 1926–30		Comparison of sex change
	1926–30	1963–7	1926–30	1963–7	Male	Female	
0–14	0	0·4	0	0·6	—	—	
15–24	2	0·8	3	1·4	−60	−47	Both sexes affected
25–34	10	5·8	12	7·6	−42	−63	
35–44	24	18·2	29	27·0	−24	−5	Males only affected by change
45–54	36	29·8	44	44·6	−17	+1	
55–64	61	35·6	49	47·8	−42	−2	
65–74	91	30·0	61	33·8	−67	−45	Both sexes affected
75+	54	16	44	13·8	−70	−69	

The people who died in 1963–7 at ages under 35 were children during the war or had been born after it, and both sexes showed a drop of about 50 % in death-rate compared with the pre-war level. One or more of the causes of multiple sclerosis productive of death in early life must have been reduced in both sexes after the war. Males dying at ages between 35 and 65 also showed falls in death-rate in 1963–7 by 17–42 % whereas females dying at those ages showed no appreciable change in the rate compared with that in 1926–30. This means that some change in the mode of life or environment peculiar to males in middle life and resulting from or following the war caused a diminution of incidence of the disease to lower levels than hitherto. At ages over 65 a considerable fall in death-rates since the war has occurred for each sex, rather greater for males than females.

MULTIPLE SCLEROSIS IN COUNTY BOROUGHS AND COUNTIES OF ENGLAND AND WALES IN 1958–67

From 1958 multiple sclerosis was distinguished as a subdivision of the Registrar General's Supplementary Abridged List, no. 32 (2), and table 21 in the Statistical Reviews recorded the numbers of deaths by sex at all ages in each county borough, Metropolitan borough and administrative county. This has made it possible to aggregate the deaths during the 10 years 1958–67 and obtain sufficient frequencies in the larger towns and counties to reveal differences which are of statistical significance. No details of age at death are available.

Table 3 shows the actual numbers of deaths of males (D_1) and of females (D_2)

Table 3. *Multiple sclerosis mortality in county boroughs in* 1958–67

(Grouped according to industry divisions in table A of Census of 1951 giving proportions per 10,000 persons in textiles (Order X, subdivided into cotton and wool, 110–12, rayon, hosiery, etc., 113, 114, 118, 134, others in X); metals, engineering, shipbuilding, electrical (Orders V, VI, VIII).

Group A: towns with 10 % or more in textiles (cotton, wool). B: towns with 5–9 % in textiles (cotton, wool) and over 10 % in metals and machinery, etc. C: towns with 10 % or more in textiles (rayon and other). D: towns with 25 % or more in metals, etc. (textiles less than 5 %). E: towns with 15–24 % in metals, etc. (textiles less than 5 %). F: towns with 10–14 % in metals, etc. (textiles less than 5 %). G: inland towns with less than 10 % in metals, etc. (textiles under 5 %). H: seaside resorts with industry as in G.)

	Males			Females				Both sexes, significant excess areas
	No. of deaths (D_1)	Expected (E_1)	Ratio $100D_1/E_1$	No. of deaths (D_2)	Expected (E_2)	Ratio $100D_2/E_2$	d^2/E	P
Group A. Textile towns with 10 % or more in cotton or wool manufacture								
Blackburn	12	7·5	160	14	11·5	122	—	*
Bolton	16	11·1	144	22	17·5	126	4·8	0·03
Bradford	22	20·0	110	39	32·5	120	—	—
Burnley	13	5·6	232	21	8·5	247	15·7	$< 0·001$
Bury	4	4·3	93	11	6·7	164	—	*
Dewsbury	7	3·7	189	2	5·8	34	—	*
Halifax	9	6·7	134	17	10·4	163	4·8	0·03
Huddersfield	16	9·7	170	17	14·4	118	3·8	0·05
Oldham	12	7·7	156	14	12·0	117	—	—
Preston	11	7·8	141	7	11·2	63	—	—
Rochdale	9	6·0	150	12	9·4	120	—	—
Stockport	13	9·9	131	33	13·6	200	16·5	$< 0·001$
Wakefield	2	4·2	48	10	6·6	152	—	—
Group B. Textile towns with 5–9 % in cotton or wool and over 10 % metals, etc.								
Coventry	15	21·5	70	14	34·2	41	—	—
Darlington	6	6·0	100	12	9·5	129	—	—
Doncaster	6	6·1	98	8	9·6	83	—	—
Manchester	67	45·6	147	83	21·7	116	6·2	0·01
Salford	26	10·8	241	18	17·0	106	9·4	0·007
Totals of A and B	266	194·2	137	354	252·1	139	—	—
Group C. Textile towns with 10 % or more in rayon, etc., factories								
Derby	12	9·1	132	11	14·2	77	—	—
Leicester	16	18·9	85	24	29·9	80	—	—
Nottingham	11	21·9	51	22	34·7	63	—	—
Totals of C	39	49·9	78	57	78·8	72	—	—
Group D. Towns with 25 % or more working in metals, machinery, etc.								
Barrow-in-Furness	4	4·5	89	7	7·1	99	—	—
Birmingham	71	76·9	92	116	122·0	95	—	—
Gateshead	2	7·4	27	11	11·4	96	—	—
Lincoln	7	5·3	130	7	8·3	84	—	—
Newport (Mon.)	7	7·4	95	6	11·7	51	—	—
Rotherham	6	6·0	100	9	9·4	96	—	—
Sheffield	51	32·7	153	59	54·3	109	6·1	0·015
Smethwick	6	4·8	125	4	7·7	52	—	—
Sunderland	10	13·2	76	6	20·7	29	—	—
Warrington	8	5·6	155	7	8·7	80	—	*

Table 3. (*cont.*)

	Males			Females			Both sexes, significant excess areas	
	No. of deaths (D_1)	Expected (E_1)	Ratio $100D_1/E_1$	No. of deaths (D_2)	Expected (E_2)	Ratio $100D_2/E_2$	d^2/E	P
Walsall	6	8·3	72	11	13·1	84	—	—
West Bromwich	1	6·7	15	11	10·7	103	—	—
West Hartlepool	5	5·4	92	11	8·5	118	—	—
Wolverhampton	2	10·3	19	18	16·4	110	—	—
Totals of D	186	194·5	96	283	310·0	91	—	—
Group E. Towns with 15–24% working in metals, machinery, etc.								
Birkenhead	7	10·1	69	16	15·8	101	—	—
Cardiff	12	18·0	67	20	29·6	70	—	—
Dudley	8	4·5	178	7	7·1	79	—	*
Ipswich	5	8·3	60	17	11·1	151	—	*
Middlesborough	11	10·8	102	18	17·1	105	—	—
Newcastle-on-Tyne	25	18·5	135	39	29·0	134	5·5	0·02
Plymouth	16	15·0	94	11	23·6	46	—	—
Portsmouth	12	15·2	79	24	23·9	100	—	—
Southampton	15	14·1	104	13	22·6	58	—	—
South Shields	4	6·9	58	7	6·2	113	—	—
Swansea	13	11·8	110	15	18·7	80	—	—
Worcester	7	4·6	152	3	7·4	41	—	*
Totals of E	135	137·8	98	190	212·1	89	—	—
Group F. Towns with 10–14% working in metals, machinery, etc.								
Bootle	3	5·8	52	3	9·8	31	—	—
Chester	3	4·2	71	8	6·5	123	—	—
Croydon	16	19·1	84	39	30·1	129	—	*
Kingston-on-Hull	23	21·1	109	31	33·1	94	—	—
Leeds	37	35·8	103	45	56·3	80	—	—
Liverpool	47	51·7	91	57	81·2	70	—	—
Merthyr Tydfil	3	4·1	73	4	6·5	62	—	—
Tynemouth	2	5·0	40	5	7·9	63	—	—
Totals of F	134	146·8	91	192	231·4	83	—	—
Group G. Towns (inland) with less than 10% working in metals, etc.								
Barnsley	9	5·3	170	3	8·3	36	—	*
Bath	5	5·8	86	11	9·1	121	—	—
Burton-on-Trent	4	1·7	135	5	2·8	179	6·5	0·03
Bristol	18	30·3	32	46	47·6	98	—	—
Canterbury	3	2·2	136	4	3·5	114	—	—
Carlisle	2	4·9	41	12	7·8	154	—	—
East Ham	2	5·3	38	6	8·4	71	—	—
Exeter	9	5·7	158	15	8·9	169	6·1	0·015
Gloucester	4	4·9	82	8	7·7	104	—	—
Great Yarmouth	14	2·6	389	9	5·8	155	19·1	< 0·001
Grimsby	7	6·7	104	7	10·7	55	—	—
Northampton	7	7·6	92	6	12·0	50	—	—
Norwich	4	8·3	48	14	13·1	107	—	—
Oxford	5	7·5	67	12	11·9	101	—	—
Reading	8	8·4	95	9	13·3	88	—	—
Stoke-on-Trent	12	19·0	63	16	30·2	53	—	—
St Helens	7	7·5	93	12	11·7	103	—	—
West Ham	2	7·0	86	5	12·6	40	—	—
York	9	7·3	123	17	11·5	148	—	*
Totals of G	131	148·0	89	217	236·9	91	—	—

Table 3 (*cont.*)

	Males			Females			Both sexes, significant excess areas	
	No. of deaths (D_1)	Expected (E_1)	Ratio $100D_1/E_1$	No. of deaths (D_2)	Expected (E_2)	Ratio $100D_2/E_2$	d^2/E	P
Group H. Seaside resorts with less than 10% working in metals, etc.								
Blackpool	9	10·4	87	16	16·2	99	—	—
Bournemouth	19	10·4	183	8	16·4	49	—	*
Brighton	18	11·3	159	26	18·0	144	7·4	< 0·01
Eastbourne	7	4·3	163	16	6·8	235	12·8	< 0·001
Hastings	6	4·6	130	10	7·3	137	—	—
Southend-on-Sea	15	11·4	132	22	17·4	126	—	—
Southport	8	5·7	140	13	8·9	146	—	—
Wallasey	1	7·2	14	15	11·4	132	—	—
Totals of H	83	65·3	127	126	102·4	122	—	—

* $P < 0·10$ for one sex.

in each town compared with the numbers expected by applying the national death-rates to the estimated populations in each year (E_1 and E_2). Comparative mortality ratios not standardized by age (CMR = $100D/E$) are given for each sex, and as a measure of statistical significance of the combined rate the value of

$$d^2/E = (D_1+D_2-E_1-E_2)^2/(E_1+E_2)$$

has been shown wherever the excess of actual over expected deaths is significant at the 5% level ($P < 0·05$ for $d^2/E > 3·8$).

The towns have been classified into a few industrial groups based on the Census of Industries 1951 (table A of the Report), using the orders and subdivisions of the official list used by the Registrar General. Since it appeared that multiple sclerosis mortality in 1958–67 was notably higher in the textile towns where 10% or more of the working population was engaged in cotton or wool manufacture (Order X, 110–112), those towns were first identified as group A. The next group, B, consists of towns with 5–9% of the population in cotton or wool processing combined with at least 10% in occupations with other heavy machinery such as metal manufacture, ship-building and engineering, including electrical goods (Orders V, VI, VIII).

After the war there was a rapid growth in the manufacture of man-made fibres in the textile industry, and this occurred particularly in Leicester, Nottingham and Derby, which have been identified as group C since the machinery in these factories was different from that employed in the cotton and wool mills (Order X, 113, 114, 118, 123).

The remaining groups, D–G and H, were based on the percentage of the population engaged in industry orders V, VI, VIII, as defined above, these towns having less than 5% in textile work and proportions in the other heavy industries diminishing from over 25% to under 10%. The classification and definition of the eight groups is shown at the head of Table 3. Examination of the other industries in the residue of the seven groups (e.g. chemicals, transport) failed to show any association with multiple sclerosis mortality, but it was found that group H, consisting

of seaside resorts, has such an association for reasons which probably have nothing to do with industry.

In Table 3 the deaths from multiple sclerosis in 1958–67 for each sex are compared with the calculated numbers obtained by applying the national rates to the estimated populations in each of the 82 county boroughs, and within each industrial group the towns are arranged alphabetically. The old textile towns in group A employed in 1951 large proportions of their workers, both men and women, in cotton and wool mills, 7 being in Lancashire, 5 in Yorkshire and 1 in Cheshire. In 11 of the 13 the deaths in the 10 years exceeded the number expected amongst males (with comparative mortality ratios (CMR) ranging from 110 to 232 per 100) and in 11 of the towns this was true also of females (CMR's 117–247). In 5 towns the excess

Table 4. *Frequency of actual/expected (A/E) values above and below* 100 *for multiple sclerosis mortality in county boroughs*, 1958–67

		Males				Females			
		No. with $100A/E$		Significance		No. with $100A/E$		Significance	
Group by industry†	No. in group	< 100	⩾ 100	χ^2	P	< 100	⩾ 100	χ^2	P
A	13	2	11	5·56(+) (A+B)	0·02 (A+B)	2	11	4·61(+) (A+B)	0·025 (A+B)
B	5	2	3			2	3		
C	3	2	1			3	0		
D	14	9	5	1·14(−)	0·29	10	4	1·51(−)	0·22
E	12	6	6	0		6	6	0	
F	8	6	2	2·0(−)	0·17	6	2	2·53(−)	0·12
G	19	12	7	1·32(−)	0·26	8	11	1·90(−)	0·23
H	8	2	6	2·0(+)	0·17	2	6	2·53(+)	0·11

† For explanation of groups see Table 3.

in the two sexes together was significant ($P < 0{\cdot}05$), Burnley and Stockport showing the most notable excess. Every town registered an excess over expectation either in males or females (see also footnote to Table).

Group B consisted of five towns having at least 5 % but less than 10 % of their populations working in textiles but 10 % or more in metals or other heavy industry, and of these three show an excess over expected mortality, statistically significant in Manchester and Salford. Combining groups A and B, there were 266 deaths of males from multiple sclerosis compared with 194 expected (CMR 137) and in females there were 354 compared with 254 (CMR 139).

Contrasting with the above, the three towns in group C with 10 % or more engaged in manufacture of the newer fabrics from man-made fibres had 96 deaths compared with 129 expected, a large deficiency being apparent for both males and females.

The next groups, D–F, consisting of towns with less than 5 % working in textiles and 10 % or more in the other heavy industries, have total deaths of males 106, 135, 134, all below expectation (CMR's 96, 98, 91) and of females 283, 190, 192, also below the expected numbers (CMR's 91, 89, 83), with only two towns, Sheffield and Newcastle-on-Tyne, showing a significant excess. Sheffield has a high proportion

of people engaged in manufacture of miscellaneous metal goods such as cutlery (in Order VIII), matched only by Warrington, Smethwick and West Bromwich.

Group G consists of 19 inland towns with less than 10 % in the heavy industries, registering 131 deaths of males (CMR 89) and 217 of females (CMR 91), and three of them show significant excess over expectation, namely Burton-on-Trent, Exeter and Great Yarmouth. The first of these is remarkable in that 23 % of its working population are engaged in brewing and manufacture of drinks (Order XII, 163–8), a proportion some ten times as great as in any other county borough. Great Yarmouth has 6½ % engaged in making wireless apparatus (Order VI, part), the largest proportion in any county borough, Southend-on-Sea in group H ranking next with 5½ % so occupied.

Table 5. *Increase or decrease in multiple sclerosis mortality from* 1958–62 *to* 1963–7 *in the county boroughs*

Groups of towns		Males: No. of deaths 1958–62	Males: No. of deaths 1963–7	Males: Rise or fall	Males: %	Females: No. of deaths 1958–62	Females: No. of deaths 1963–7	Females: Rise or fall	Females: %
Textiles									
Cotton, wool	A	77	69	−8	−12·4	132	87	−45	−23.7
	B	57	63	+6		69	66	−3	
Rayon, etc.	C	15	24	+9	+60	23	34	+11	+48
Other towns	D–H	371	314	−57	−15	541	467	−74	−14

Group H comprises eight seaside resorts and is the only group other than the cotton and wool textile towns (A, B) to show an average excess of deaths over the numbers expected (CMR's 127 for males and 122 for females). The reason for this is probably the choice by some chronic invalids with multiple sclerosis to move to these resorts, where they die of the disease. Two of the towns show a large excess of mortality in each sex, namely Brighton and Eastbourne.

Table 4 shows clearly the contrast between the cotton and wool towns (A, B) and other groups in their association with multiple sclerosis. Out of 18 towns only 4 for each sex had mortality ratios below 100, and the χ^2 test for such a distribution produced probabilities of 0·02 for males and 0·025 for females, both significant. All of the other groups produced negative or zero associations with mortality, except the seaside resorts (H), for which the relation was insignificantly positive, with $P > 0{\cdot}1$ for each sex.

The contrast in Table 4 between groups A–B and C–G could result if work over long periods in cotton and wool mills, with their peculiar noise and vibration, was a factor in initiating multiple sclerosis in some of the people of both sexes who were exposed to those conditions of work. The absence of any suggestion of such an effect arising in more than an occasional town with heavy metal and machinery production suggests that there is a special kind of noise and vibration in the cotton and wool factories which affects the central nervous system, but that this is not present in the factories producing rayon fabrics. This hypothesis is not yet supported by other evidence but the indications are strong enough to suggest that workers in

textile mills who show early symptoms of multiple sclerosis should be moved as a precaution to other kinds of work.

In view of the progressive changes which have taken place in the textile industry since the war, the deaths in 1958–62 have been compared with those in the next 5 years, 1963–7, in Table 5.

In the cotton and wool towns (A, B) deaths of males scarcely changed, but those of females fell from 201 to 153, a reduction for females of about 24 %. In the towns with rayon factories (C) the deaths of males rose from 15 to 24 and of females from 23 to 34, so the mortality of females in the textile towns seems to have followed the shift in numbers working from the old to the new processes, and seems to support the supposition that there was a smaller risk in the new than in the old. In all the other groups there was a total fall of deaths in males by 15 % and in females by 14 %.

Table 6. *Multiple sclerosis in Metropolitan boroughs in* 1958–64

Metropolitan boroughs	Males			Females			Both sexes areas with significant excess,	
	No. of deaths (D_1)	Expected (E_1)	Ratio $100D_1/E_1$	No. of deaths (D_2)	Expected (E_2)	Ratio $100D_2/E_2$	d^2/E	P
Battersea	2	5·1	39	13	8·1	160	—	**
Bermondsey	2	2·5	80	5	4·0	125	—	—
Bethnal Green	1	2·3	43	5	4·6	109	—	—
Camberwell	7	8·6	81	13	13·4	97	—	—
Chelsea	3	2·4	125	4	3·7	108	—	—
Deptford	2	3·4	59	—	5·3	0	—	—
Finsbury	2	1·6	125	2	2·5	80	—	—
Fulham	3	5·4	56	11	8·6	128	—	—
Greenwich	4	4·2	95	5	6·6	79	—	—
Hackney	8	8·0	100	11	12·6	79	—	—
Hammersmith	2	5·3	38	3	8·3	36	—	—
Hampstead	2	4·8	42	7	7·5	93	—	—
Holborn	1	1·0	100	2	1·6	125	—	—
Islington	9	11·0	82	8	17·3	46	—	—
Kensington	1	8·3	12	16	13·1	122	—	—
Lambeth	17	11·7	143	23	18·3	126	(3·3)	0·06
Lewisham	12	10·9	110	11	17·1	64	—	—
Paddington	4	5·5	73	7	8·6	81	—	—
Poplar	2	5·6	36	3	8·7	34	—	—
St Marylebone	3	3·4	88	8	5·4	88	—	—
St Pancras	7	6·2	113	8	5·7	140	—	—
Shoreditch	1	2·8	36	2	3·1	65	—	—
Southwark	1	4·5	11	6	6·7	89	—	—
Stepney	2	4·5	44	4	7·1	56	—	—
Stoke Newington	3	2·5	120	5	4·0	125	—	—
Wandsworth	29	16·8	163	82	26·4	311	10·6	0·001
Westminster	4	4·2	95	7	6·6	106	—	—
Woolwich	2	7·1	28	6	11·2	54	—	—
City of London	—	—	—	—	—	—	—	—
London Administrative County	137	156·3	88	280	248·7	113	—	—
Total A.C. without Wandsworth	108	139·5	72	198	222·3	89	—	—

** For females, $d^2/E = 3{\cdot}0$, $P = 0{\cdot}09$.

Table 7. *Multiple sclerosis in administrative counties in* 1958–67

Administrative county outside county boroughs	Males			Females			Both sexes, areas with significant excess	
	No. of deaths (D_1)	Expected (E_1)	Ratio $100D_1/E_1$	No. of deaths (D_2)	Expected (E_2)	Ratio $100D_2/E_2$	d^2/E	P
Bedfordshire	30	23·3	129	41	36·6	112	—	—
Berkshire	23	37·7	61	45	43·6	103	—	—
Buckinghamshire	22	34·2	70	32	53·7	60	—	—
Cambridge and Ely	10	19·6	51	26	30·0	87	—	—
Cheshire	82	65·2	120	122	102·4	119	7·3	< 0·01
Cornwall	29	23·7	122	60	36·7	163	13·5	< 0·001
Cumberland	11	15·5	73	21	24·3	87	—	—
Derby	41	52·7	78	78	82·7	73	—	—
Devon	45	36·8	122	63	58·2	108	—	—
Dorset	22	22·2	99	30	34·9	87	—	—
Durham	64	67·1	75	71	105·4	86	—	—
Essex	125	112·5	111	110	76·7	143	11·6	< 0·001
Gloucester	30	38·9	77	60	61·1	98	—	—
Hampshire	54	65·8	82	113	103·3	109	—	—
Hereford	5	9·3	54	12	14·5	83	—	—
Hertford	48	57·9	83	85	91·0	93	—	—
Huntingdon and Peterborough	7	11·2	62	15	17·6	85	—	—
Kent	108	107·6	101	215	169·1	126	7·7	< 0·01
Lancashire	181	155·5	116	293	244·4	120	13·7	< 0·001
Leicester	29	29·5	98	9	11·4	79	—	—
Lincoln (Holland)	6	7·3	82	20	15·2	132	—	—
Lincoln (Kesteven)	14	9·7	144	31	35·9	86	—	—
Lincoln (Lindsey)	27	22·9	118	53	46·3	114	—	—
Middlesex	113	110·4	108	196	173·4	113	†	—
Norfolk	37	27·8	133	47	43·7	108	—	—
Northampton	15	20·8	72	30	32·6	92	—	—
Northumberland	45	34·1	132	54	53·6	101	—	—
Nottingham	33	41·9	78	66	65·7	100	—	—
Oxford	7	12·7	55	29	23·1	126	—	—
Rutland	3	1·8	166	0	2·8	0	—	—
Shropshire	15	21·5	70	47	43·7	108	—	—
Somerset	46	40·4	114	57	63·6	91	—	—
Stafford	49	70·1	70	58	110·1	53	—	—
Suffolk East	17	11·2	152	38	25·5	149	9·1	0·003
Suffolk West	7	9·4	74	20	14·8	135	—	—
Surrey	109	91·9	119	158	144·4	130	3·9	0·05
Sussex East	28	70·4	40	51	63·5	80	—	—
Sussex West	34	28·8	118	57	45·2	126	3·9	0·05
Warwick	58	40·6	143	72	63·8	113	6·3	0·01
Westmorland	5	5·6	89	7	8·7	79	—	—
Isle of Wight	4	6·6	61	17	10·3	165	‡	—
Wiltshire	24	30·2	80	44	47·5	93	—	—
Worcester	25	31·3	80	48	49·2	98	—	—
Yorkshire, E. Riding	18	16·1	112	37	25·3	145	4·5	0·04
Yorkshire, N. Riding	17	28·5	60	41	44·8	91	—	—
Yorkshire, W. Riding	123	113·8	108	175	178·8	98	—	—

For females $d^2/E = 2·9$. ‡ For females $d^2/E = 10·4$.

Table 7 (*cont.*)

Administrative county outside county boroughs	Males: No. of deaths (D_1)	Males: Expected (E_1)	Males: Ratio $100D_1/E_1$	Females: No. of deaths (D_2)	Females: Expected (E_2)	Females: Ratio $100D_2/E_2$	Both sexes, areas with significant excess: d^2/E	Both sexes, areas with significant excess: P
Anglesey	6	3·7	162	6	5·5	91	—	—
Brecknock	2	3·7	51	5	16·1	31	—	—
Caernarvon	13	8·5	153	103	13·3	75	—	—
Cardigan	6	3·7	162	7	5·9	119	—	—
Carmarthen	11	11·7	94	7	16·4	43	—	—
Denbighshire	10	12·1	83	15	19·0	79	—	—
Flintshire	28	10·6	264	17	16·6	102	11·6	$< 0·001$
Glamorgan	38	52·6	72	63	83·7	75	—	—
Merioneth	2	2·7	74	2	4·3	47	—	—
Monmouth	13	23·6	55	23	37·1	62	—	—
Montgomery	3	3·0	100	0	4·8	0	—	—
Pembroke	9	6·2	142	7	9·8	72	—	—
Radnorshire	1	1·3	77	2	2·1	95	—	—

MULTIPLE SCLEROSIS IN ADMINISTRATIVE COUNTIES AND METROPOLITAN BOROUGHS

Until 1964 London County comprised 29 metropolitan boroughs and the city, so Table 6 shows the distribution of deaths in the various divisions from 1958, when multiple sclerosis was first identified in the Registrar General's Abbreviated List of causes of death, to 1964, when the parts of Greater London were redefined. The format of the Table is the same as in Table 3. In the whole area during the 7 years there were 137 deaths of males compared with 156 expected from national rates, giving a CMR of 88 (not standardized for age variations in the populations), and there were 280 deaths of females compared with 249 (CMR 113). The crude ratio of female to male deaths was 2·04, compared with 1·41 in the county boroughs.

In the borough of Wandsworth 111 deaths were recorded by the Registrar General compared with only 43 expected, a highly significant excess ($P < 0·001$). In Lambeth, with 40 deaths compared with 30 expected, the excess was just below the conventional level of significance, and in Battersea this was true for females. No other borough showed any remarkable excess over expectation. The very high rate in Wandsworth seems to be partly accounted for by the presence in the borough of the Royal Hospital and Home for Incurables, which takes patients resident in other districts with chronic disease such as multiple sclerosis, who may then die at the institution after a long stay and be counted in the statistics as residents of Wandsworth. This may be the main factor increasing the death-rates in that borough, but it is curious that the three boroughs with high rates contiguous with Middlesex, Croydon and Surrey are also exposed to the maximum continuous noise of aircraft arriving at and leaving the London airports. Excluding Wandsworth, because of its special hospital, the numbers of deaths in the area defined above, with Lambeth and Battersea, were 257 of males and 429 of females, giving CMR's 108 and 115, compared with only 72 and 89 in London County except

Wandsworth. If Wandsworth is included these become 88 and 112. As will be seen in considering Table 7, however, the four counties of West Sussex, Kent, Essex and East Suffolk, which are affected, though in lesser degree, by aeroplane noise from military training activities, have just as high mortality rates, and this lends doubtful support to the hypothesis that noise is the important factor.

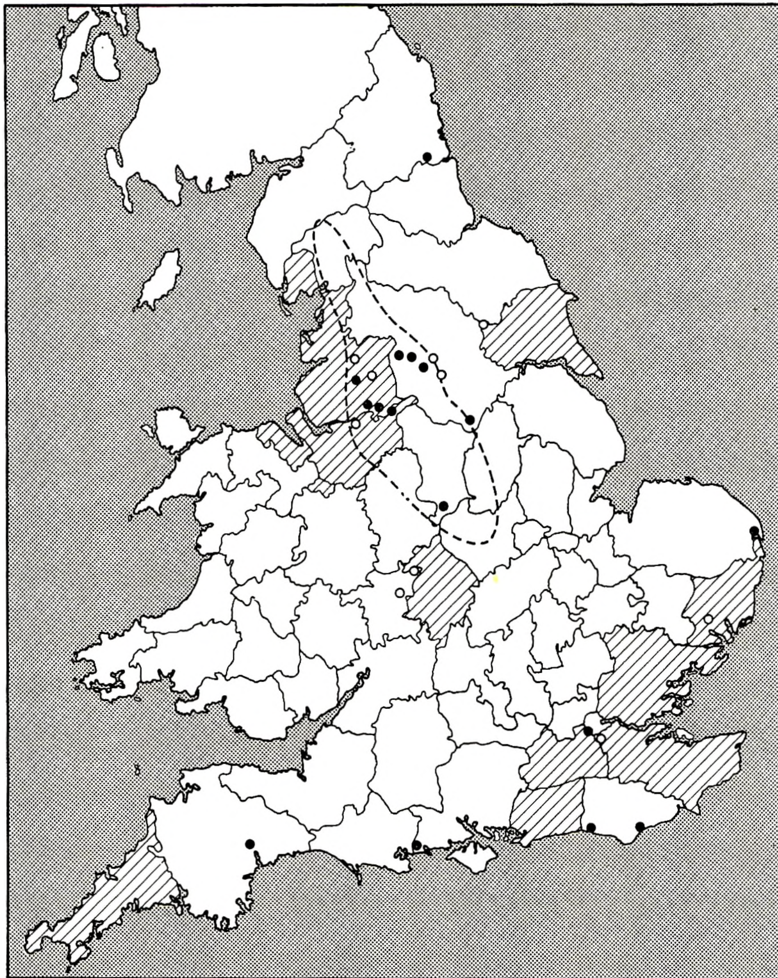


Fig. 1. Multiple sclerosis in 1958–67. ●, County boroughs with significant excess ($P < 0{\cdot}05$). ▨, Administrative counties with significant excess. ○, County boroughs with excess for one sex ($P < 0{\cdot}10$). – – –, Boundary of area with daily sunshine below $3\frac{1}{2}$ hr.

Table 7 shows the deaths in 1958–67 in the other 59 administrative counties excluding county boroughs. Those with significant excess over expectation at the conventional level ($P < 0{\cdot}05$) in the sexes together were Lancashire, East Riding and Cheshire in the north with Flintshire in North Wales; Surrey, West Sussex, Cornwall and Kent in the south; and Essex, East Suffolk and Warwick elsewhere. Middlesex gave a rate just below the significance limit for females and the Isle of

Wight gave a highly significant excess for females. Fig. 1 depicts the county boroughs and counties with high mortality.

In view of the statistical relationship with latitude in North America and Australia and presumed relation with amount of solar radiation, the distribution of the mean annual hours of bright sunshine during years 1931–60 as recorded by the Meteorological Office (1963) has been examined. In Fig. 1 the area enclosed by the broken line represents approximately the part of England where less than 3½ hr. per day of bright sunshine was registered as the 30-year average. Out of the 20 county boroughs located in this area with lowest sunshine 14 have notably high mortality from multiple sclerosis. The area with over 3½ hr. but less than 4 hr.

Table 8. *Multiple sclerosis in hospital regions*, 1963–7

	Males			Females		
Hospital region	No. of deaths	Mean annual rate	Ratio to national	No. of deaths	Mean annual rate	Ratio to national
Manchester	212	19·5	140	299	23·8	119
Metropolitan S.W.	123	15·6	112	217	25·4	127
South Western	107	16·0	115	169	22·0	110
Leeds	113	15·0	108	179	21·9	109
Wessex	70	15·2	109	106	21·8	109
Sheffield	161	14·3	103	213	18·6	93
East Anglia	48	12·4	89	95	23·4	117
Metropolitan S.E.	106	13·0	93	193	21·7	108
Metropolitan N.E.	104	13·3	96	178	20·9	104
Metropolitan N.W.	138	13·5	97	211	19·0	95
Liverpool	67	12·6	90	102	17·5	87
Newcastle-on-Tyne	95	12·4	89	128	16·4	82
Birmingham	147	12·1	88	206	16·4	82
Welsh	76	12·6	90	102	14·9	75
Oxford	85	8·0	57	74	16·2	81
England and Wales	1,614	13·93	100	2,452	20·00	100

average covered most of the north-east of England, including the high-mortality localities of East Riding and Newcastle-on-Tyne. In contrast, most of the south-east of England and south coast recorded averages over 4½ hr. per day but nevertheless included some counties with high mortality, as already noted. Table 8 shows the distribution in 1963–7 of mortality according to sex in the Hospital Regions which incorporate all classes of area, urban and rural.

Supposing that noise and vibration of particular kinds such as textile machinery and aircraft is one factor in the aetiology of multiple sclerosis and lack of solar radiation and warmth is another, the workers in the northern textile towns would have been exposed to both factors, whilst the south-eastern area exposed to the excessive air-traffic noise would suffer from the noise factor with the effects offset by about 1 hr. per day more sunshine than in the north. This would be consistent with the high death-rates in Manchester and Leeds Hospital regions and in the

South-West Metropolitan region (shown in Table 8), but it would leave unexplained the high rate in Cornwall.

It must be emphasized that the hypothesis that vibratory noise is a factor in causation is purely speculative and is not supported at present by any other evidence.

MULTIPLE SCLEROSIS DEATH-RATES IN 30 COUNTRIES IN 1951–60

The World Health Organization (1966) published details by sex and age in years from 1951 for 30 countries where the information was available, and in Table 9 mean annual death-rates have been calculated at ages 25–44, 45–64, 65–74 for each sex. There are 20 countries in Europe with age distinction and 2 without, and 8

Table 9. *Mean annual death-rates from multiple sclerosis per million living in* 1951–60 *or years specified in* 22 *countries of Europe and* 8 *other countries*

	Males				Females				Both sexes	
Countries (Europe, ranked in order of mean annual rate)†	All ages	25–44	45–64	65–74	All ages	25–44	45–64	65–74	Mean rate, all ages	Ratio F/M rates
Europe										
Scotland	27·0	25	66	65	35·0	36	80	59	31·0	1·33
Northern Ireland	23·5	26	66	52	35·0	41	87	59	29·3	1·49
Ireland	26·5	—	—	—	27·5	—	—	—	27·0	1·04
Belgium (1)	27·0	18	42	57	22·0	18	39	49	24·5	0·81
Switzerland	17·0	15	39	38	30·5	29	69	51	23·8	1·79
German F.R. (2)	19·0	16	40	38	25·0	25	47	40	22·0	1·32
Austria (1)	16·0	6	29	38	29·0	20	58	43	21·5	1·83
Denmark	20·5	21	49	45	22·0	25	52	32	21·3	1·07
Czechoslovakia (3)	17·7	14	44	43	23·3	25	53	39	20·5	1·31
England and Wales	15·0	15	33	29	22·0	22	48	32	18·5	1·47
Netherlands	15·5	18	39	41	21·0	23	55	39	18·3	1·35
Norway	16·0	16	36	41	16·5	17	34	36	16·3	1·03
Hungary (3)	13·7	12	34	39	14·7	13	27	31	14·2	1·07
Sweden	11·0	12	6	16	13·0	14	27	16	12·0	1·18
Portugal	14·0	—	—	—	10·0	—	—	—	12·0	0·70
Poland (3)	10·7	11	42	66	11·7	12	19	59	11·2	1·07
France (4)	8·0	7	17	21	12·0	12	25	22	10·0	1·50
Finland	10·0	10	22	43	9·0	8	16	33	9·5	0·90
Italy	6·5	5	13	26	7·0	6	14	16	6·8	1·08
Roumania (3)	5·3	6	33	10	6·7	8	11	15	6·0	1·26
Greece (3)	2·7	4	4	9	2·7	2	7	5	2·7	1·00
Bulgaria (3)	2·7	3	7	4	2·3	3	5	2	2·5	0·85
Other countries										
U.S.A.	9·0	7	19	27	9·0	10	21	21	8·5	1·12
Canada	10·0	10	23	34	11·0	15	27	25	10·5	1·10
Iceland	3·0	—	—	—	10·0	—	—	—	6·5	3·33
Israel	5·0	6	14	11	4·0	4	14	4	4·5	0·80
Japan	1·0	0	2	5	0·5	1	1	3	0·7	0·50
Australia	5·5	4	15	15	8·0	6	19	22	6·8	1·45
New Zealand	7·5	6	22	27	12·0	11	34	31	9·8	1·60
Venezuela	0·5	0	3	11	1·0	2	2	1	0·75	2·00

† (1) 1956–60, (2) 1952–60, (3) 1961–3, (4) 1956–63.

other countries, giving 30 in all with crude rates at all ages for each sex. In the last columns are given the mean crude rates without age distinction and the ratio of female to male rate. The European countries are ranked in order of the rates per million persons, which range from 31·0 in Scotland to 2·5 in Bulgaria.

Table 10. *Countries of Europe, and others, ranked according to latitude, with longitude, population density and (where data were available) mean annual consumption of total fuel, tea and coffee*

Countries (Europe ranked in order of latitude of capital	Capital city		Population density	Annual consumption per capita			Multiple sclerosis death-rate (Table 9)
	Lat.	Long.		Total fuel, 1955–8	Coffee, 1965–6	Tea, 1965–6	
Europe, Iceland, Israel							
Iceland	65	22° W.	3	—	—	—	6·5
Finland	61	25° E.	34	1147	10·2	0·40	9·5
Norway	60	11° E.	1	1504	9·6	0·29	16·3
Sweden	59	15° E.	46	2709	12·9	0·46	12·0
Scotland	56	3° W.	171	—	—	—	31·0
Denmark	55	13° E.	257	2478	11·5	0·70	21·3
Northern Ireland	54	6° W.	261	—	—	—	29·3
Ireland	53	6° W.	49	—	0·2	9·37	27·0
Netherlands	52	5° E.	796	2451	7·1	1·85	18·3
Poland	52	21° E.	206	—	—	—	11·2
England and Wales	51	0	860	—	—	—	18·5
United Kingdom				4872	1·6	9·66	(20·0)
Belgium	51	4° E.	724	3528	—	—	24·5
German F.R.	51	12° E.	502	3265	4·9	0·33	22·0
Czechoslovakia	50	15° E.	246	—	—	—	20·5
Austria	49	16° E.	214	1637	2·8	0·24	21·5
France	49	2° E.	190	1288	4·9	1·19	10·0
Switzerland	47	7° E.	291	1296	7·1	0·51	23·8
Hungary	45	19° E.	107	—	—	—	14·2
Roumania	44	16° E.	191	—	—	—	6·0
Italy	42	12° E.	406	615	2·4	0·10	6·8
Bulgaria	42	15° E.	164	—	—	—	2·5
Greece	38	23° E.	148	—	—	—	2·7
Israel	32	35° E.	196	—	2·5	1·40	4·5
North America and Japan							
U.S.A.	41	(73° W.)	550	5428	7·0	0·73	8·5
Canada	50	(74° W.)	4	4298	3·9	2·44	10·5
Japan	35	(140° E.)	605	770	0·5	—	0·7
Mean	49	10° E.	259	2990	5·33	1·36	14·05
No. of countries	26	23	26	15	16	15	26

(Figures in parentheses are not included in the means.)

The male rates for early death at 25–44 rank rather differently from the order in the table; thus Belgium moves down from 3rd to 4th place and Austria from 6th to 18th, whilst Denmark moves up from 7th to 3rd, Netherlands from 10th to 4th and Norway from 11th to 5th. In Denmark, Norway and Holland deaths of males evidently tend to occur at an earlier age, but this does not apply to females.

Scotland and Northern Ireland rank first or second in each sex–age group, whilst Canada and U.S.A. rank about 15th at all ages and also at early ages.

For the sex ratio of female to male rates at all ages, values over 1·5 appear in Iceland, Austria, Switzerland and New Zealand, and the lowest values, below 1·0, in Finland, Belgium, Portugal, Bulgaria, Israel and Japan.

In Table 10 the European countries and Israel are ranked according to the geographical latitude of their capital cities, ranging from Iceland (65°) to Israel (32°), and it is apparent that the multiple sclerosis death-rate (repeated in the final column) tends to fall on moving farther south. The correlation with latitude is + 0·43 for males and + 0·50 for females. There is also a relation with longitude of the capital cities, the death-rates tending to fall as the degrees East of Greenwich increase, giving correlations of – 0·29 for males and – 0·34 for females, as shown in Table 11.

Table 11. *Correlation between multiple sclerosis death-rates in countries and climatological and environmental factors*

Factors in Table 10	No. of countries	Correlation with death-rate in Table 7: Males	Females
Latitude of capital city	26	+ 0·430	+ 0·500
Longitude of capital city	23	– 0·294	– 0·337
Population density	26	+ 0·374	+ 0·351
Fuel consumption	15	+ 0·144	—
Tea consumption	15	+ 0·340	+ 0·360
Coffee consumption	16	—	+ 0·126

Death-rates exceeding 18 per million occur in a region extending from the United Kingdom, Ireland, the Netherlands, Denmark and West Germany (lat. 51–56°, long. 6° W.–14° E.), to Switzerland, Austria and Czechoslovakia (lat. 46–50°, long. 7–16° E.). Rates below 18 occur in countries to the north of this area – Norway, Sweden, Finland and Iceland (lat. 56–65°, long. 22° W.–15° E.) – and to the south and south-east of it, namely France (lat. 46–50°, long. 2° E.), Italy, Bulgaria, Roumania, Hungary, Poland (lat. 41–45°, long. 12–21° E.) and Portugal, Greece and Israel (lat. 31–40°, long. 9° W.–35° E.). Though sunshine records are lacking for these countries, the average length of daylight certainly increases down the table from Britain and Scandinavia to Greece, consonant with the hypothesis that lack of solar radiation is a factor in causation.

The correlation with longitude indicates, however, that the climatic connexion with multiple sclerosis is not a simple one. It appears that the mean annual air temperature, which depends on extent and altitude of the land mass, distance from the ocean and prevailing winds, is also a factor of importance. The countries with death-rates exceeding 18 per million correspond approximately with an area bounded by the isotherms of 45° and 55° F. and the meridians of longitude between 10° W. and 16° E. This extends from Britain across western Europe in a south-easterly direction, and although it lies between the lines of latitude at 60° and 40° its axis is not parallel with those lines. North and south of the high mortality

wedge, and to the east of its termination, the death-rates become much lower. It is possible that the lower rates in the Balkan countries east of the 16th meridian may be due in part to less complete recognition of the disease on death certificates.

Table 10 gives data for most of the countries for population density per square mile and annual consumption per person of fuel (coal and oil), coffee and tea. Table 11 shows that population density correlates with multiple sclerosis mortality to the extent of +0·37 for males and +0·35 for females, and this might result from some effects of one or more urbanization factors such as noise. Fuel consumption per person has no appreciable relation with male mortality. Tea consumption correlations are +0·34 for males and +0·36 for females, but coffee consumption shows no association with the death-rate.

REFERENCES

ACHESON, E. D., BACHRACH, C. A. & WRIGHT, F. M. (1960). Some comments on the relationship of the distribution of multiple sclerosis to latitude, solar radiation, and other variables. *Acta Psychiatrica et Neurologica Scandinavica* **35** (Suppl. 147), 132–47.

BARLOW, J. S. (1960). Correlation of the geographic distribution of multiple sclerosis with cosmic ray intensities. *Acta Psychiatrica et Neurologica Scandinavica* **35** (Suppl. 147), 108–31.

EADIE, M. J., SUTHERLAND, J. M. & TYRER, J. H. (1965). Multiple sclerosis and poliomyelitis in Australia. *British Medical Journal* i, 1471–3.

KURLAND, L. T. (1952). Frequency and geographic distribution of multiple sclerosis as indicated by mortality statistics and morbidity surveys in United States and Canada. *American Journal of Hygiene* **55**, 457–76.

KURLAND, L. T. & REED, D. (1964). Geographic and climatic aspects of multiple sclerosis; a review of current hypotheses. *American Journal of Public Health* **54**, 588–97.

LIMBURG, C. C. (1950). *Multiple Sclerosis and the Demyelinating Diseases*, chap. 2. Baltimore: Williams and Wilkins.

METEOROLOGICAL OFFICE (1963). *Averages of Bright Sunshine in Great Britain and Northern Ireland.* M.O. 743, H.M.S.O.

MILLER, H. (1968). Epidemiological aspects of multiple sclerosis. *Proceedings of the Royal Society of Medicine* **61**, 937–40.

POSKANZER, D. C., SHAPIRA, K. & MILLER, H. (1963). Multiple sclerosis and poliomyelitis. *Lancet* ii, 917–21.

REGISTRAR GENERAL (1957). *Census of* 1951. Industries. H.M.S.O.

REGISTRAR GENERAL (1958–67). *Annual Statistical Reviews.* Tables. H.M.S.O.

SUTHERLAND, J. M., TYRER, J. H. & EADIE, M. J. (1962). The prevalence of multiple sclerosis in Australia. *Brain* **85**, 149–64.

WESTLUND, K. B. & KURLAND, L. T. (1953*a*). Studies on multiple sclerosis in Winnipeg, Manitoba and New Orleans, Louisiana; prevalence. Comparison between patient groups in Winnipeg and New Orleans. *American Journal of Hygiene* **57**, 380–96.

WESTLUND, K. B. & KURLAND, L. T. (1953*b*). Studies on multiple sclerosis in Winnipeg, Manitoba and New Orleans, Louisiana. Controlled investigation of factors in life history of Winnipeg patients. *American Journal of Hygiene* **57**, 397–407.

WORLD HEALTH ORGANIZATION (1966). *Epidemiological and Vital Statistics Report* **19**, 1–52.

J. Hyg., Camb. (1971), **69**, 391
Printed in Great Britain

The comparative susceptibility of hysterectomy-produced, colostrum-deprived pigs and naturally born, enzootic-pneumonia-free pigs to enzootic pneumonia

BY R. F. W. GOODWIN AND P. WHITTLESTONE
School of Veterinary Medicine, University of Cambridge

(*Received* 11 *February* 1971)

SUMMARY

Hysterectomy-produced, colostrum-deprived (HPCD) pigs and naturally born, enzootic-pneumonia-free (EPF) pigs were compared with respect to their susceptibility to two strains of enzootic pneumonia induced by intranasal inoculation of suspensions of ground pneumonic tissue. All but one of the HPCD pigs developed enzootic pneumonia, whereas the EPF pigs commonly failed to develop the disease; secondly, the pneumonic lesions were more extensive in the HPCD pigs.

When the dose of inoculum was increased in EPF pigs, the resulting pneumonic areas were larger.

In a small, in-contact experiment the disease was also more readily transmitted to HPCD pigs than to EPF pigs.

INTRODUCTION

The hysterectomy-produced, colostrum-deprived (HPCD) pig is particularly useful in research on infectious diseases, and such animals were used extensively in this laboratory to elucidate the aetiology of enzootic pneumonia. Now that the disease has been shown to be caused by *Mycoplasma suipneumoniae* (Goodwin, Pomeroy & Whittlestone, 1965, 1967), other aspects of this important condition – such as its epidemiology and immunology – can be studied more precisely. Although we have already used HPCD pigs to investigate some aspects of natural and artificial immunity in enzootic pneumonia (Goodwin, Hodgson, Whittlestone & Woodhams, 1969*a*, *b*), these animals are clearly different from naturally born pigs, and they may give misleading results if used in experiments designed to elucidate the behaviour of the disease in the field. Furthermore, HPCD pigs are expensive to produce and maintain in strict isolation, and hence naturally born pigs from an enzootic-pneumonia-free herd (EPF pigs) could have a cost advantage if large groups were to be used experimentally. Before using EPF pigs in greater numbers, however, we wished to know how they compared with HPCD pigs in their susceptibility to enzootic pneumonia.

MATERIALS AND METHODS

The HPCD pigs were produced in a manner close to that described by Betts, Lamont & Littlewort (1960). The EPF pigs came from two herds: both of these were formed originally from HPCD pigs and their offspring, and they had been maintained as closed, isolated herds thereafter. The lungs of routinely slaughtered pigs from both herds are regularly checked, and they have been found to be free from lesions of enzootic pneumonia. All the pigs were housed during experiments in a specially designed isolation building, as previously described (Goodwin, Pomeroy & Whittlestone, 1968).

Two strains of enzootic pneumonia were used: the J strain (Goodwin & Whittlestone, 1963) and the CZ strain (Goodwin *et al.* 1968). The pigs were inoculated by slow intranasal instillation of suspensions of ground pneumonic lesions in broth. The diagnosis of enzootic pneumonia in the experimental cases was based on the examination of touch preparations for organisms with the morphology of *M. suipneumoniae* (Whittlestone, 1967) and on the nature of the gross lesions and the histological picture as previously summarized (Goodwin *et al.* 1969*a*). In addition, 11 cases of pneumonia induced with the J strain and 12 cases induced with the CZ strain were cultured in other work (Goodwin *et al.* 1968); all the 11 J cases and 10 of the CZ cases yielded *M. suipneumoniae*, but no other mycoplasmas. The scoring system for recording the extent of the consolidated lesions was related to the fact that, in enzootic pneumonia, such lesions occur almost entirely in the apical and cardiac lobes of the lung, in the intermediate lobe, and the leading edges of the diaphragmatic lobes. Ten points were allocated to each apical or cardiac lobe, five points to the intermediate lobe and five points to each leading edge of the diaphragmatic lobes; thus, if all this tissue were totally consolidated (which would be an unusually severe case) the pneumonic score would be 55.

RESULTS

Inoculation of HPCD pigs

A total of 42 pigs were infected in a series of 24 separate experiments, using 18 different litters; 36 of these pigs received the J strain, and 6 received the CZ strain of enzootic pneumonia. The dose ranges and the scores for the macroscopic lesions produced are shown in Table 1. Only one pig failed to develop any macroscopic lesions; this was one of the four pigs given the smallest dose. The remainder all showed lesions which were confirmed as cases of enzootic pneumonia microscopically, although in eight of the pigs there was also some foreign-body giant-cell reaction. The average pneumonic score for all 42 pigs was 13·4. Hence, the HPCD pigs almost invariably developed macroscopic lesions and, in the main, substantial areas of the lungs were affected.

The average score for the CZ strain pneumonias was 23 but, as only six pigs were involved, and as they received some of the biggest doses, it would not be justifiable to conclude that the CZ strain was more pathogenic.

If the dose of lung inoculated into individual pigs is plotted against the pneu-

monic score, there is no clear dosage effect. However, as the four pigs which received the lowest dose (0·03–0·04 g. of pneumonic tissue) had a mean score of 5, and as there was a slight tendency for the mean score to increase with dose in the other three groups, this might indicate some relationship between the dose given intranasally and the extent of the lesions. This suggestion was taken up later, by observing the effect of increasing the dose of inoculum in EPF pigs.

Table 1. *Relation between dose of pneumonic tissue and extent of lesions in 42 hysterectomy-produced, colostrum-deprived pigs*

Dose range of tissue (g.)	No. of pigs	Lesion score*	
		Range	Average
0·03–0·04	4†	0–8·5	5
0·15–0·5	20	2–38	13·1
	(2)	(21–32)	(26·5)
0·58–1·17	12	1–27	14·4
2·67–8·0	6	7–34	17·8
	(4)	(7–34)	(21·7)

The figures in parentheses refer only to pigs inoculated with the CZ strain. The main figures refer to all the pigs; that is, those given either the CZ strain or the J strain. Apart from two pigs inoculated with the J strain in the group of 20, which were killed after 12 days, all the pigs were killed between 16 and 26 days after inoculation.

* See Materials and Methods. † One pig had no pneumonia when killed.

Inoculation of EPF pigs

In the first part of the work using EPF pigs, 33 animals were inoculated in eight separate experiments; 26 pigs received the CZ strain, and 7 received the J strain of enzootic pneumonia. The dose ranges, the scores for the macroscopic lesions produced, and the time intervals between infection and slaughter are shown in Table 2. It can be seen that 10 pigs did not have gross pneumonic lesions when killed; the gross lesions in the other 23 were confirmed as cases of enzootic pneumonia microscopically. Although 12 of these 33 pigs were killed longer after infection than any of the HPCD pigs in Table 1, four of the six EPF pigs with pneumonia killed late still had quite active lesions, and the six pigs without lung lesions showed no indication of any previous lesions that might have healed completely by the time they were killed; furthermore, there were more negative results among the six pigs killed after 45 days than among those killed after 61 days. The average score for all 33 pigs was 6·2, the average score for the 23 pigs showing lesions was 8·9, and the average score for the 21 pigs that were killed at similar intervals after inoculation to the HPCD pigs in Table 1 was 7·4. Thus, only 70 % of the EPF pigs had macroscopic lesions, and for those that were positive and killed at a comparable time after inoculation to the pigs in Table 1, the average score was only 69 % of the mean score for all the HPCD pigs. As the incidence and extent of the lesions in EPF pigs was less than is desirable in experimental work, an attempt was made to increase both the incidence and the extent of the lesions by giving larger doses of inoculum.

Effect of increasing dose

Pigs from three litters were distributed into three groups, which were given increasing doses of inoculum prepared from the J strain of enzootic pneumonia (Table 3). The inoculum for each pig in group 2 was divided into two doses which were given 1½ hr. apart; the inoculum for each pig in group 3 was divided into three doses, which were given with 1½ hr. intervals. It can be seen that as the dose of the inoculum was increased, the average score for the extent of the lesions also

Table 2. *Relation between dose of pneumonic tissue and extent of lesions in 33 naturally born, enzootic-pneumonia-free pigs*

Dose range of tissue (g.)	No. of pigs	Post infection interval when killed (days)	Pigs without pneumonia	Lesion score*	
				Range	Average
0·01–0·04	5	18–19	1	0–18·5	8·5
0·55–1·14	4	17–19	1	0–7	2·3
3·0–4·33	12	19–20	2	0–27·5	8·8
	6	45	4	0–23	6·1
	6	61	2	0–8	1·9

* See Materials and Methods.

Table 3. *Effect of increasing the dose of inoculum on the extent of pneumonic lesions in naturally born, enzootic-pneumonia-free pigs*

Group	Dose ranges of inoculum		No. of pigs	Lesion score*	
	Volume (ml.)	Tissue (g.)		Range	Average
1	3·5–5	0·58–0·83	4†	0–16·5	5·9
2	19–25	3·17–4·17	4	6–10·5	7·4
3	62–66	10·33–11·0	3	3·5–16	11·7

All the pigs received the same inoculum, a 1 in 6 dilution of the J strain, and all were killed 20 days later. Two uninoculated control pigs killed on the same day had no pneumonia.

* See Materials and Methods. † One pig had no pneumonia when killed.

increased. Even so, when very large doses of inoculum were given (average 64 ml. containing 10·6 g. of pneumonic tissue) the lesions were not quite as extensive as the average for all the HPCD pigs in Table 1. All the lung lesions were confirmed as cases of enzootic pneumonia microscopically.

Inoculation of HPCD and EPF pigs in parallel

While the work summarized in Tables 1 and 2 was still in progress, it was becoming apparent that there was probably a substantial difference in susceptibility between HPCD pigs and EPF pigs, and this was confirmed when the two series were completed. In the meantime, we wished to establish that a similar difference occurred when HPCD and EPF pigs were inoculated in parallel on the same day with the same inoculum under similar experimental conditions.

A comparison of this type was obtained by giving 10 ml. of the inoculum used for

the EPF pigs in Table 3 to two HPCD pigs in parallel. When killed on the same day as the EPF pigs, their lesions (which were confirmed as enzootic pneumonia microscopically) had scores of 16 and 18, giving a mean of 17. This was considerably greater than the average for any of the EPF groups, even those that received over 60 ml. of the same inoculum, although three out of the 11 EPF pigs had lesions of about this extent. This experiment confirmed the difference in susceptibility to the J strain of enzootic pneumonia, and a similar comparison was made using the CZ strain.

Table 4. *Parallel inoculation of hysterectomy-produced, colostrum-deprived pigs and naturally born, enzootic-pneumonia-free pigs, with subsequent in-contact exposure of pigs of the same type*

HPCD pigs			EPF pigs		
Pig no.	Lesion score	Days after infection when killed	Pig no.	Lesion score	Days after infection when killed
2910	21	21	2912	1·5	21
2911	32		2913	6	
2932	3·5	59	2922	5·5	31
2933	1·5		2923	3	
2917	0	Controls*	2936	1·5	62
2918	0		2937	2	
			2939	0·5	
			2941	9	
			2924	0	Control†
	In contact			In contact	
2930	0	23‡	2934	0	29‡
2931	17	37‡	2935	1	
			2938	0	
			2940	0	

The scoring system for lesions is described under Materials and Methods.
* Killed on same day as 2910 and 2911.
† Killed two days after 2922 and 2923.
‡ Days after first contact when killed.

In this other experiment not only were HPCD pigs inoculated in parallel with EPF pigs, but a comparison was made of the natural transmission of the disease in these two types of pig. For the primary infection all the pigs received 0·5 g. of pneumonic tissue as 3 ml. of a 1 in 6 dilution. Two pigs from each group were killed after 21 days, when the HPCD pigs were found to have much more extensive lesions (Table 4); all four cases were confirmed as enzootic pneumonia microscopically, as were the other cases in this experiment, apart from 2941 (see below) and 2939. Although the results for the 12 pigs killed after 45 and 61 days in Table 2 had indicated that the pneumonic lesions in EPF pigs were not less extensive because they developed more slowly – so that they might become comparable with the lesions in HPCD pigs at a different time after infection – this possibility was re-examined by killing two more HPCD pigs at 59 days, two more EPF pigs at 31 days, and a further four EPF pigs at 62 days (see Table 4). Again, there was no

evidence to suggest a delayed development of more extensive lesions of enzootic pneumonia in EPF pigs, for the pneumonic lesions in pig 2941 did not, for the most part, resemble enzootic pneumonia microscopically. The pneumonic lesions in the others remained small despite the longer post-inoculation intervals.

Having established that EPF pigs were considerably less susceptible to a single intranasal inoculation, we wished to see whether this difference also obtained with in-contact infection, for it had been observed in the field that enzootic pneumonia was often not a highly infective disease, in that apparently susceptible pigs could be housed with affected pigs for varying periods of time without developing pneumonia.

Two HPCD pigs (2930 and 2931) were added to the isolation room the day following the removal of pigs 2910 and 2911; they were thus living continuously with pigs 2932 and 2933 for 23 and 37 days, respectively (Table 4). One of the two pigs housed in contact (2931) developed substantial areas of enzootic pneumonia; the other pig (2930), although having no gross lesions, had a catarrhal tracheal exudate containing organisms with the morphology of *M. suipneumoniae*.

Four EPF pigs (2934, 2935, 2938 and 2940) were added to the second isolation room 2 days after the removal of pigs 2922 and 2923; they were thus closely housed with the EPF pigs 2936, 2937, 2939 and 2941 for 29 days before being killed (Table 4). Only one of the four added pigs (2935) had any macroscopic lesions; these were two very small areas in each cardiac lobe; the histological picture was not inconsistent with the early changes in enzootic pneumonia, but no mycoplasmas could be seen in touch preparations. The low degree of transmission in the pigs placed in contact, if indeed enzootic pneumonia had been induced in these pigs, could not be explained by the presence of inactive lesions in the experimentally inoculated pigs; for the two pigs (2922 and 2923) that were probably representative of the degree of infection remaining in the inoculated EPF group showed active lesions histologically with many organisms of the *M. suipneumoniae* type in touch preparations.

DISCUSSION

These experiments show a considerable difference between HPCD pigs and EPF pigs in their susceptibility to intranasal inoculation with two strains of enzootic pneumonia. First, HPCD pigs almost invariably developed gross lung lesions, whereas the failure rate in EPF pigs was considerably higher; secondly, the pneumonic areas were more extensive in the HPCD pigs. It is clearly important to appreciate this difference in susceptibility to intranasal inoculation in experimental work, particularly in studies concerned with natural or artificial protection against this disease; for a degree of immunity or protection that might be inadequate for the more susceptible HPCD pig could be sufficient for the more natural EPF pig.

It seems that the increased resistance of EPF pigs might be partly overcome by increasing the dose of intranasal inoculum, but such heavy dosing could soon exhaust stocks of tested pneumonic tissue.

Why the respiratory tract of EPF pigs should be more resistant to enzootic pneumonia is not clear. Although it is theoretically possible that the herds which

supplied the EPF pigs had at some time been infected subclinically with *M. suipneumoniae*, there is no evidence to suggest that such infection had occurred and both the herds are still free from enzootic pneumonia clinically and pathologically. It is more likely therefore to be a non-specific effect, possibly associated with natural suckling or with some other aspect of the more normal rearing of these animals.

Most of the expenses of this work were met by a grant from the Agricultural Research Council.

REFERENCES

BETTS, A. O., LAMONT, P. H. & LITTLEWORT, M. C. G. (1960). The production by hysterectomy of pathogen-free, colostrum-deprived pigs and the foundation of a minimal-disease herd. *Veterinary Record* **72**, 461.

GOODWIN, R. F. W., HODGSON, R. G., WHITTLESTONE, P. & WOODHAMS, R. L. (1969*a*). Immunity in experimentally induced enzootic pneumonia of pigs. *Journal of Hygiene* **67**, 193.

GOODWIN, R. F. W., HODGSON, R. G., WHITTLESTONE, P. & WOODHAMS, R. L. (1969*b*). Some experiments relating to artificial immunity in enzootic pneumonia of pigs. *Journal of Hygiene* **67**, 465.

GOODWIN, R. F. W., POMEROY, A. P. & WHITTLESTONE, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Veterinary Record* **77**, 1247.

GOODWIN, R. F. W., POMEROY, A. P. & WHITTLESTONE, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *Journal of Hygiene* **65**, 85.

GOODWIN, R. F. W., POMEROY, A. P. & WHITTLESTONE, P. (1968). Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. *Journal of Hygiene* **66**, 595.

GOODWIN, R. F. W. & WHITTLESTONE, P. (1963). Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. *British Journal of Experimental Pathology* **44**, 291.

WHITTLESTONE, P. (1967). Mycoplasma in enzootic pneumonia of pigs. *Annals of the New York Academy of Sciences* **143** (art. 1), 271.

J. Hyg., Camb. (1971), **69**, 399
Printed in Great Britain

The association of viruses with clinical pertussis*

BY MARGUERITE S. PEREIRA
Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London, NW9 5HT

AND J. A. N. CANDEIAS
Department of Microbiology and Immunology, Institute of Biomedical Sciences, University of São Paulo, Brazil

SUMMARY

This study describes the results of attempts to grow viruses from per-nasal swabs taken from 136 children with clinical pertussis.

Altogether 37 strains of a variety of different viruses were isolated. Adenovirus was the most frequent, making up 30 % of the total. Besides these, herpes simplex, measles, influenza A2, influenza B, mumps, poliovirus and respiratory syncytial virus were detected.

Bordetella pertussis was isolated from 22 % of the cases.

It appears that a pertussis-like syndrome can be caused by many agents besides *Bord. pertussis* and an accurate diagnosis requires laboratory confirmation.

INTRODUCTION

The clinical diagnosis of pertussis is based on a respiratory disease syndrome with a characteristic whooping cough. In the past it has been a common and often serious illness of childhood, particularly in those under 1 year of age. The use of a specific vaccine made of *Bordetella pertussis* has considerably modified this picture in many countries. In Britain, for example, the number of cases notified has fallen from 174,000 in 1952 to under 4,000 in 1969. However since notifications had not continued to decline in the last few years doubts have been raised as to the continued efficacy of pertussis vaccine, especially since there were reports of the disease in vaccinated children.

A study to investigate this was undertaken in Britain in 1966 and in a preliminary report (1969) the bacteriological findings described indicated that *Bord. pertussis* was isolated from 792 of 3564 households where a possible case of whooping cough had been reported. The incidence of viruses in these cases has so far not been reported.

Links between viruses and whooping cough have been sought by many workers in the past. In Lapin's monograph on whooping cough published in 1943 he comments on the long history suggesting a virus aetiology for pertussis. Feyrter in 1927 found nuclear inclusions in the lungs of a child who had died of pertussis. He

* This work was done while one of the authors (M.S.P.) was in receipt of grants nos. 68/809 and 68/810 from the Foundation for the Support of Research of the State of São Paulo.

noted the striking similarities of the pulmonary lesion with those found in pneumonia after measles. This resemblance was emphasized by McCordock (1932), who saw intranuclear inclusions in the lungs of 35 fatal whooping cough cases, and similar lesions were reported by Goodpasture, Auerbach, Swanson & Cotter in 1939 in five infants who died from what he called a new virus infection following measles and whooping cough. However, filtrates of infectious material from cases did not transmit whooping cough nor did filtrates of *H. pertussis*, so his comment was: 'practically all investigators are now convinced that whooping cough is not due to a virus'.

However, since then there have been several reports of viruses causing a pertussis-like illness. Chany and his colleagues reported in 1958 their results of virus studies with children with pneumonia. They presented evidence associating adenoviruses with whooping cough and pneumonia and compared their histological findings with those reported by Goodpasture *et al.* in 1939. They commented on the difficulty of distinguishing some of their cases of proven adenovirus infection from whooping cough and measles. Farber & Vawter in 1961 reported on a fatal case of 'clinical pertussis' where inclusions typical of adenovirus were found in sections of lung.

In 1964 Olson, Miller & Hanshaw reported on the isolation of adenovirus type 12 from four children with a pertussis-like syndrome, and in 1966 Collier, Connor & Irving reported the isolation of adenovirus type 5 from a child who died after a pertussis-like illness. In this case several siblings had colds at the same time and one younger brother was observed to have a paroxysmal cough with a whoop, typical of pertussis. None of these children had antibody to *Bord. pertussis* but antibody to adenovirus was detected and the lungs in the fatal case showed typical adenovirus inclusions. Finally Connor (1970) found evidence of infection with adenovirus in 11 out of 13 children with a pertussis syndrome in Florida. Thus when a virus has been associated with pertussis it has almost always been adenovirus. The association of other viruses does not seem to have been reported, but it seemed possible that other viruses could be implicated, especially as the first strain of the Hong Kong/68 variant of influenza A2 to be isolated in Britain was from a child diagnosed as having pertussis and only when attempts to grow *Bord. pertussis* failed was the possibility of a virus aetiology investigated.

The results reported here were obtained from a study in São Paulo, Brazil, over a period of 8 months in 1969–70.

MATERIALS AND METHODS

Pernasal swabs were taken from 136 children with clinical pertussis and after the inoculation of media for bacteriology the swabs were inoculated into HeLa cells, rhesus monkey kidney cultures (from Flow Laboratories) and into fertile hens' eggs. Standard procedures for the detection and identification of viruses were followed.

RESULTS

Altogether 37 strains of virus were grown from 37 (27 %) of the children (Table 1). A wide variety of different viruses were represented, nearly all of which are commonly associated with respiratory infection. Suitable systems for isolating rhinovirus were not available and none were detected.

Table 1. *Virus isolations from cases of clinical pertussis, São Paulo* 1969–70

Virus isolated	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Total
Adenovirus	3	3	3	1	—	1	—	—	11
Herpes simplex	1	3	—	1	2	1	1	—	9
Measles	—	1	—	2	1	—	1	1	6
Influenza A 2	—	1	1	1	—	—	—	—	3
Para influenza	—	1	1	—	—	1	—	—	3
Influenza B	—	—	1	—	—	1	—	—	2
Mumps	—	—	1	—	—	—	—	—	1
Poliovirus	—	—	1	—	—	—	—	—	1
Resp. syncytial	—	—	—	1	—	—	—	—	1
Total	4	9	8	6	3	4	2	1	37
No. of specimens examined	6	12	26	14	21	31	16	10	136

Table 2. *Frequency and type of* 37 *viruses isolated*

Virus	Type	Number	Total	%
Adenovirus	1	4	11	30
	2	1		
	3	3		
	5	3		
Herpes simplex	—	—	9	24
Measles	—	—	6	16
Influenza A 2	—	—	3	8
Para influenza	1	1	3	8
	3	2		
Influenza B	—	—	2	5
Mumps	—	—	1	3
Polio	2	—	1	3
Resp. Syncytial	—	—	1	3

The number of viruses isolated was considerably greater during the three winter months, July–September. The average number of cases of suspected pertussis in those months was 17 and a virus was isolated in 44 %.
During the other 5 months the number of cases averaged 17, but in this period the percentage of viruses isolated was only 17 %.

Table 2 shows the frequency with which different viruses were detected. Adenoviruses were the most frequent virus isolated and formed 30 % of the total strains isolated. The serotypes were those commonly associated with infections in childhood. The next most frequent was herpes simplex. Whether these were aetiological

agents in the disease process is uncertain, but since most of the children were young it is possible that some of the illnesses anyway were primary infections with this virus.

The isolation of measles virus was perhaps surprising. These strains were isolated in rhesus monkey kidney after 2–3 passages and were identified by the characteristic inclusions in stained cover-slip preparations and finally by specific neutralization tests. One cannot exclude the possibility that they had been carried as latent viruses in the monkey kidney cultures as has been shown by Ruckle (1958) to occur in a proportion of rhesus monkeys. Against this is the fact that measles virus was not found in other cultures in the same batch, neither inoculated nor controls.

Table 3. *Isolation of viruses and* Bord. pertussis *in 136 cases of clinical pertussis*

	No. of patients	No. of virus isolations	%
Bord. pertussis isolated	29	5*	17
Bord. pertussis not isolated	107	32	30

* Three adeno-, 1 polio-, 1 herpes virus.

Of the six strains isolated, four were from children who had been ill for 10 days with fever and cough, one from a child with fever, cough and sore throat, and one from a child 11 days after onset of illness with fever, cough and a discrete rash.

The three strains of influenza A2 were all similar to the Hong Kong/68 variant and were isolated one in each of the three winter months. These strains provided an indication of the prevalence of this virus in the population as no epidemic was recorded. This pattern was not unlike that observed in Britain earlier that year when this variant was isolated during an unusually prolonged period of several months but did not cause any well-defined epidemic.

The isolations of influenza B viruses were also widely separated in time.

The strain of mumps virus was isolated from a child in whom parotitis was not a feature; but it is of course well known that a considerable proportion of mumps infections are similarly without parotitis.

The genetic markers of the strain of poliovirus were not investigated and it is not known whether it was a vaccine or a wild strain.

Table 3 shows the proportion of cases where *Bord. pertussis* was grown, where a virus was isolated and where they were associated. The 21 % isolation rate for *Bord. pertussis* is curiously enough almost identical with that reported in the study done in Britain on a much greater scale where *Bord. pertussis* was isolated from 792 in 3564 households, just over 22 %. The virus isolation rate in cases where *Bord. pertussis* was not isolated was nearly twice as great as in cases where it was isolated.

DISCUSSION

It appears from these results that many viruses besides adenovirus may be associated with a syndrome easily confused with pertussis. Some of the viruses isolated may well have been unconnected with the current infection but it was impossible to determine which of these was merely incidentally detected in the respiratory tract. Even a serological response would not confirm the association since double infections occur frequently and either of the isolated agents could have provoked the clinical syndrome observed.

The large number of different viruses detected suggests that the diagnosis of pertussis cannot be made accurately on clinical grounds and must be confirmed bacteriologically.

We should like to express our thanks to Dr Sebastião Timo Iaria for the material used in the present work, which was collected in order to develop his research programme on isolation and type identification of *Bord. pertussis*.

We should like to thank Miss Maria Aparecida de Rezende Araujo for her excellent technical assistance.

REFERENCES

Chany, C., Lépine, P., Lelong, M., Le Tan Vinh, Satgé, P. & Virat, J. (1958). Severe and fatal pneumonia in infants and young children associated with adenovirus infections. *American Journal of Hygiene* **67**, 367.

Collier, A. M., Connor, J. D. & Irving, W. R. (1966). Generalised type 5 adenovirus infection associated with the pertussis syndrome. *Journal of Pediatrics* **69**, 1073.

Connor, J. D. (1970). Evidence for an etiologic role of adenoviral infection in pertussis syndrome. *New England Journal of Medicine* **283**, 390.

Farber, S. & Vawter, G. F. (1961). Clinical Pathological Conference. *Journal of Pediatrics* **58**, 876.

Feyrter, F. (1927). Über die pathologische Anatomie der Lungenveranderungen beim Keuchhusten. *Frankfurter Zeitschrift für Pathologie* **35**, 213.

Goodpasture, E. W., Auerbach, S. H., Swanson, H. S. & Cotter, E. F. (1939). Virus pneumonia of infants secondary to epidemic infections. *American Journal of Diseases of Children* **57**, 997.

Lapin, G. H. (1943). In *Whooping Cough*. Springfield, Illinois: Charles C. Thomas.

McCordock, H. A. (1932). Intranuclear inclusions in pertussis. *Proceedings of the Society for Experimental Biology and Medicine* **29**, 1288.

Olson, L. C., Miller, G. & Hanshaw, J. B. (1964). Acute infectious lymphocytosis presenting as a pertussis-like illness. Its association with adenovirus type 12. *Lancet* i, 200.

Report (1969). Efficacy of whooping cough vaccines used in the United Kingdom before 1968. *British Medical Journal* iv, 329.

Ruckle, G. (1958). Studies with the monkey intra-nuclear inclusion agent (MINIA) and foamy agent derived from spontaneously degenerating monkey kidney cultures. *Archiv für die Gesamte Virusforschung* **8**, 139.

J. Hyg., Camb. (1971), **69**, 405
Printed in Great Britain

Colonization resistance of the digestive tract in conventional and antibiotic-treated mice

BY D. VAN DER WAAIJ, J. M. BERGHUIS-DE VRIES AND J. E. C. LEKKERKERK-VAN DER WEES
Radiobiological Institute TNO, Lange Kleiweg, Rijswijk, The Netherlands

(*Received* 1 *March* 1971)

SUMMARY

The effect of oral administration of antibiotics on the intestinal flora of conventional mice and their resistance to colonization by orally introduced *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was studied. Colonization resistance (CR) was expressed as the log of the oral bacterial dose followed by a persistent take in 50 % of the contaminated animals. The intestinal flora was virtually eliminated by the antibiotics and this elimination was accompanied by a precipitous fall of CR. CR gradually returned to normal values during the period of repopulation of the intestinal tract by the organisms surviving the treatment. Antibiotic treatment resulted in the disappearance of Enterobacteriaceae, enterococci, staphylococci and yeasts and, under appropriate housing conditions, the animals remained free of these organisms indefinitely. Germ-free mice contaminated with the intestinal flora of an antibiotic-treated animal and their offspring housed in a germ-free isolator showed high values of CR. Their intestinal flora consisted of anaerobic bacteria only. Apparently, these anaerobes are responsible for CR in these and in conventional mice.

INTRODUCTION

In a previous paper the selective elimination of Enterobacteriaceae from the digestive tract of mice by administration of an antibiotic mixture was reported (van der Waaij, 1968). Provided precautions were taken to prevent gross contamination, the animals remained free of Enterobacteriaceae species. Even when placed in conventional animal rooms, it took weeks before the animals became recolonized and they were, apparently, resistant to colonization of their intestinal tract by small numbers of ingested Enterobacteriaceae. We propose to call this resistance colonization resistance (CR).

When it was found that elimination of organisms depends on numbers of orally administered cells, CR could be quantified. In the present investigation, CR was studied quantitatively before, during and after antibiotic treatment. As indicated in Fig. 1, the period before antibiotic treatment is called phase I, the actual treatment period phase II and the period in which organisms surviving the treatment repopulate the intestinal tract phase III; phase IV begins when repopulation is completed. The results showed CR to be equal in animals of phases I and IV and

to be severely decreased in phases II and III. This suggested that CR might be due to organisms present in phase I and surviving the antibiotic treatment. In order to analyse the mechanism of CR, germ-free mice were contaminated with the flora of one phase IV mouse. These animals and their offspring were found to be highly resistant to colonization. Apparently, their intestinal flora induces CR and it is supposed to produce a hypothetical factor, colonization resistance factor (CRF). For convenience, these mice are called CRF-mice. Their intestinal flora, which consisted of anaerobic bacteria only, was subjected to a detailed study to be reported separately (Wensinck & Ruseler-van Embden, 1971).

MATERIALS AND METHODS

Mice

Conventional females from a non-inbred *Pseudomonas aeruginosa*-free Swiss mouse stock (ND 2) (van der Waaij, Zimmerman & van Bekkum, 1963), varying in age between 9 and 12 weeks and weighing between 25 and 35 g., were used. In a few experiments CRF-mice, the offspring of germ-free (GF) mice contaminated with the flora of one phase IV mouse (see Introduction), were used. These animals were maintained under GF conditions and were of the same stock and age and weight range as the conventional animals.

Antibiotic sensitivity of faecal organisms

Applying the sensitivity test as used in the selective decontamination of monkeys (van der Waaij, de Vries & Lekkerkerk, 1970) the combination of streptomycin and neomycin was found to be optimal; therefore these antibiotics were used in the present experiments. The mixture was supplied in a concentration of 5 mg. of each antibiotic per ml. of drinking water to which pimaricin was also added (100 μg./ml.).

Housing and isolation conditions

These will be described for each of the four phases of the selective decontamination procedure. Phase I: animals were housed singly on wire mesh 1 cm. above three sheets of filter paper in autoclaved polycarbonate cages and supplied with autoclaved pellets and drinking water acidified to pH 3 after being autoclaved. Cages were placed on racks closed with plastic sheets (van der Waaij & Sturm, 1968). Phase II: the cages were transferred to peracetic acid-sterilized laminar cross-flow benches and placed at a distance of 10 cm. one from another; the autoclaved drinking water to which antibiotics were added was not acidified. Phase III: the same conditions as phase II but antibiotics were not supplied and drinking water was acidified. During manipulations inside the cabinet long peracetic acid-sterilized neoprene gloves were worn. The phase II and III procedures have been described in detail before (van der Waaij & Andreas, 1971). Phase IV: the same conditions as phase I.

The offspring of GF mice contaminated with the faecal flora of one phase IV mouse (CRF-mice) were housed in a GF plastic isolator. When used for quantitative

study of CR the animals were removed from the isolator and housed as the animals of phase III.

Evaluation of CR

Overnight cultures of mouse-derived streptomycin- and neomycin-resistant strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in brain–heart infusion (Difco) were tenfold serially diluted. The phase I and IV mice received 10^9, 10^7 and 10^5 cells per dose of 0·1 ml., the phase II mice 10^3 and 10^2 and the phase III mice 10^8, 10^6 and 10^4 cells. The bacteria were administered to the mice by introducing the cone of a 2 ml. injection syringe into the mouth and slowly delivering 0·1 ml. of the suspensions. Each challenge dose was given to 20 mice and different mice were challenged in the four phases. Faeces were cultured at intervals for the presence of the organisms introduced.

Faeces cultures

Fresh faeces of phase III and IV mice were cultured weekly in brain–heart infusion; subcultures were made on brain–heart infusion agar, Endo agar, staphylococcus medium no. 110 and aesculin-azide agar which was prepared according to Sneath (1956). Faeces of animals contaminated with streptomycin- and neomycin-resistant strains of *E. coli*, *Ps. aeruginosa* or *Kl. pneumoniae* to evaluate CR were cultured 2–3 times a week in brain–heart infusion containing streptomycin (10 mg./ml.) and bacitracin (1 mg./ml.); subcultures were made on Endo agar. The endogenous Gram-negative flora of phase I mice was sensitive to 100 μg./ml. of streptomycin.

RESULTS

Effect of the phase II antibiotic treatment on the intestinal flora

More than 200 conventional mice were treated and all became free of Enterobacteriaceae, enterococci, staphylococci, yeasts and fungi. They were free of *Ps. aeruginosa* when entering phase I.

CR in phases I–IV

The results in Fig. 2 show that the number of phase I and IV animals excreting the three contaminants gradually decreases and that this decrease is more rapid as the dose is lower. In contrast, persistent colonization with the three organisms takes place in all phase II animals.

In order better to compare the CR of mice to different organisms, CR was expressed as the log of the oral dose of an organism resulting in a colonization in 50 % of the animals for at least 2 weeks. Thus, the CR of phase I mice to *E. coli* was 7, that of phase II mice to *Ps. aeruginosa* < 2 and that of phase IV mice to *Kl. pneumoniae* > 9. From a comparison of CR in phases II and IV it is clear that CR increases to normal values during phase III. Recovery in this phase is shown in Fig. 1 in which CR was measured with *E. coli*.

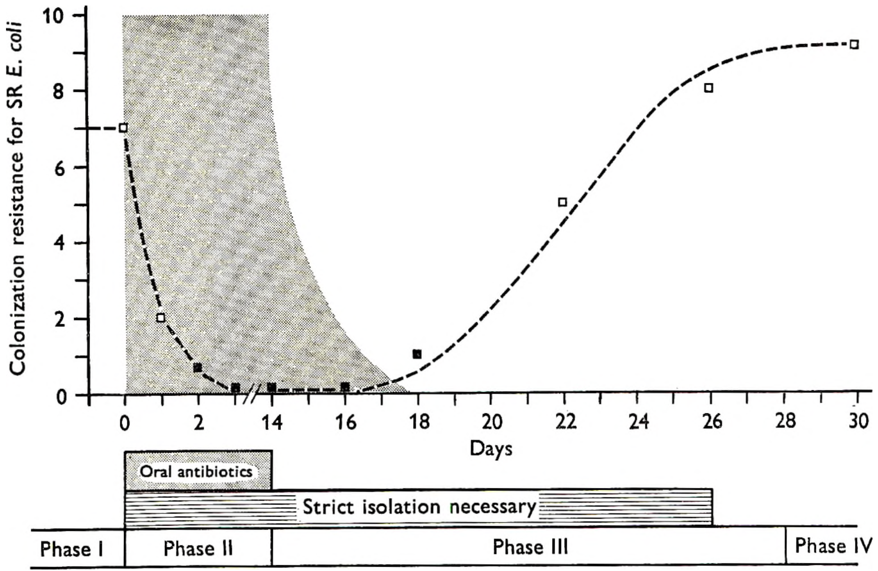


Fig. 1. Colonization resistance of mice to *Escherichia coli* in phases I–IV. Twenty mice per group. □, CR ⩾ 2; ■, CR < 2.

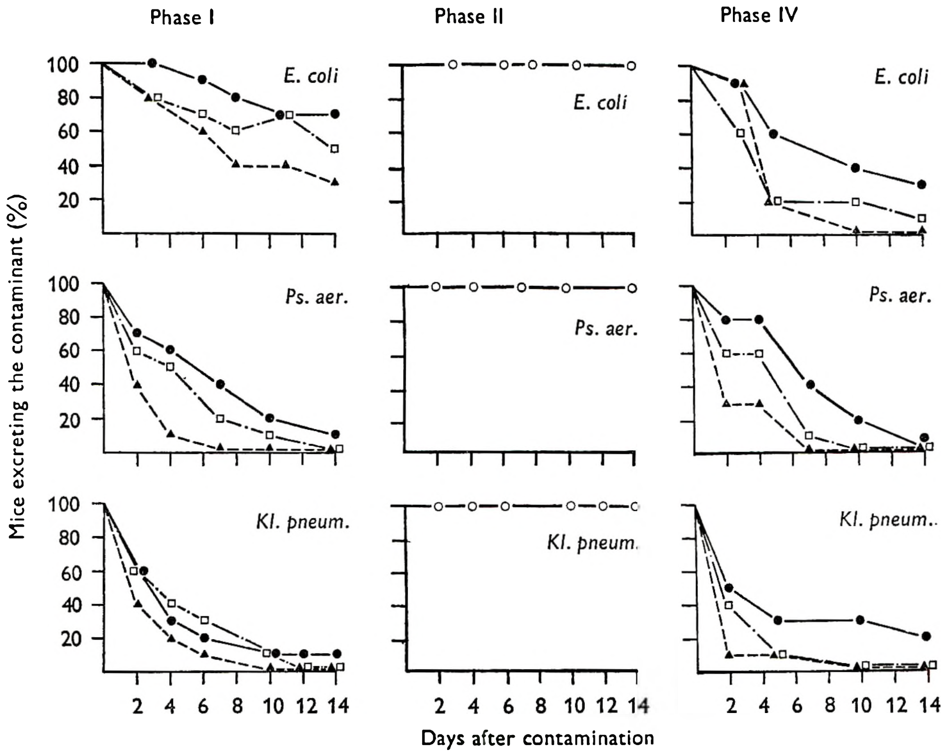


Fig. 2. Percentage of mice excreting *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* at different intervals after oral contamination in phase I, II and IV. Oral dose: ●, 10^9; □, 10^7; ▲, 10^5; ○, 10^2. Mice were contaminated 2 days after the onset of antibiotic treatment.

CR in CRF-mice

As shown in Fig. 3, the pattern of elimination of *E. coli* by CRF-mice closely resembles elimination by phase IV mice, their CR to this organism being about 9. Taking into account that the flora of CRF-mice came from one phase IV mouse and that the flora of this animal represents the fraction of the conventional intestinal flora which survived antibiotic treatment, the results with CRF-mice suggest that their flora consists of organisms responsible for CR in conventional mice.

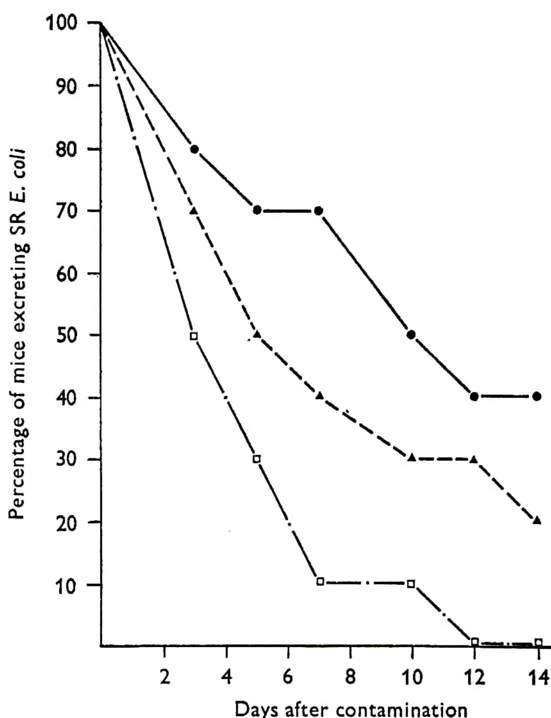


Fig. 3. Percentage of CRF-mice excreting *Escherichia coli* following three different oral doses. Oral dose: ●, 10^9; ▲, 10^7; □, 10^5.

DISCUSSION

The term 'colonization resistance' (CR) seems appropriate for the process leading to elimination of orally introduced organisms. By expressing CR as the log of the oral dose of bacteria which produces a long-lasting take in 50% of the contaminated animals elimination of different organisms can be quantitatiyely compared. Values of CR in conventional mice were found to be slightly different for the strains of *E. coli*, *Kl. pneumoniae* and *Ps. aeruginosa* tested. Immediately after the onset of oral administration of antibiotics CR decreases and the values remain very low during the period of antibiotic treatment. Decrease and subsequent recovery of CR in the post-treatment phase coincide with profound changes of the intestinal flora. The antibiotics virtually eliminate this flora and the intestinal tract is gradually repopulated by organisms surviving the treatment. Comparable

results were obtained when the effect of antibiotic treatment on susceptibility to infection with pathogenic organisms was studied. Single oral doses of streptomycin were followed within 24 hr. by a striking increase of susceptibility to *Salmonella enteritidis* (Bohnhoff & Miller, 1962). Susceptibility gradually decreased to that of untreated mice and decrease was even more rapid when normal mice were housed together with streptomycin-treated animals. Furthermore, though coliform bacilli multiplied extensively and persisted in all parts of the gastro-intestinal tract of germ-free mice, their numbers fell precipitously when these mice were fed the intestinal contents of healthy pathogen-free mice (Schaedler, Dubos & Costello, 1965). These data clearly demonstrate the important role of the resident intestinal flora in host resistance to orally introduced organisms.

So far, no definite conclusions have been drawn about the intestinal residents responsible for CR and the mechanism by which resistance is brought about. Our results suggest that CR is due to those anaerobes in the conventional intestine which survive the antibiotic treatment and subsequently repopulate the intestinal tract. Studies of the caecal flora of the offspring of germ-free mice contaminated with these anaerobes (CRF-mice) show that five species are represented. Lactobacilli and *Bacteroides*, the predominant anaerobes of conventional mice, are absent and the major constituents are tapered rods belonging to the genus *Clostridium* (Wensinck & Ruseler-van Embden, 1971). The inability of lactobacilli and *Bacteroides* to reduce coli populations in gnotobiotic mice (Schaedler *et al.* 1965) supports the view that in conventional animals CR is also induced by one or more of the five anaerobes present in CRF-mice. Results from preliminary experiments with germ-free mice contaminated with various combinations of these species strongly suggest that CR is due to the presence of a large, Gram-labile tapered *Clostridium*.

The elimination of orally introduced organisms has been ascribed to mechanical and chemical factors. The normal flora has been shown to accelerate the propulsion of gastro-intestinal contents (Abrams & Bishop, 1966, 1967), to reduce the volume of the caecum characteristically enlarged in germ-free and antibiotic-treated animals (Savage & Dubos, 1968; van der Waaij, 1969) and to produce volatile acids which are thought to inhibit the multiplication of *Salmonella* in the large bowel (Meynell, 1963; Bohnhoff, Miller & Martin, 1964*a*, *b*). In this respect it is important to note that the caecum volume of CRF-mice is normal and that the anaerobes isolated produce several volatile acids *in vitro* (Wensinck & Ruseler-van Embden, 1971). It is unlikely that the immune system contributes to CR: both the high values in CRF-mice, born and raised in the germ-free isolator, and the immediate drop of CR in conventional animals after the onset of antibiotic treatment, are difficult to explain when CR is supposed to be antibody-dependent.

REFERENCES

ABRAMS, G. D. & BISHOP, J. E. (1966). Effect of the normal microbial flora on the resistance of the small intestine to infection. *Journal of Bacteriology* **92**, 1604.

ABRAMS, G. D. & BISHOP, J. E. (1967). Effect of the normal microbial flora on gastro-intestinal motility. *Proceedings of the Society for Experimental Biology and Medicine* **126**, 301.

BOHNHOFF, M. & MILLER, C. P. (1962). Enhanced susceptibility to *Salmonella* infection in streptomycin-treated mice. *Journal of Infectious Diseases* **111**, 117.

BOHNHOFF, M., MILLER, C. P. & MARTIN, W. R. (1964*a*). Resistance of the mouse's intestinal tract to experimental *Salmonella* infection. I. Factors which interfere with the initiation of infection by oral inoculation. *Journal of Experimental Medicine* **120**, 805.

BOHNHOFF, M., MILLER, C. P. & MARTIN, W. R. (1964*b*). Resistance of the mouse's intestinal tract to experimental *Salmonella* infection. II. Factors responsible for its loss following streptomycin treatment. *Journal of Experimental Medicine* **120**, 817.

MEYNELL, G. G. (1963). Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the normal gut. *British Journal of Experimental Pathology* **44**, 209.

SAVAGE, D. C. & DUBOS, R. (1968). Alterations in the mouse cecum and its flora produced by antibacterial drugs. *Journal of Experimental Medicine* **128**, 97.

SCHAEDLER, R. W., DUBOS, R. & COSTELLO, R. (1965). Association of germfree mice with bacteria isolated from normal mice. *Journal of Experimental Medicine* **122**, 77.

SNEATH, P. H. A. (1956). Cultural and biochemical characteristics of the genus *Chromobacterium*. *Journal of General Microbiology* **15**, 70.

WAAIJ, D. VAN DER (1968). The persistent absence of Enterobacteriaceae from the intestinal flora of mice following antibiotic treatment. *Journal of Infectious Diseases* **118**, 32.

WAAIJ, D. VAN DER (1969). Similarities between GF mice and mice with an antibiotic decontaminated digestive tract. In *Germfree Biology*, p. 181. New York: Plenum Press.

WAAIJ, D. VAN DER & ANDREAS, A. H. (1971). Prevention of airborne contamination and cross-contamination in germ-free mice by laminar flow. *Journal of Hygiene* **69**, 83.

WAAIJ, D. VAN DER & STURM, C. A. (1968). Antibiotic decontamination of the digestive tract of mice. Technical procedures. *Laboratory Animal Care* **18**, 1.

WAAIJ, D. VAN DER, VRIES, J. M. DE & LEKKERKERK, J. E. C. (1970). Eliminating bacteria from monkeys with antibiotics. In *Infections and Immunosuppression in Subhuman Primates*, p. 21. Copenhagen: Munksgaard.

WAAIJ, D. VAN DER, ZIMMERMAN, W. M. T. & BEKKUM, D. W. VAN (1963). An outbreak of *Pseudomonas aeruginosa* infection in a colony previously free of this infection. *Laboratory Animal Care* **13**, 46.

WENSINCK, F. & RUSELER-VAN EMBDEN, J. G. H. (1971). The intestinal flora of colonization resistant mice. *Journal of Hygiene* **69**, 413.

J. Hyg., Camb. (1971), **69**, 413
With 2 plates
Printed in Great Britain

The intestinal flora of colonization-resistant mice

By F. WENSINCK and J. G. H. RUSELER-VAN EMBDEN
Laboratory of Medical Microbiology, Medical Faculty, Rotterdam, The Netherlands

(*Received* 1 *March* 1971)

SUMMARY

To facilitate the isolation of anaerobes from cultures in which swarming organisms were present Roux flasks were adapted to provide anaerobic conditions. Using these flasks, five species of anaerobes were isolated from the caecum of mice resistant to colonization with *Escherichia coli* and other Gram-negative organisms. These species accounted for the majority of organisms seen in films of caecal material. No aerobes were isolated and the flora of these mice apparently consisted of a limited number of anaerobes representing part of the intestinal flora of conventional mice. Three of the species were clostridia and another was identified as *Propionibacterium acnes*. One species resembled *Catenabacterium* but was not identified. One of the *Clostridium* species could only be isolated when the medium was supplemented with yeast extract and vitamins. This species sporulated on media with ammonium phosphate as the source of nitrogen; tryptone completely suppressed sporulation.

INTRODUCTION

When mice colonized with a purely anaerobic intestinal flora were challenged by oral administration of graded doses of *Escherichia coli* and other Gram-negative organisms, colonization resistance (CR) was observed, i.e. the bacteria were eliminated from the intestinal tract in periods of time related to the challenge dose. These mice were the offspring of germ-free mice contaminated with the intestinal flora of a conventional mouse treated with antibiotics and were called CRF-mice. The effects of oral administration of antibiotics on the intestinal flora of conventional mice and the resulting changes of CR were studied by van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees (1971). The present paper deals with the intestinal flora of CRF-mice. By examination of Gram-stained films of caecal contents the following organisms were recognized: (*a*) Gram-positive, semi-circular rods, almost exclusively arranged in clusters; (*b*) Gram-positive straight rods, occasionally with oval, terminal spores; (*c*) Gram-positive, irregularly stained rods and (*d*) tapered rods. Many tapered rods were large, cigar-like organisms and others very slender, Gram-negative fusiform rods. Large tapered rods were Gram-positive or negative and some showed demarcated Gram-positive areas. In view of the morphological differences between large, Gram-variable and slender Gram-negative rods it seemed likely that the population of tapered rods consisted of at

least two species. From these observations it was concluded that at least five species were represented in the intestinal flora.

With conventional anaerobic techniques, a *Clostridium* species was isolated but spreading growth of tapered rods interfered with the isolation of other organisms. A method was therefore devised to facilitate the early recognition of colony formation. Essentially, it consisted in using Roux flasks adapted to provide anaerobic conditions. Using these flasks, five species of anaerobes were isolated. One of them, a large, Gram-labile tapered rod, was often present in primary cultures but disappeared on subculture. This suggested that growth depended on metabolites of one or more of the other organisms. Although extracts from primary cultures of caecal material were growth-promoting, no effort was made to isolate the active principle since a vitamin supplement was much more effective. Eventually, pure cultures were obtained on synthetic media supplemented with yeast extract and vitamins. In the present paper the method of anaerobic cultivation is described and the characteristics of the species isolated are reported.

MATERIALS AND METHODS

Mice

Groups of five CRF-mice housed in an isolation cage (van der Waaij, 1968) were received from the Radiobiological Institute TNO (Rijswijk, The Netherlands). The animals were killed with ether; a small piece of the caecal wall was cut out and put immediately on the medium in culture flasks. The material was distributed over the surface with spreaders made by bending Pasteur pipettes at right angles, the short limb being 1 cm. long.

Culture flasks

The conical neck of Pyrex-brand Roux flasks ($19 \times 10 \times 5$ cm.) was ground to fit the B 29 standard joint adapter. At a distance of about 2 cm. from the neck a socket fitting the B $14\frac{1}{2}$ joint adapter was attached to the upper part of the side wall of the flask. Flasks were also modified by folding the wall with a triangular mould to form a transversal dam separating two medium compartments (Pl. 1, fig. 1). Stopcocks and adapters were lubricated with high-vacuum grease (Glisseal; PMC, Solothurn, Switzerland). The side stopcock served to introduce gas into the flask; with the central stopcock removed the interior was easily accessible to manipulation with Pasteur pipettes.

Anaerobic conditions

A commerical mixture of 5% CO_2 in nitrogen passing through an oil lock filled with mineral oil was freed from oxygen by leading it through a column of reduced BTS catalysator (Color Chemie, Arnhem, The Netherlands) heated at about 70° C. The columns were of the type described by Drasar (1967) and Moore (1966) and contained about 500 g. of the catalysator, which was activated at intervals by passing hydrogen through the column. Before flasks were connected to the gas supply, gas was allowed to stream out freely for 2 min. The side stopcock was connected to the tubing (butane-gas tubing, Vredestein) and opened. Immediately

afterwards, the central stopcock was opened and the gas flow adjusted with the reducing valve of the gas cylinder to maintain slight pressure at the opening of this stopcock; the central stopcock adapter was then removed. When closing a flask, this adapter was replaced and gas allowed to stream through the flask for a few minutes. The stopcocks were then closed simultaneously. To prevent the adapters being forced out by gas produced by the culture the inside pressure was slightly reduced before placing flasks in the incubator. Resazurin (2 mg./l.) was added to all media as an indicator to show that anaerobiosis was maintained.

Isolation media

The medium of Hamilton & Zahler (1957) was used, with Oxoid No. 3 agar substituted for Difco agar. This medium was suited for the isolation and propagation of the intestinal anaerobes with the exception of large, Gram-labile tapered rods. These organisms were isolated on the following medium: tryptone (Oxoid), 10 g.; glucose, 1 g.; soluble starch, 4 g.; yeast extract, 5 g.; $K_2HPO_4.2H_2O$, 2 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 5 g.; cysteine HCl, 0·5 g.; distilled water 1 l. The pH was adjusted to 7·2 and 1·5 % agar was added. To the sterilized medium, the following vitamin solution was added (10 ml./l.): *p*-aminobenzoic acid, 100 mg.; biotin, 0·03 mg.; calcium pantothenate, 1·2 g.; folic acid, 100 mg.; niacin, 100 mg.; pyridoxal phosphate, 100 mg.; riboflavin, 100 mg.; thiamine HCl, 100 mg.; distilled water 1 l. The solution was adjusted to pH 6·8 and sterilized by filtration.

Sterilization of the isolation media

The media were prepared in culture flasks and sterilized in pressure cookers for 10 min. at 100° C. The cookers were allowed to cool to about 60° C. and the pressure was maintained at 1 atmosphere by introducing the gas mixture at intervals. The cookers were opened and the flasks connected to the gas supply immediately. Gas was allowed to stream through the flasks until the media had solidified. The vitamin solution was introduced into the flask when the medium had cooled to about 45° C.

Sporulation medium

This medium contained per litre of distilled water: $(NH_4)_2HPO_4$, 3 g.; glucose, 3 g.; NaCl, 4 g.; KCl, 1 g.; $MgSO_4.7H_2O$, 0·5 g.; yeast extract, 3 g.; cysteine HCl, 0·5 g. The pH was adjusted to 7·2 and 0·5 % agar was added. The medium was sterilized in the same way as the isolation media and the vitamin solution was added to the cooled medium.

Fermentation media

The Hamilton and Zahler medium was prepared with glucose omitted and 0·3 % agar added and was sterilized at 120° C. for 15 min. Concentrated carbohydrate solutions were sterilized by filtration and added to the basal media to give a final concentration of 0·5 %. The following carbohydrates were tested: arabinose, xylose; fructose, galactose, glucose, mannose; lactose, maltose, saccharose, trehalose; starch; glycerol, mannitol. The media were distributed in tubes which were placed in a boiling water bath for 20 min. and cooled before being inoculated.

Fermentation products

The pH of 1-week-old cultures on fermentation media and on the corresponding basal medium was determined potentiometrically; a difference of at least 0·5 pH unit was considered evidence of carbohydrate fermentation. Cultures grown for 1-week in an isolation medium with agar omitted were centrifuged and the supernatants analysed for lactic acid (Long, 1947) and for volatile acids by a modification of the method outlined by Skerman (1967). This method involves the extraction of volatile acids from a distillate of the acidified culture with ether. This step was modified according to Guillaume & Osteux (1955). The distillate was brought to pH 9·0 with 1 N-Na_2CO_3 and evaporated to dryness under reduced pressure at 50° C. on a rotary evaporator. The residue was dissolved in water and treated with Zeocarb 225 (British Drug Houses); the acids were then converted to their ammonium salts by addition of 1 N-NH_4OH.

Miscellaneous tests

Standard methods were used to demonstrate catalase, gelatinase, hydrogen sulphide and indole production and reduction of nitrate to nitrite (Cruickshank, 1968).

RESULTS

Primary cultures of caecal contents on the Hamilton and Zahler medium consisted of colonies and areas of spreading growth. By subculturing on this medium three morphologically different organisms forming characteristic colonies were easily obtained in pure culture. Spreading growth consisted mainly of slender, Gram-negative fusiform rods which were obtained in pure culture by passage on thoroughly dried media solidified with 2 % agar. From a comparison of several strains of these four types of organisms isolated from different groups of mice it was concluded that they represented four well-defined species provisionally called A, B, C and D. Their properties are summarized below in the same order as the organisms are mentioned in the Introduction. The isolation of a fifth species (E), a large, Gram-labile tapered rod, was difficult and is therefore described in detail. The five species were strictly anaerobic and no growth was obtained when caecal contents were incubated aerobically.

Species A

Morphology. In films of caecal material the organisms are arranged in clusters of Gram-positive semicircular rods and the same arrangement is seen in young cultures (Pl. 1, fig. 2), where the clusters are embedded in large masses of capsular material. In older cultures the organism is highly pleomorphic, Gram-negative with Gram-positive granules and shows true branching.

Surface colonies. Circular, umbonate, white, mucous, 0·5–2·0 mm.

Physiology. Strictly anaerobic, catalase negative. Acid but no gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic acid. Gelatinase, hydrogen sulphide and indole not produced, nitrate not reduced.

Identification. Morphologically, this organism closely resembles 'doughnut-shaped' rods isolated from the mouse caecum by Gordon & Dubos (1970) and identified as *Catenabacterium catenaforme* or *C. contortum.* True branching seems to exclude species A from *Catenabacterium*; it could not be assigned to one of the other rather ill-defined genera in which Gram-positive non-sporulating anaerobic rods are placed.

Table 1. *Fermentation of carbohydrates by species A–E*

	Species				
	A	B	C	D	E*
Substrate					
Arabinose	−	+	−	+	.
Xylose	−	−	−	+	.
Fructose	+	+	+	+	.
Galactose	+	+	+	+	.
Glucose	+	+	+	+	+
Mannose	+	+	+	+	.
Lactose	+	−	−	+	−
Maltose	+	+	−	−	+
Saccharose	+	+	−	+	+
Trehalose	−	+	+	−	.
Glycerol	−	−	+	−	.
Mannitol	−	+	−	−	.
Soluble starch	−	+	−	−	−
Products of glucose fermentation					
Acetic acid	+	+	+	+	+
Butyric acid	−	+	−	+	+
Lactic acid	−	−	−	−	−
Propionic acid	−	−	+	−	+
Valeric acid	−	−	−	−	+
Gas	−	+	−	+	−
Final pH	5·6	4·9	5·5	4·9	5·3

* Fermentation pattern incompletely studied with media in culture flasks.

Species B

Morphology. Straight or slightly curved rods, $0{\cdot}5 \times 4\text{–}5\,\mu$, occurring singly; Gram-positive in young cultures, non-motile, non-capsulated. Spores are oval, terminal and slightly wider than the diameter of the rod.

Surface colonies. Circular, convex, white, smooth, 1–3 mm.

Physiology. Strictly anaerobic, catalase negative. Acid and gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and butyric acids and carbon dioxide. Gelatinase, hydrogen sulphide and indole not produced, nitrate not reduced.

Identification. The organism is considered to be a *Clostridium* not belonging to one of the well-defined species.

Species C

Morphology. Gram-positive, irregularly stained, pleomorphic rods, occurring singly, in pairs or in groups and showing the arrangement of corynebacteria. Non-motile, non-capsulated.

Surface colonies. Irregular, umbonate, lobate, 1–4 mm., coloured pink after 3–5 days.

Physiology. Anaerobic when isolated but aerotolerant after a few subcultures; catalase positive. Acid but no gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and propionic acids. Acetic and propionic acids produced from lactate. Gelatin hydrolysed, hydrogen sulphide not produced, indole positive and nitrate reduced to nitrite.

Identification. The organism was identified as *Propionibacterium acnes* (*Corynebacterium acnes*).

Species D

Morphology. Gram-negative, tapered rods, $0{\cdot}5 \times 3\text{–}10\ \mu$, occurring singly and in pairs (Pl. 1, fig. 3). Motile by numerous peritrichate flagella; motility stops soon after exposure to air. Non-capsulated. Spores are not readily formed and are oval, subterminal and hardly wider than the rods.

Surface colonies. Spreading growth, translucent with irregular light reflexion. Round, white colonies, 1–2 mm., are formed on thoroughly dried media solidified with 2% agar.

Physiology. Strictly anaerobic, catalase negative. Acid and gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and butyric acids and carbon dioxide. Gelatinase negative, hydrogen sulphide and indole produced, nitrate not reduced.

Identification. Morphologically, this organism closely resembles fusobacteria and has several characters in common with the medium and thin tapered rods isolated from the mouse caecum by Gordon & Dubos (1970) and assigned by them to *Fusobacterium*. Species D differs in many respects from this genus as characterized by Baird-Parker (1960) and Barnes & Goldberg (1968). The cells are motile by peritrichate flagella, carbohydrates are actively fermented with copious gas production, the terminal pH value of glucose-fermenting cultures is low and pentoses are fermented. Moreover, species D grows well on simple media like peptone water with 0·3% agar. It could be assigned to *Clostridium* when sporulation was demonstrated on media with 0·15% fructose. Only a few percent of the cells sporulate.

Species E

Morphology. Tapered cigar-shaped rods, Gram-positive in young cultures, $1 \times 9\text{–}15\ \mu$. Cells of 24 hr. old cultures are either Gram-negative or show band-like Gram-positive areas (Pl. 2, fig. 4). Motile by numerous peritrichate flagella; motility stops immediately after exposure to air. When young cultures are observed with a pocket lens, irregular patterns of reflected light are seen which change con-

tinuously as a result of cellular movement. Spores are not readily formed and are subterminal, cylindrical, conical and do not swell the cells (Pl. 2, fig. 5). Non-capsulated.

Surface colonies. Spreading growth with irregular light reflexion; on dry media, irregular-shaped colonies, 1–3 mm., are formed within 24–48 hr. The heaped cell material is rather sticky and slightly brown-coloured.

Physiology. Strictly anaerobic, catalase negative, and extremely sensitive to air unless cultivated on the sporulation medium. Acid but no gas produced from carbohydrates (Table 1). Glucose fermented with the production of propionic and acetic acids and small quantities of butyric and valeric acids. Gelatinase negative, abundant production of hydrogen sulphide, indole negative. Preliminary studies of growth requirements have shown that even in the presence of the vitamin supplement growth is improved by yeast extract. Sporulation is completely suppressed by tryptone. On media with the ammonium ion as the source of nitrogen sporulation starts after 2–3 days; only a few per cent of the cells sporulate.

Isolation. After many unsuccessful attempts to isolate this species it was found that it is essential that vitamins be added to the medium. Moreover, the glucose concentration has to be low (0·1 %); otherwise, species E is readily overgrown. In primary cultures of caecal material the organism mutiplies in areas of spreading growth together with species D. After two or three subcultures these areas mainly consist of species D and E; subcultures are then made on the isolation medium with 0·5 % glucose and with soluble starch omitted. Species D and E grow rapidly on this medium and the mixed culture is subcultured on the sporulation medium. When a reasonable number of spores has formed (3–4 days), growth is harvested, suspended in NaCl solution (0·9 %), heated for 10 min. at 70° C. and plated out on the isolation medium with 0·5 % glucose. The resulting culture is likely to consist of species E only.

Identification. The organism is considered to be a *Clostridium* not belonging to one of the well-defined species.

DISCUSSION

It is generally agreed that methods for isolating fastidiously anaerobic bacteria should fulfil the following requirements: (*a*) the gas mixture should be freed from oxygen, (*b*) the media should be reduced before they are inoculated, and (*c*) exposure to air of material to be cultivated must be minimal (Drasar, 1967; Aranki, Syed, Kenney & Freter, 1969; Moore, Cato & Holdeman, 1969; Gordon & Dubos, 1970). The method described meets these requirements. By sterilizing the media in pressure cookers and introducing the gas mixture into the cookers while cooling and into the flasks while the media solidify, oxidation is sufficiently prevented as indicated by the absence of coloration by oxidized resazurin. The redox potential increases when vitamin solutions are mixed with the sterilized medium but the resulting pink colour disappears within a few minutes.

We consider the anaerobic flask to be useful for separating anaerobes in mixed cultures. By removing condensed water and passing the gas mixture for about 20 min. through the flasks the medium surface can be made sufficiently dry to

limit swarming – a property displayed by several anaerobic species. Moreover, the flasks can be inspected at any time and individual colonies can thus be recognized early and subcultured on the separate medium compartment of the same flask or on other flasks to which material can be transferred within one second.

The species isolated account for the majority of the organisms seen in films of caecal contents. Therefore, it seems reasonable to conclude that most if not all of the species present were cultivated. Evidently, this flora is different from the anaerobic intestinal flora of man and conventional mice in which anaerobic lactobacilli (bifidobacteria) and *Bacteroides* species predominate (Schaedler, Dubos & Costello, 1965; van Houte & Gibbons, 1966; Drasar, 1967; Lee, Gordon & Dubos, 1968; van der Wiel-Korstanje & Winkler, 1970). These organisms were not isolated from the mice studied. Instead, their intestinal flora is dominated by two species of tapered rods in which sporulation was eventually demonstrated. One of these *Clostridium* species (species E) outnumbers other organisms in films of caecal wall mucus, where it apparently multiplies. It closely resembles tapered rods shown to be present in the intestine of mice (Lee *et al.* 1968) and ground squirrels (Barnes & Burton, 1970), but as far as is known it has not been isolated and characterized before. A preliminary survey indicates that this *Clostridium* species is a member of the intestinal flora of conventional laboratory rodents. Moreover, results from experiments with germ-free mice contaminated with various combinations of the five species described strongly suggest that colonization resistance is due to the presence of this *Clostridium*.

We would like to thank Miss P. H. J. Kustermans for skilful technical assistance. Sporulation in species D was demonstrated by Mr J. M. J. C. Scheres (Experimental Gerontology Unit TNO, Rijswijk, The Netherlands).

REFERENCES

Aranki, A., Syed, S. A., Kenney, E. B. & Freter, R. (1969). Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Applied Microbiology* **17**, 568.

Baird-Parker, A. C. (1960). The classification of Fusobacteria from the human mouth. *Journal of General Microbiology* **22**, 458.

Barnes, E. M. & Burton, G. C. (1970). The effect of hibernation on the caecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). *Journal of Applied Bacteriology* **33**, 505.

Barnes, E. M. & Goldberg, H. S. (1968). The relationship of bacteria within the family Bacteroidaceae as shown by numerical taxonomony. *Journal of General Microbiology* **51**, 313.

Cruickshank, R. (ed.) (1968). *Medical Microbiology*, 11th ed. Edinburgh: E. and S. Livingstone Ltd.

Drasar, B. S. (1967). Cultivation of anaerobic intestinal bacteria. *Journal of Pathology and Bacteriology* **94**, 417.

Gordon, J. H. & Dubos, R. (1970). The anaerobic bacterial flora of the mouse cecum. *Journal of Experimental Medicine* **132**, 251.

Guillaume, J. & Osteux, R. (1955). Détermination des acides aliphatiques volatils de C_1 à C_6 dans les liquides biologiques par chromatographie sur papier. *Comptes Rendus de l'Académie des Sciences (Paris)* **241**, 501.

Hamilton, R. D. & Zahler, S. A. (1957). A study of *Leptotrichia buccalis*. *Journal of Bacteriology* **73**, 386.

Fig. 1

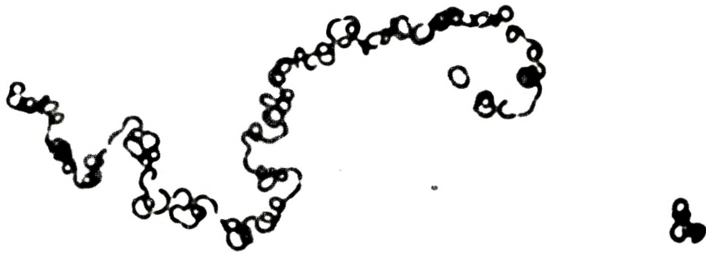


Fig. 2

Fig. 3

Fig. 4

Fig. 5

F. WENSINCK AND J. G. H. RUSELER-VAN EMBDEN

Houte, J. van & Gibbons, R. J. (1966). Studies of the cultivable flora of normal human feces. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **32**, 212.

Lee, A., Gordon, J. & Dubos, R. (1968). Enumeration of the oxygen sensitive bacteria usually present in the intestine of healthy mice. *Nature, London* **220**, 1137.

Long, C. (1947). The stabilization and estimation of lactic acid in blood samples. *Biochemical Journal* **40**, 27.

Moore, W. E. C. (1966). Techniques for routine culture of fastidious anaerobes. *International Journal of Systematic Bacteriology* **16**, 173.

Moore, W. E. C., Cato, E. P. & Holdeman, L. V. (1969). Anaerobic bacteria of the gastro-intestinal flora and their occurrence in clinical infections. *Journal of Infectious Diseases*, **119**, 641.

Schaedler, R. W., Dubos, R. & Costello, R. (1965). Association of germfree mice with bacteria isolated from normal mice. *Journal of Experimental Medicine* **122**, 77.

Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd ed. Baltimore: Williams and Wilkins.

Waaij, D. van der (1968). The persistent absence of Enterobacteriaceae from the intestinal flora of mice following antibiotic treatment. *Journal of Infectious Diseases* **118**, 32.

Waaij, D. van der, Berghuis-de Vries, J. M. & Lekkerkerk-van der Wees, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic treated mice. *Journal of Hygiene* **69**, 405.

Wiel-Korstanje, J. A. A. van der & Winkler, K. C. (1970). Medium for differential count of the anaerobic flora in human feces. *Applied Microbiology* **20**, 168.

EXPLANATION OF PLATES

Plate 1

Fig. 1. Roux flask adapted to anaerobic cultivation.

Fig. 2. Species A. Gram-stained film of 24 hr. culture (× 1400).

Fig. 3. Species D. Gram-stained film of 24 hr. culture (× 1400)

Plate 2

Fig. 4. Species E. Gram-stained film of 12 hr. culture (× 1400).

Fig. 5. Species E. Malachite green-safranine stained film of 3-day-old culture on sporulation medium (× 1400).

J. Hyg., Camb. (1971), **69**, 423
Printed in Great Britain

A mathematical model of common-cold epidemics on Tristan da Cunha

By B. J. HAMMOND
National Institute for Medical Research, Mill Hill, London, N.W.7
AND D. A. J. TYRRELL
Medical Research Council Common Cold Unit, Salisbury, England

(*Received* 3 *March* 1971)

SUMMARY

Records of seven common-cold outbreaks on the island of Tristan da Cunha are compared with the corresponding time courses given by the mathematical model of Kermack & McKendrick (1927) and with an alternative model that directly involves a constant average duration of individual infection. Using computer simulation techniques the latter model is shown to be preferred and is then closely matched to the field data to obtain values for the model parameters. Consideration is then given to the intensity of epidemics predicted by the model and to the distribution of the actual epidemics relative to the theoretical epidemic threshold.

INTRODUCTION

The occurrence of common-cold epidemics among the islanders of Tristan da Cunha has already been discussed in a previous paper (Shibli, Gooch, Lewis & Tyrrell, 1971). For the purpose of that study daily records of the development of upper respiratory infection were kept by individuals involved in each of the seven outbreaks that affected the community between 1964 and 1968. Several observations were made based upon a collation of these records but the data may also be used to provide information regarding the time course of each epidemic as a whole. For example, Fig. 1 shows the development of the outbreak in April 1966 and has been obtained from the individual record cards in the following manner.

The total number of individuals reporting the onset of symptomatic upper respiratory infection was found for each day and these totals then accumulated day-by-day to give the left-hand curve of Fig. 1. In general, any ordinate of this curve gives the number of individuals who have become involved in the epidemic during the corresponding period, but this number of individuals is uniquely fixed for only the beginning and end of each day. At intermediate times there is uncertainty as to the appropriate numbers that should be taken, but limits to this uncertainty are set by the opposing assumptions that the daily totals are achieved immediately at the beginning and end of each day. The representation of these two extreme assumptions in Fig. 1 gives rise to rectangles which are one unit wide and of heights equal to the daily totals. The figure thus embodies a statement of these uncertainty

limits. Identical arguments apply to the right-hand curve, which is composed of the daily totals of individuals reporting a last day of infection. Any ordinate of the latter curve gives the number of individuals who have recovered from symptomatic infection at the appropriate time whilst the numerical difference between the two curves gives the number of individuals who remain infected.

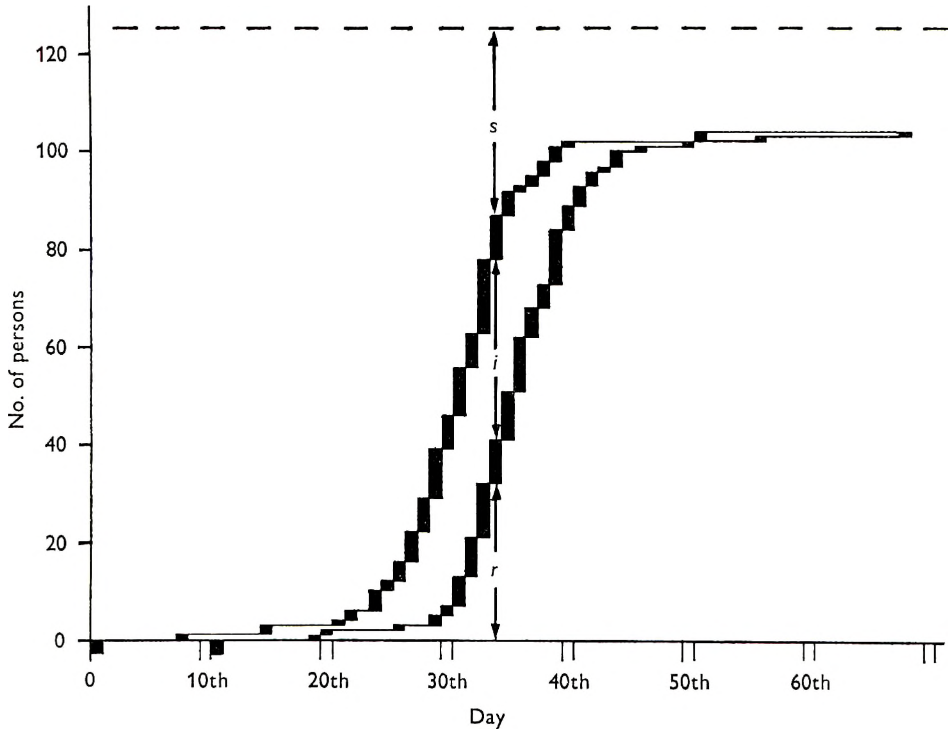


Fig. 1. The epidemic of April 1966 constructed by means described in the text. The number of individuals who have recovered from infection, the number who are infected and the number of susceptibles remaining are given by the quantities r, i and s respectively. The number of susceptibles at time zero is not known but is represented by the horizontal dashed line. The dates of ship arrivals are indicated by rectangles below the abscissa.

The data for all seven epidemics, when prepared as for Fig. 1, show broadly similar characteristics although the form of the smaller outbreaks tends to be less distinctive. However, the data seem sufficiently consistent to serve as a set of empirical functions against which the performance of a mathematical model of the epidemic process could be checked, and it is with this approach that the present paper is concerned.

Formulation of mathematical models

The smooth progress of the two curves in Fig. 1 suggests that the main phase of an epidemic in the present situation could well be described by a deterministic process. At the beginning and end of the epidemic, however, when the number of infected individuals is small, the curves show the sort of irregularity associated with a more stochastic process. In these circumstances an approach using a deter-

ministic model was adopted and the epidemic data were suitably truncated to exclude the two extreme phases.

Certain characteristics of the Tristan community allow the corresponding epidemic model to be somewhat simplified. Firstly, the moderate size and free social mixing of the community (Shibli *et al.* 1971) suggests that topographical or sociological groupings may not significantly affect the progress of the epidemic; and secondly, the isolation of the community rules out the effects of emigration and immigration. The Tristanian community would in fact seem to approach very closely the ideal homogeneous and closed society whose theoretical consideration has provided a basis for epidemic theory since its inception.

In conformity with the classic deterministic approach, the following three classes of individuals are recognized within the community at any time: (*a*) the class, numbering s individuals, who are susceptible to infection; (*b*) the class, numbering i individuals, who are infected and are also assumed to be infectious; (*c*) the class, numbering r individuals, who have recovered from infection and are assumed to be immune from reinfection. The number of individuals who are susceptible at the outbreak of infection, $s(o)$, must also be considered, and this unknown number is represented in Fig. 1 as the horizontal dashed line, which then allows all three of the above classes to be related to the epidemic field data.

Statements concerning the transfer of individuals between classes are now required and a suitable law for the process of infection is derived from the hypothesis that the rate of infection is proportional to the probability of contact between infected and susceptible individuals. In a randomly mixing society the deterministic infection rate is then given by Iis, where I is a constant which may be termed 'infectivity'. The rate of recovery may be taken as proportional to the number of infected individuals at any time, that is equated to Ri, where R is a recovery rate constant. These laws of infection and recovery then give the differential equations which constitute the model due to Kermack & McKendrick (1927):

MODEL 1

$$ds/dt = -Iis,$$
$$di/dt = Iis - Ri,$$
$$dr/dt = Ri.$$

An alternative to the above model arises with a modified recovery law that corresponds with the assumption of a constant duration of infection. In this case the recovery rate at a time $(t+D)$ may be equated to the infection rate at time t, where the time interval, D, represents the average duration of individual infection. The function Iis, when delayed by a period D, may be written as $I[is]$, then

MODEL 2

$$\begin{aligned} ds/dt &= -Iis, \\ di/dt &= Iis - I[is], \quad \text{where} \quad \begin{cases} [is] = i(t-D)\,s(t-D),\ t \geqslant D \\ [is] = 0,\ t < D \end{cases} \\ dr/dt &= I[is] \end{aligned}$$

METHODS

Preliminary examination of the above models was carried out using a Pace TR 48 analogue computer and more detailed studies on the second model were then undertaken by coupling the analogue computer to a Honeywell DDP 516 digital computer to form a hybrid computer system. The main object of the later studies was to determine the sets of values for $s(o)$, I and D that gave rise to best agreement with the seven recorded epidemics.

To aid the search for best agreement a special hybrid program was written which allowed use of the teletype keyboard for prespecification of eight values for each of the model parameters, and which then supervised the implementation of the 512 resulting combinations. For each combination of the parameter values the program compared the performance of the model with the epidemic under study by calculating the sum-square-error between the appropriate variables of the model and the daily epidemic data. These error values were automatically printed out in eight by eight arrays corresponding with increasing values of the first parameter from column to column and increasing values of the second parameter from row to row. The third parameter was incremented between successive arrays. This format allowed the error to be visualized as a three-dimensional function and rapid location of the minimum was made possible. Determination of the parameter values to two significant figures usually demanded repetition of the procedure with successively finer increments between the parameters. A further facility of the hybrid program allowed sets of values for $s(o)$, I and D to be specified using the computer teletype and the resultant time-courses of the variables s, i and r were then automatically typed out.

RESULTS

Implementation of the Kermack and McKendrick model on the analogue computer revealed a discrepancy between the performance of the model and the form of the recorded epidemics. This is illustrated in Fig. 2(*d*), where the dashed lines show the solution of the model which gives minimum error when compared with the corresponding epidemic data. The failure of the model evidently arises from the recovery law, which imposes an unduly high recovery rate in the early part of an epidemic. The second model was shown by the analogue computer to promise better agreement with the epidemic records and this model was therefore pursued in preference to the former. Using the hybrid computer as previously described, least-squared-error solutions were obtained for five of the seven epidemics and the daily values given by these solutions are shown in Fig. 2 together with their corresponding sets of field data.

The time courses of the two remaining epidemics were not satisfactorily matched by model 2 owing to discontinuities which appear in the later phases of each epidemic. The form of these discontinuities is apparent in Fig. 3 and their possible cause was considered in some detail. First, the epidemics were split into five components corresponding with the five social groups distinguished during a sociological survey in 1965 (P. A. Munch, personal communication). However, no significant difference

in the time courses of these components could be distinguished. Secondly, the locations of houses in which new infections occurred were annotated on a map of the island's residential area, a different map being used for each day. By scanning the completed sequence of maps the spread of the epidemics could be followed and several possibly significant groupings of houses could be distinguished. However, examination failed to reveal any relevant differences between the epidemic components associated with various combinations of these groups. It became apparent from these first two investigations that neither sociological nor topographical grouping could be indicted as the cause of the observed discontinuities.

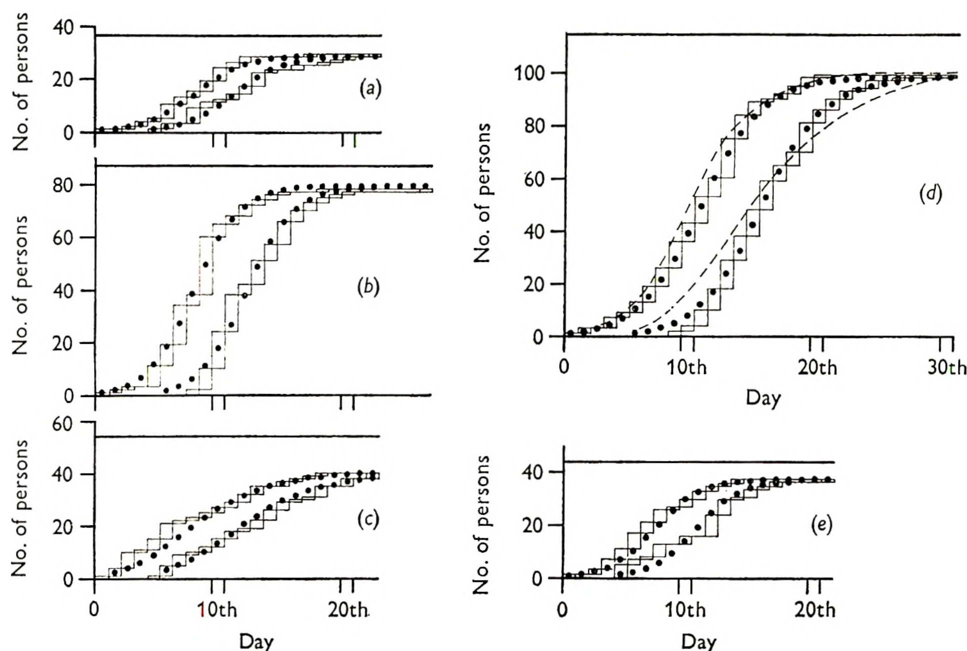


Fig. 2. The epidemic records for September 1964, January 1965, January 1966, April 1966 and October 1967 correspond with letters (*a*)–(*e*) consecutively. The dashed lines in (*d*) represent the solution of model 1 that is in closest agreement with the data. The solid circles in each figure give the solutions of model 2 that are in closest agreement with each set of field data and the upper horizontal lines show the corresponding levels of susceptibles at time zero.

Another cause of irregular epidemics could be a sudden change in infectivity related, for example, to temperature or even possibly to an increase in the inherent virulence of the infective agent itself. However, when such a change was implemented in the analogue computer simulation, agreement with the data was only marginally improved and the hypothesis of changed infectivity was abandoned. A final hypothesis considered the data of Fig. 3 to arise from two independent epidemics involving different viral agents. It may be noted in this respect that both epidemics were associated with the arrival at Tristan da Cunha of two ships and that contact with infected passengers has been identified as the origin of common-cold epidemics on the island (Shibli *et al.* 1971). In the case of the May 1967 outbreak one ship arrived 2 days before the first notified infection and another ship

arrived 1 week later; in February 1968 two ships arrived at the island on the day preceding the first notification of infection. The hybrid simulation of model 2 was extended to include a second and concurrent epidemic whose form was determined by an independent set of the parameters $s(o)$, I and D. The start of the second epidemic was delayed by a period whose duration gave a seventh parameter that it was necessary to adjust in matching the double infection model to the irregular epidemics. The daily values corresponding with the least-squared-error solutions that were eventually obtained are shown in Fig. 3.

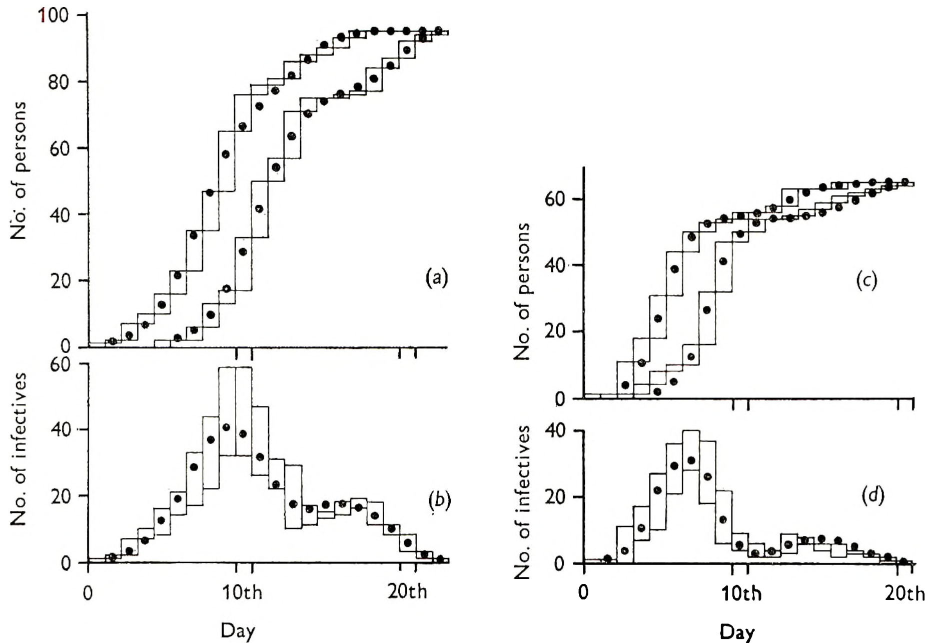


Fig. 3. The epidemic records for May 1967 and February 1968 correspond with (*a*) and (*c*) respectively. The lower figures (*b*) and (*d*) in each case give the number of individuals infected at any time. The solid circles in each figure give the solutions of model 2 that are closest in agreement with each set of field data.

Table 1.

Date of epidemic	Ident no.	$s(o)$ (inds.)	I (ind./day)	D (days)	N'	$s(o)\,ID$
Sept. 1964	1	36	0·018	3·2	0·78	2·1
Jan. 1965	2	87	0·0072	4·0	0·91	2·5
Jan. 1966	3	54	0·0086	3·6	0·74	1·7
Apr. 1966	4	114	0·0043	4·6	0·87	2·3
May 1967	5*a*	88	0·0084	3·4	0·90	2·5
May 1967	5*b*	16	0·074	5·5	1·00	6·5
Oct. 1967	6	44	0·016	3·2	0·84	2·3
Feb. 1968	7*a*	56	0·22	2·8	0·96	3·4
Feb. 1968	7*b*	10	0·067	4·2	0·95	2·8

The values of $s(o)$, I and D are the parameter values for model 2 that give best agreement with the records of the corresponding epidemics; in stating units 'individuals' is abbreviated to 'inds'. Double infections are indicated by the use of letters following a common reference number. The last two columns give values of epidemic constants as discussed in the text.

The values of $s(o)$, I and D that give best agreement between model 2 and the seven recorded epidemics are listed in Table 1, where statement to two significant figures is made possible by the sensitivity of the error-squared function to changes in parameter values. The sensitivity was different for each epidemic but at worst gave a 10 % change in the sum of squares of errors corresponding with unit change in the second significant figure of each parameter. Typically the change was in excess of 50 %. The method used to determine these values required the model to be expressed in terms of numbers of individuals, but it is of more general value to

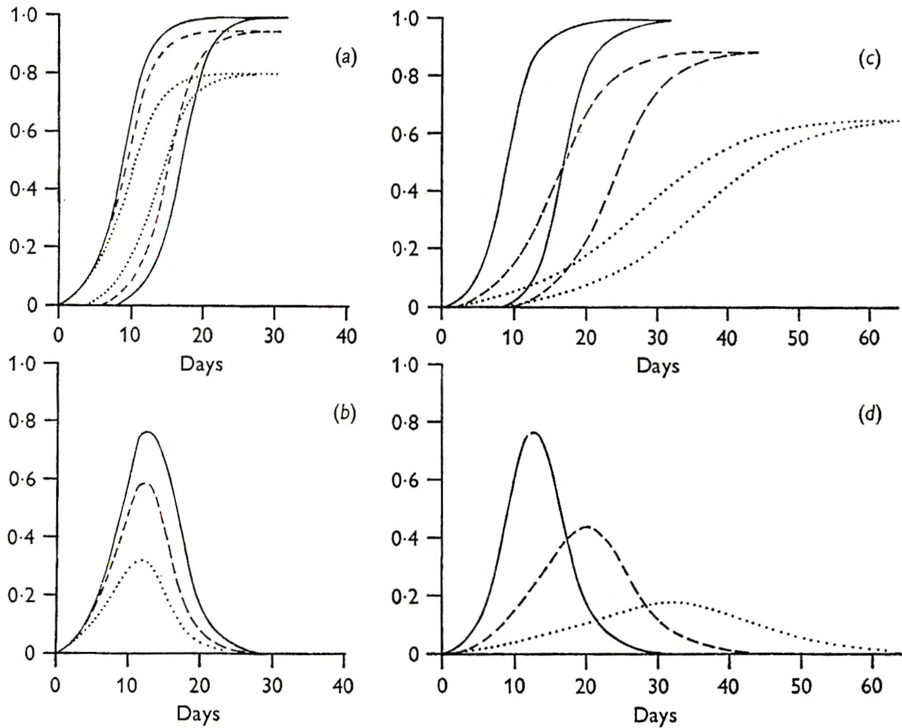


Fig. 4. Theoretical epidemics produced by model 2. In (*a*) and (*c*) the left-hand curves give the number who have been infected as a fraction of the initial number of susceptibles; the right-hand curves give the fraction who have recovered. The difference between any pairs of curves gives the fraction who remain infected at any time and these values are plotted in the lower figures. In (*a*) and (*b*) the product $s(o)I$ is kept at 1/day and D has the values, 4 days (—), 3 days (– – –) and 2 days (. . . .). In (*c*) and (*d*) the value of D is kept constant at 4 days and the product $s(o)I$ has the values 1·0/day (—), 0·6/day (– – –) and 0·4/day (. . . .).

consider the model in a normalized form where the variables are expressed as fractions of the initial number of susceptibles $s(o)$. Thus, let

$$s' = s/s(o), \qquad ds'/dt = -f(t),$$

$$i' = i/s(o), \quad \text{then} \quad di'/dt = f(t)-f(t-D),$$

$$\text{and } r' = r/s(o), \qquad dr'/dt = f(t-D),$$

$$\text{where } f(t) = s(o)Ii's'.$$

It becomes apparent from the normalized model that the resulting temporal forms of the predicted epidemics are dependent solely upon two factors: first, the product of infectivity with initial number of susceptibles, and secondly, the average duration of individual infection. As is shown in Fig. 4, both these factors affect the size of an epidemic, but in addition the value of $s(o)I$ has a profound effect upon the time at which an epidemic reaches its peak.

DISCUSSION

Mathematical epidemiology really began with the work of Kermack & McKendrick (1927), and in particular with the simple deterministic model reproduced as model 1 in this paper. Considering a closed homogeneous society and this simple three-state infection, the time course of an epidemic is determined by the laws that are assumed for the rates at which individuals enter and leave the infected state. The product law describing the rate at which new infectives are generated is based upon the probability of a susceptible coming into effective contact with an infectious individual in a randomly mixing society, and this law has been adopted by most later workers in application to both deterministic and stochastic models. On the other hand, the law stating that the rate of removal is proportional to the number of infectives has been less readily accepted and alternatives have arisen; for example, in models of the Reed-Frost variety, first examined by Abbey (1952). In these models infectives are removed after a given period of infection and the recovery rate at any time is thus implied by the duration of individual infection. The fundamental laws of infection and recovery are obviously of vital importance to the body of mathematical epidemiology as summarized by Bailey (1957) and Bartlett (1960). These studies have been largely theoretical and the data from Tristan da Cunha now make it possible to test more thoroughly than was previously possible whether the fundamental laws adequately describe real epidemics.

In view of the above comments, the basic field data were processed so that as much as possible of the relevant information was retained for comparison with the performance of the models. In particular this allowed the simultaneous matching of the time courses for all three groups of individuals rather than the more common procedure of using some selected feature such as infection rate as the sole criterion for the performance of the model. In addition, Figs. 2 and 3 show, as previously described, the uncertainty arising from the use of daily totals of first and last days of infection. It is also known that, owing to less than complete co-operation of the islanders, the data sets are not complete records of an epidemic. The degree of co-operation has been estimated by one of the island's Medical Officers as about 80 %, but provided that under-reporting does not distort the shape of the epidemic record it does not affect the general agreement between model and data. This retention of agreement arises as a property seen in the equations of the normalized model whereby epidemics of different sizes but of identical shape are obtained provided that any change in $s(o)$ is compensated by an opposite change in I. In this respect it should be noted that no allowance for under-reporting has been made in obtaining the values given in Table 1, and in particular that the values given for I will be proportionately high.

Despite a measure of uncertainty in the field data, an inconsistency has been shown between the data and the epidemic model of Kermack & McKendrick. This inconsistency is removed when the recovery law is replaced by one based directly on a constant duration of infection and embodied in the revised formulation of model 2. The revised law would also seem to be more reasonable on the common-sense ground that the progress of a patient's infection is independent of other infectives and that in summating the infectives the individual infections will only lose their identities as the number of infectives rises. In considering the improved agreement with present epidemic data provided by model 2 it should, however, be borne in mind that the extent and number of epidemics are too small for statistical validification of the model.

An expression for the final size of an epidemic as predicted by the new model may readily be obtained by integration of its first equation,

$$\int_{s(o)}^{s(\infty)} 1/s\,ds = -I\int_{0}^{\infty} i\,dt,$$

but

$$\int_{0}^{\infty} i\,dt = ND,$$

where N is the final number of individuals involved in the epidemic, and

$$s(\infty) = s(o)-N.$$

Thus

$$\frac{ln\,(1-N')}{N'} = -s(o)ID,$$

where N' is the intensity of the epidemic, i.e. the proportion of susceptibles affected, and is given by $N/s(o)$. This result may be compared with that for model 1 obtained by Kendall (1956), which in present terminology gives

$$\frac{ln\,(1-N')}{N'} = \frac{-s(o)I}{R}.$$

These two predictions become identical if the duration of infection in model 2 is taken to correspond with the reciprocal of the removal rate constant in model 1. This relationship may be readily accepted since each quantity then implies the same removal rate of infectives over any time increment during the central phase of an epidemic.

The relationship between epidemic intensity and the triple product $s(o)ID$ is plotted in Fig. 5 and clearly shows an epidemic threshold at unity value of the triple product. This threshold, below which epidemics do not occur, is equivalent to that first discussed by Kermack & McKendrick (1927) in relation to their deterministic model and subsequently demonstrated in stochastic models by Whittle (1955) and Kendall (1956). The intensities and triple products of the present epidemics are given in Table 1 and these values locate each epidemic in Fig. 5, where the closeness of each point to the calculated intensity curve reflects the overall accuracy of the hybrid simulation by which $s(o)$, I and D were determined. The epidemics are clustered well above the theoretical threshold; if the secondary epidemics of May 1967 and February 1968 are discounted as involving too few individuals for

realistic analyses, the mean value of $s(o)ID$ for the epidemics is 2·40 with a standard deviation of 0·48.

A population may be described as subcritical to a particular infection at any time that its triple product lies below the threshold value of unity; that is, when the number of its susceptibles is less than $1/ID$. However, the birth of new susceptibles, coupled with any loss of immunity by previously infected individuals, will steadily increase the number of susceptibles. The regular progress of the triple product may be envisaged as the movement of a point along the abscissa of Fig. 5,

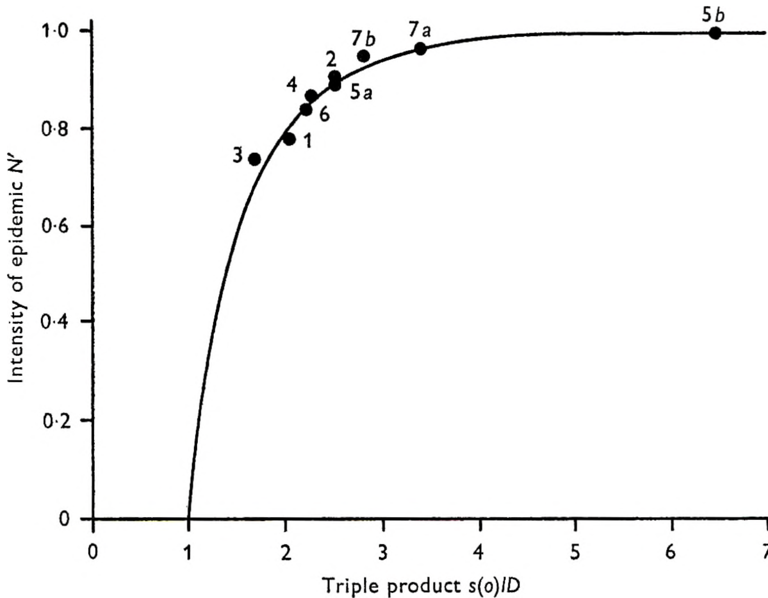


Fig. 5. The solid line gives the intensity of an epidemic predicted by model 2 as a function of the triple product $s(o)ID$. The solid circles show the positions of the analysed epidemics, which may be identified by the numbers given in Table 1.

the point reaching unity as the population achieves the critical condition and passing beyond it as the population becomes increasingly supercritical. The velocity of the point is given by the product ID, which for the present epidemics may be determined from the values given in Table 1. Excluding the secondary epidemics the velocities range from 0·020 to 0·058 per susceptible. An epidemic may be caused by the chance presentation of an infective agent when its corresponding point is at any position beyond the threshold and it is therefore surprising to find that the epidemics are not evenly distributed beyond the threshold. Furthermore, the clustering of the epidemics is not explained by assuming more frequent presentation of infective agent since in this case the epidemics would tend to cluster near to the threshold.

An explanation of this behaviour could be that theoretical epidemics of an intensity less than 0·7 involve fewer infectives than is required in practice for the maintenance of the level of infection. The predicted reduction in the number of infectives as intensity is decreased may be seen in Fig. 4 and the effect would seem

sufficiently marked to cast doubt on the applicability of the present deterministic infection law, and hence the model, under conditions which would lead to low-intensity epidemics. The present results therefore seem to argue for the existence of an empirical threshold below which infections follow stochastic paths to extinction without causing an epidemic. In the case of common-cold epidemics on Tristan da Cunha this empirical threshold lies between one-and-a-half times and twice the level of the theoretical threshold.

We hope to make further checks on the validity of the model, in particular to determine whether it adequately describes the epidemics that occur in other closed communities, such as residential schools, and also to obtain independent evidence on epidemic intensities by serological studies.

The authors wish to acknowledge the contribution made to the present work by Mr F. G. Tattam of the National Institute for Medical Research, who originated and developed the hybrid computer program. A debt is also owed to the MRC Tristan da Cunha Working Party for their assistance in this study. Finally, we wish to express our gratitude to the Tristanian community, whose co-operation made the present study possible.

REFERENCES

ABBEY, H. (1952). An examination of the Reed-Frost theory of epidemics. *Human Biology* **24**, 201.

BAILEY, N. T. J. (1957). *The Mathematical Theory of Epidemics*. London: Griffin.

BARTLETT, M. S. (1960). *Stochastic Population Models*. London: Methuen.

KENDALL, D. G. (1956). Deterministic and stochastic epidemics in closed populations. *Proceedings of the 3rd Berkeley Symposium on Mathematical Statistics and Probability* **4**, 149.

KERMACK, W. O. & MCKENDRICK, A. G. (1927). A contribution to the mathematical theory of epidemics. *Proceedings of the Royal Society* A **115**, 700.

SHIBLI, M., GOOCH, S., LEWIS, H. E. & TYRRELL, D. A. J. (1971). Common colds on Tristan da Cunha. *Journal of Hygiene* **69**, 255.

WHITTLE, P. (1955). The outcome of a stochastic epidemic. *Biometrika* **42**, 116.

J. Hyg., Camb. (1971), **69**, 435
Printed in Great Britain

Postnatal decline of maternally acquired viral antibodies of different specificities

By M. J. CLOONAN, R. A. HAWKES and L. H. STEVENS

Virology Section, Department of Pathology, the Prince Henry Hospital, N.S.W., Australia, and the Department of Paediatrics, the Prince of Wales Hospital, N.S.W., Australia

(*Received* 9 *March* 1971)

SUMMARY

The rates of decline (half-lives) of maternally acquired antibodies of two different specificities in a group of infants were found to be highly variable, ranging from 18 to 192 days for parainfluenza type 3 antibody (54 infants) and from 15 to 251 days for influenza A2 antibody (nine infants). For antibodies of both specificities approximately 75% of the half-lives were between 15 and 60 days. With parainfluenza type 3 antibody, and possibly with influenza A2 antibody, the half-lives were inversely proportional to the initial antibody titre of the babies' sera. This relationship could be described by a rectangular hyperbola. Babies with high antibody titres at birth lost this antibody rapidly whereas in babies with low initial titres antibody declined over a longer period.

The half-lives of parainfluenza type 3 antibody and influenza A2 antibody were compared with that of rubella antibody in the same group of infants (previously published). Maternally acquired viral antibodies of different specificities did not necessarily decline at similar rates in any given child. In nine infants, maternally acquired antibodies of two different specificities (rubella and parainfluenza type 3) declined at significantly different rates in the same child. It is suggested that although the half-life of antibody of a given specificity is related to its concentration in the serum, it is independent of the level of serum antibodies of other specificities.

INTRODUCTION

In a previous study we have shown that the rate of postnatal decline (half-life) of maternally acquired rubella antibody in a large group of infants was highly variable, with half-lives in different children ranging between 14 and 70 days (Cloonan, Hawkes & Stevens, 1970). A significant relationship was found between the half-life and the rubella antibody titre at or near birth ('initial titre') which could be described by a rectangular hyperbola, such that babies having high titres at birth lost their antibody rapidly, whereas in those with low initial titres antibody declined at a slower rate.

The unexpected nature of these findings prompted further investigations of the decline of maternally acquired viral antibodies of other specificities present in the sera of the same group of infants. These studies have considered, in particular,

(i) whether antibodies of different specificities decline at a similar rate in a given child, (ii) whether the half-lives are independent and related only to the initial level of specific antibody in the serum or whether they are influenced by levels of antibodies of other specificities which might also be present in the serum.

To facilitate comparison with the previous study in which rubella antibody was measured by the haemagglutination-inhibition test, two haemagglutinating viruses, influenza A2 and parainfluenza type 3 were used as test antigens. This study reports the postnatal decline of maternally acquired antibodies to these viruses, and describes the relationship between the rates of antibody decline and initial antibody titres for both antibodies. The results are discussed in relation to those obtained previously with rubella antibody.

MATERIALS AND METHODS

Study group and sera for the study

The babies in this study and the collection of test sera have been described previously (Cloonan *et al.* 1970). Although there were 120 babies in the original group, sufficient serum was left from only 102 babies for the influenza A2 tests and from only 70 babies for the parainfluenza type 3 tests. Previous tests on mothers' sera had indicated that, though only nine babies could be expected to have influenza A2 antibody of maternal origin in their sera at birth, parainfluenza type 3 antibody could be expected in the sera of 54 babies.

Viral antigens

The influenza A2 strain (A2/NSW/61) was obtained from Dr M. F. Warburton of the Commonwealth Serum Laboratories, Melbourne. The parainfluenza type 3 (Crowe) strain was isolated in our laboratory during September 1969.

Serological techniques

Influenza A2 antibody was determined by the haemagglutination-inhibition (HI) test (Fazekas de St Groth & Webster, 1966), modified by removing non-specific inhibitors from the sera by treatment with trypsin and potassium periodate (Davenport & Minuse, 1964) and by testing the sera in a micro-system. Antibody to parainfluenza type 3 virus was also determined by the HI test (Chanock & Johnson, 1964). Many of the sera contained agglutinins for guinea-pig red cells; these were removed by prior absorption of the sera with guinea-pig red cells (final concentration 20 %) for 2 hr. at 4° C. Non-specific inhibitors were removed by the trypsin and periodate method. A micro-system was also used for the titration of this antibody.

The babies whose sera were to be tested on a given day were selected by an appropriate ranking procedure in order to minimize bias, and all sera from a given baby were tested at the same time. The influenza A2 and parainfluenza type 3 tests were performed separately and, for both, ten samples of a standard positive serum were included in each test as a check on the within-test and between-test

variability. Negative control sera were included in each test as a check on the removal of non-specific inhibitors from the sera.

Determinations of antibody half-lives were made only on those babies possessing antibody in at least three but usually four to five sequential sera. The antibody titres were punched onto data cards and were analysed in an IBM 360 Model 50 computer. Using these antibody titres, a linear regression of log. titre against time (days from birth) was computed; the print out included the rate of antibody decline (half-life) for each baby. The computer program used previously (Cloonan *et al.* 1970) was modified in that: (i) it contained the rubella data (half-lives and initial antibody titres) and the input was a second set of data (in this case influenza A2 or parainfluenza type 3 titres) on which half-life calculations were performed; (ii) it carried out a test of significance of regression on all sets of data using the linear equivalent of the model proposed by Brambell, Hemmings & Morris (1964); (iii) it permitted a test of the relationship between the initial titre of either influenza A2 or parainfluenza type 3 antibody and the half-life of rubella antibody, after allowing for the effect of the initial titre of rubella antibody on the half-life of rubella antibody.

RESULTS

Antibody status of babies

Fifty-four babies possessed parainfluenza type 3 antibody and nine had antibodies to influenza A2. Of the 54 babies having parainfluenza type 3 antibody, 43 also possessed rubella antibody, thus permitting comparisons between these two groups. Although the small size of the influenza A2 group limited its usefulness, there were five babies in which antibodies of all three specificities (rubella, parainfluenza type 3 and influenza A2) were detected.

Rate of antibody decline (half-life)

Frequency distributions of the half-life values obtained with both antigens are shown in Fig. 1. In both cases half-life values were spread over a wide and similar range. In the larger parainfluenza type 3 group there was a range of half-life values from 18 to 192 days, with a mean of 58 days, and 75% of the values lay between 15 and 60 days. In most cases the straight line obtained from the regression equation was a good fit to the observed values (Fig. 2). However, during the first month of life, four babies had a fourfold rise, and one baby had a stationary titre, of parainfluenza type 3 antibody. After this, antibody declined in an exponential manner. These five babies were not members of the group of ten babies in which similar variations in rubella antibody titre occurred early in life (Cloonan, *et al.* 1970).

Antibody status in twins

Both members of each of four pairs of twins possessed parainfluenza type 3 antibody. In these pairs both the initial titre at birth and the half-life of parainfluenza type 3 antibody were similar.

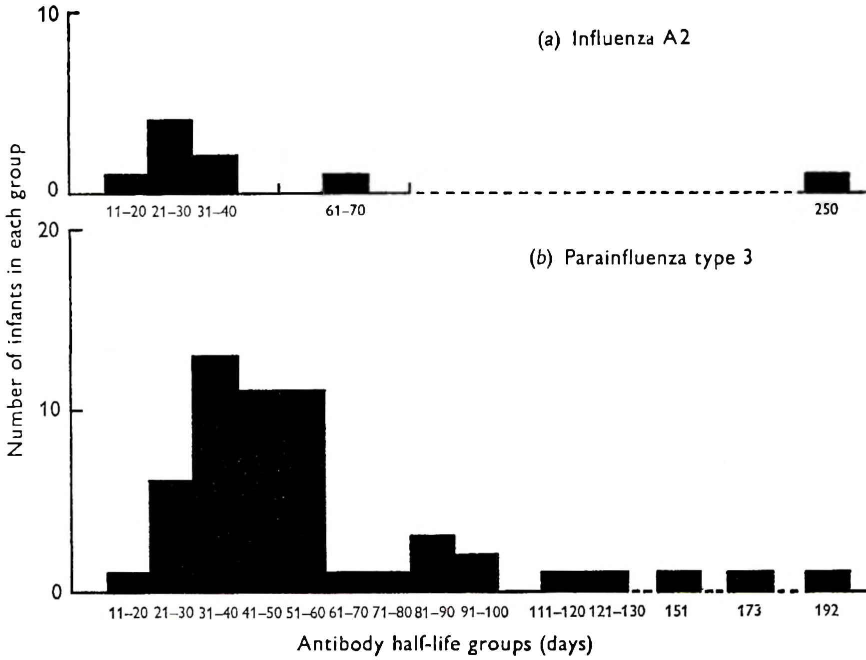


Fig. 1. Frequency distribution of half-life values. (*a*) Influenza A2 antibody, (*b*) parainfluenza type 3 antibody.

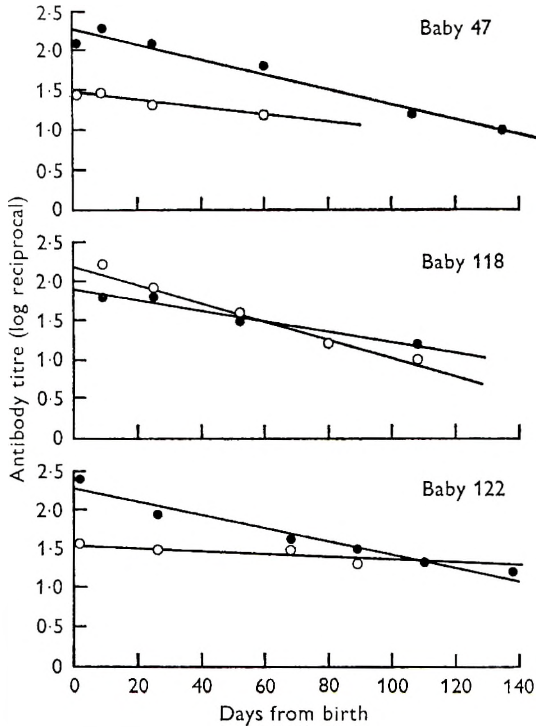


Fig. 2. Examples of postnatal decline of maternally acquired viral antibodies in three infants. ●, Rubella antibody; ○, parainfluenza type 3 antibody.

Sex of the babies

There was no statistically significant difference between the mean parainfluenza type 3 antibody half-lives of the sexes, and the frequency distributions of the half-lives in the sexes were also similar.

Relationships between initial antibody titre, birth weight and gestational age

There was no statistically significant relationship between initial parainfluenza type 3 antibody titre and birth weight, nor between initial antibody titre and gestational age. Though a linear trend in these relationships was evident, the scatter of points was considerable.

Relationship between antibody titres of infants at birth and those of their mothers

A significant linear correlation between the baby's initial parainfluenza type 3 antibody titre and that of its mother was found ($r = 0{\cdot}30$, $P < 0{\cdot}05$). Eleven (21 %) of the babies had a titre at least twofold greater (7) or lower (4) than that of their mothers; these differences were never greater than sixfold.

Duration of detectable maternally acquired antibody

From the regression equation for each baby it was possible to determine the time for the antibody titre to fall to 1/10 (the limit of detectability of both antibody

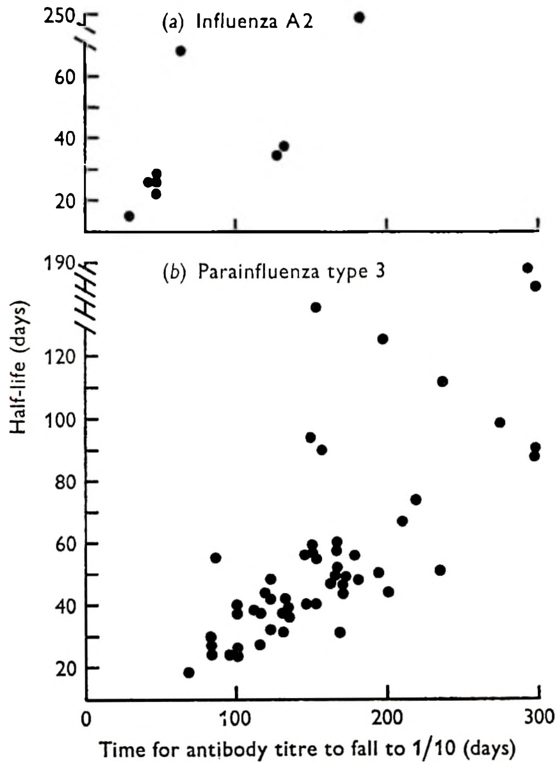


Fig. 3. Relationship between half-life and time calculated for antibody to decline to a titre of 1/10. (*a*) Influenza A2 antibody, (*b*) parainfluenza type 3 antibody.

tests). The relationship between half-life and the number of days for the titre to reach 1/10 for both antigens is shown in Fig. 3. It can be seen that, in both instances, there is a positive relationship between half-life and the duration of detectable antibody. From the experimental results it was observed that, with parainfluenza type 3 antibody, 13 % of the babies had detectable antibody at 6 months of age and 5 % had antibody at nine months.

Relationship between half-life and initial antibody titre

Using the linear equivalent of the model proposed by Brambell *et al.* (1964) to describe the relationship between the catabolism of IgG and its concentration in the serum, it was found that, in the parainfluenza type 3 group, there was a significant relationship between the antibody half-life and the initial titre ($r = 0{\cdot}43$, $P < 0{\cdot}001$). This took the form of a rectangular hyperbola (Fig. 4) such that the higher the initial titre of parainfluenza type 3 antibody, the greater the rate of decline of this antibody. In the smaller influenza A2 group the relationship between the half-life and initial titre was similar but was not significant (Fig. 4).

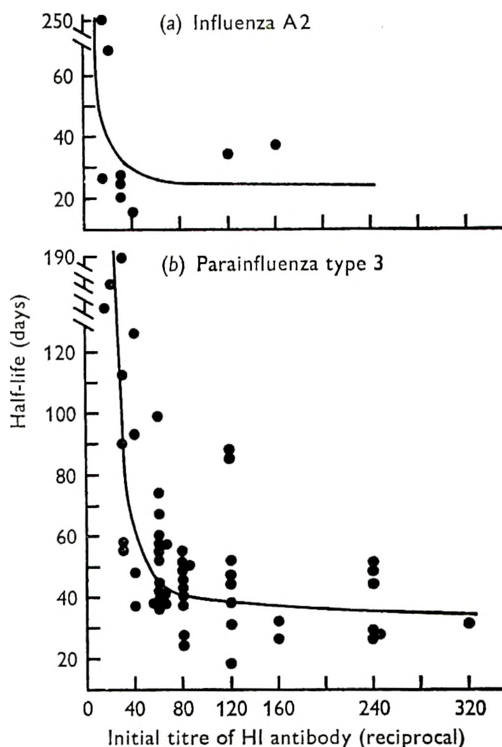


Fig. 4. Relationship between half-life and initial antibody titre in infants. (*a*) Influenza A2 antibody, (*b*) parainfluenza type 3 antibody. The curves (Brambell *et al.* 1964) are:

$$(a)\ \text{half-life} = \frac{\text{initial titre}\ \ln 2}{0{\cdot}03\ (\text{initial titre} - 8{\cdot}35)} \qquad (b)\ \text{half-life} = \frac{\text{initial titre}\ \ln 2}{0{\cdot}02\ (\text{initial titre} - 20{\cdot}71)}$$

Comparison between half-lives within a given baby

Since the computer print-out for each baby included the half-life and its variance, it was possible to compare the half-lives, for a particular infant, of parainfluenza type 3 antibody and of rubella antibody (as reported previously – Cloonan *et al.* 1970) using the t test. It was found that in 21 % of the babies having both antibodies, the half-lives were significantly different (t test, $P < 0{\cdot}05$). It should

Table 1. *Decline of antibodies in infants whose rubella and parainfluenza type* 3 *half-lives were significantly different*

	Antibody half-life (days)	
Baby no.	Rubella	Parainfluenza type 3
8	24·6	56·2
16	42·9	27·3
23	33·9	59·6
30	33·3	18·4
36	31·7	99·0
47	32·8	55·5
101	58·4	27·1
118	46·3	25·8
122	36·2	191·7

Table 2. *Decline of antibodies in infants who possessed antibodies of all three specificities (rubella, parainfluenza type* 3 *and influenza A* 2) *at birth*

	Antibody half-life (days)		
Baby no.	Rubella	Parainfluenza type 3	Influenza A 2
45	33·0	31·1	21·5
46	27·1	39·6	26·1
62	36·4	30·9	33·9
74	30·2	40·1	14·9
118	46·3	25·8	68·2

be noted that in the repeated application of the t test to all 43 babies a significant difference in half-lives would be expected to occur by chance in two or three babies. However, in this study significant differences were obtained in nine babies, and these results are summarized in Table 1. In five babies the half-life of parainfluenza type 3 antibody exceeded that of rubella, whereas in the remaining four the reverse occurred. The actual results with three of these babies are presented graphically in Fig. 2.

There were five babies for whom the half-lives of antibodies of all three specificities (rubella, influenza A 2 and parainfluenza type 3) were determined. Whereas three of the babies had similar half-lives for all three antibodies, two babies had markedly different values (Table 2). Because of the small size of this group, statistical tests to assess the significance of the differences were not applied.

Relationship between initial titre of parainfluenza type 3 antibody and half-life of rubella

Using the results from those babies having both rubella and parainfluenza type 3 antibodies, and an appropriate regression analysis, no significant relationship was found between the initial titre of parainfluenza type 3 antibody and the half-life of rubella antibody, after allowing for the effect of the initial rubella titre on the rubella half-life.

DISCUSSION

We have previously reported a high degree of variability in the rate of decline of maternally acquired rubella antibody in the babies used in the present study (Cloonan *et al.* 1970). For that antibody the range of half-lives was between 14 and 70 days, with the exception of three babies whose values were between 74 and 259 days. A similar degree of variability has now been demonstrated with two other viral antibodies. In the case of influenza A2 antibody the half-lives ranged from 15 to 251 days, and for parainfluenza type 3 antibody from 18 to 192 days (Fig. 1). This study therefore confirms our previous suggestion that the range of half-life values for maternally acquired antibody must be extended beyond the generally accepted range of 20–30 days (Brambell, 1970).

This study has also shown that with parainfluenza type 3 antibody, and possibly with influenza A2 antibody, the half-life is inversely proportional to the initial antibody titre of the baby's serum. A relationship of this type has previously been demonstrated between the level of passively acquired IgG in the serum and its half-life both in animals (Brambell *et al.* 1964; Brambell, 1966) and in man (Waldmann & Strober, 1969). These results confirm our earlier finding with rubella antibody, and indicate that, for an antibody of a given specificity, babies having high antibody titres at birth lose this antibody rapidly whereas in babies with low initial titres antibody declines over a longer period of time. Since maternally acquired antibody in the infant may provide protection against virus infection, it would seem that children with moderate to low initial antibody titres may possess effective congenital immunity for longer periods than those having comparatively high antibody titres at birth.

It has been tacitly assumed that maternally acquired antibodies of different specificities decline at the same rate in a given infant, and that this rate is the same as that of total maternally acquired IgG. This study has shown, however, that for some of the infants maternally acquired antibodies of two different specificities (rubella and parainfluenza type 3) declined at significantly different rates in the same child (Table 1). In addition, there were two babies (Table 2) who had very different rates of decline for all three antibodies (rubella, influenza A2 and parainfluenza type 3). It would seem that antibody of a given specificity declines at a rate which is proportional to its initial titre in the serum and which is independent of the titre of other serum antibodies. This independence is supported by two other observations. In the present study, no relationship was found to exist between the initial titre of antibody of one specificity (parainfluenza type 3) and the rate of decline of another antibody (rubella). In associated studies on the same

group of infants, the IgG concentrations were determined in the initial serum (Wells, 1969). There was no correlation between the half-lives of either of these viral antibodies and the initial IgG concentration.

A feature of these results was that, for each antibody, there was considerable variability in the half-lives for each initial antibody titre (Fig. 4). This also occurred in the previous study with rubella antibody (Cloonan *et al.* 1970). The postnatal decline of maternally acquired antibodies is due partly to the catabolism of the antibodies (Schultze & Heremans, 1966) and partly to dilution of antibodies as a result of expansion of the volume occupied by the plasma and extravascular protein pools as a result of the infants' growth (Trevorrow, 1959; Trevorrow & Washburn, 1970). The latter factor may well account for some of the observed variability in half-lives of antibody of a single specificity, since it is most likely that the rate of growth in the months following birth varied considerably from infant to infant.

In view of the unforeseen nature of these findings, it should be emphasized that all possible care was taken both with the serological tests (conduct of the tests, incorporation of negative and positive control sera) and with the statistical aspects of the study (selection of babies, recording and processing of results). It has been assumed in this study that the influenza A 2 and parainfluenza type 3 antibody in the infants' initial sera is entirely maternally acquired IgG. It could be argued that babies with long antibody half-lives might have experienced a postnatal infection with one of the viruses used in this study or one which was serologically related. This does not appear likely. In both babies having an influenza A 2 half-life of 60 days or longer, the antibody titre fell to less than 1/10, while in 9 of the 14 babies with a parainfluenza type 3 antibody half-life of 60 days or more, the titres also fell to less than 1/10. In the remaining five cases, sera had not been collected for a sufficiently long period to enable observation of such a decline.

We thank Dr C. McGilchrist, Department of Mathematics, University of New South Wales, for advice and assistance in the statistical analysis of the results; Dr R. W. Thomas, Department of Mechanical Engineering, University of New South Wales, for writing the modified computer programs, and for assisting in the computing; Mr K. Deason, Department of Medical Illustration, University of New South Wales, for preparing the illustrations; and Professor G. N. Cooper, Department of Medical Microbiology, University of New South Wales, for constructive criticism of the manuscript.

REFERENCES

Brambell, F. W. R. (1966). The transmission of immunity from mother to young and the catabolism of immunoglobulins. *Lancet* ii, 1087.

Brambell, F. W. R. (1970). *The transmission of passive immunity from mother to young.* Amsterdam: North Holland.

Brambell, F. W. R., Hemmings, W. A. & Morris, I. G. (1964). A theoretical model of γ-globulin catabolism. *Nature, London* **203**, 1352.

Chanock, R. M. & Johnson, K. M. (1964). In *Diagnostic Procedures for Viral and Rickettsial Diseases.* Chap. 14. *Parainfluenza viruses*, p. 482. Ed. E. H. Lennette and N. J. Schmidt. New York: American Public Health Association.

CLOONAN, M. J., HAWKES, R. A. & STEVENS, L. H. (1970). Postnatal decline of maternally acquired rubella antibodies. *Journal of Hygiene* **68**, 461.

DAVENPORT, F. M. & MINUSE, E. (1964). In *Diagnostic Procedures for Viral and Rickettsial Diseases*. Chap. 13. *Influenza viruses*, p. 462. Ed. E. H. Lennette and N. J. Schmidt. New York: American Public Health Association.

FAZEKAS DE ST GROTH, S. & WEBSTER, R. G. (1966). Disquisitions on original antigenic sin. 1. Evidence in man. *Journal of Experimental Medicine* **124**, 331.

SCHULTZE, H. E. & HEREMANS, J. F. (1966). *Molecular Biology of Human Proteins*, Vol. 1. *Nature and Metabolism of Extracellular Proteins*. Amsterdam: Elsevier.

TREVORROW, V. E. (1959). Concentration of gamma-globulin in the serum of infants during the first 3 months of life. *Pediatrics* **24**, 746.

TREVORROW, V. E. & WASHBURN, A. H. (1970). Gamma-globulin synthesis in the first month of life. *American Journal of Diseases of Children* **119**, 296.

WALDMANN, T. A. & STROBER, W. (1969). Metabolism of immunoglobulins. *Progress in Allergy* **13**, 1.

WELLS, J. V. (1969). The nature and metabolism of serum immunoglobulins in clinical syndromes. M.D. thesis, University of New South Wales.

J. Hyg., Camb. (1971), **69**, 445
Printed in Great Britain

Sero-epidemiological survey on Yaba and 1211 virus infections among several species of monkeys

BY Y. TSUCHIYA AND I. TAGAYA
Department of Enteroviruses, National Institute of Health, Nakato, Musashimurayama, Tokyo, 190–12, Japan

(*Received* 9 *March* 1971)

SUMMARY

The distribution of neutralizing antibody to Yaba virus and 1211 agent in the sera of three Asian and one African monkey species was examined.

Cynomolgus (*Macaca irus*), bonnet (*M. radiata*) and rhesus (*M. mulatta*) monkeys possessed antibody to Yaba virus at incidences of 19·9, 8·4 and 0 %, respectively. In African green monkeys (*Cercopithecus aethiops*) the incidence was as high as 76·4 %.

As for 1211 agent, no Asian monkeys had neutralizing antibody and 5·5 % of African green monkey sera neutralized the virus.

INTRODUCTION

Since the discovery by Bearcroft & Jamieson (1958) of a contagious dermal disease in rhesus monkeys kept in open pens at a medical institute in Yaba, Lagos, Nigeria, no outbreak of the same disease in monkeys has been reported despite the increasing number of monkeys that has been used for various scientific studies and viral vaccine production. The causative agent, Yaba virus, has properties common to members of the poxvirus group (Andrewes *et al.* 1959; Niven *et al.* 1961; Woodroofe & Fenner, 1962) and, when experimentally inoculated, caused histiocytic tumour* in Asiatic macaques such as *M. mulatta*, *M. irus*, *M. nemistrinus* and *M. fuscata* (Ambrus, Feltz, Grace & Owens, 1963; Niven *et al.* 1961; Kato, Tsuru & Miyamoto, 1965) and man (Grace & Mirand, 1963). African monkeys are, in general, resistant to the virus (Ambrus *et al.* 1963).

In the meantime, the occurrence of dermal tumours similar to Yaba tumours in rhesus monkeys was reported and the causative agent was identified also as a poxvirus (Hall & McNulty, 1967; Casey, Woodruff & Butcher, 1967). This virus was tentatively named as 1211 agent (Nicholas & McNulty, 1968), Yaba-related virus (Crandell, Casey & Brumlow, 1969) or Yaba-like disease virus (Hull, 1968). The histological appearances of this tumour and Yaba tumour were quite different from each other in that the former consisted of epidermal cells with vacuolated nuclei while the latter consisted of histiocytes without nuclear vacuolation (Casey *et al.* 1967; Hall & McNulty, 1967). The causative agents of both types of tumour,

* We use the word 'tumour' in the widest sense as suggested by Bearcroft & Jamieson (1958), Andrewes *et al.* (1959) and Niven *et al.* (1961).

however, shared common antigens in complement-fixation and neutralization tests (Nicholas & McNulty, 1968; Crandell *et al.* 1969).

The present experiments were carried out to determine the natural hosts and geographical distribution of both viruses among monkeys. The results presented revealed that at least the African green monkey is a natural host of Yaba virus and Asian monkeys also acquire natural infection with Yaba virus or a closely related virus. It was also demonstrated that at least the Asian monkeys examined were not natural hosts of 1211 agent. Sufficient evidence to support the view that the African green monkey is a natural host of 1211 agent could not be obtained.

MATERIALS AND METHODS

Cell cultures and media

A cynomolgus monkey kidney cell line JINET was used. The properties of JINET cells and media used for the growth and maintenance of these cells have been described (Tsuchiya, Takayama & Tagaya, 1969).

Viruses

Yaba virus was propagated and titrated in JINET cells as previously described (Tsuchiya *et al.* 1969). The 1211 virus was a generous gift from Dr W. P. McNulty of the Oregon Regional Primate Research Centre, U.S.A., and was propagated and titrated in JINET cells. The latter virus was assayed by the plaque method.

Monkey sera

Cynomolgus monkey (*M. irus*) sera were collected in our laboratory by heart puncture at the time of nephrectomy. Some of the sera were provided by the Department of Veterinary Science of our institute. Indian bonnet (*M. radiata*) and rhesus (*M. mulatta*) monkey sera were obtained through the courtesy of Dr R. A. Feldman of the Christian Medical College and Hospital, Vellore, South India (present address: N.C.D.C., Atlanta, Ga., U.S.A.). African green monkey (*Cercopithecus aethiops*) sera were donated by the Chemo-serotherapy Institute, Kumamoto. All the sera were heated at 56° C. for 30 min.

Antisera

Yaba virus antisera were collected from cynomolgus monkeys bearing multiple tumours at the time of bleeding. The 1211 virus antisera were obtained by immunizing rabbits. The rabbits were inoculated subcutaneously and intravenously with 0·5 ml. each of fluorocarbon-treated virus 4 times at intervals of 2–3 days and booster injections were given by both routes 1 month after the last injections. The sera were collected by heart puncture under anaesthesia 8 days after the booster injections. All the sera were inactivated at 56° C. for 30 min.

Neutralization test

Neutralization was carried out by mixing equal amounts of appropriately diluted virus and serum. The virus–serum mixtures were incubated at 36° C. for 1 hr. and then 4° C. overnight. In the case of Yaba virus neutralization tests, 0·2 ml. amounts of virus–serum mixtures were inoculated into replicate tube cultures whose growth medium was replaced with 1 ml. amounts of fresh maintenance medium just before inoculation. In 1211 virus neutralization tests, 0·2 ml. amounts of inoculum were allowed to adsorb onto drained monolayers grown in 2 oz. prescription bottles for 3 hr. at 36° C., after which nutrient agar medium was added in 5 ml. amounts. The titres of the sera were expressed as the reciprocals of the highest dilution which caused more than 50 % reduction of the cellular foci of Yaba virus or plaques of 1211 virus.

Plaque assay of 1211 *virus*

The plaque method used in these experiments was the same as that reported previously for the variola–vaccinia subgroup of poxviruses (Tsuchiya & Tagaya, 1970). As plaque formation by 1211 agent was also found to be enhanced by the

Table 1. *Effects of DEAE-dextran and $MgCl_2$ on plaque formation by* 1211 *agent*

Additive		Plaque titre*
None		$4·8 \times 10^4$
DEAE-dextran	100 μg/ml	$7·0 \times 10^4$
$MgCl_2$	25 mM	$1·2 \times 10^5$
	35 mM	$1·4 \times 10^5$
	45 mM	$8·8 \times 10^4$
DEAE-dextran	100 μg/ml	$1·4 \times 10^5$
Plus $MgCl_2$	25 mM	

* Plaque titre is expressed in p.f.u./ml. Additives were contained in agar overlay medium.

addition of DEAE-dextran and $MgCl_2$ in the agar overlay medium (Table 1) as in the case of other poxviruses (Tsuchiya & Tagaya, 1970), both drugs were incorporated in the agar medium at concentrations of 100 μg./ml. and 25 mM respectively. The plaques were counted 8–14 days after inoculation.

RESULTS

Yaba virus-neutralizing antibody in various monkey species

Screening tests for Yaba virus-neutralizing antibody were carried out on 265 Asian monkey and 55 African green monkey sera at a serum dilution of 1/4. The geographical origins of the monkeys from which the sera were collected and the results of the screening tests are summarized in Table 2. As shown in the Table, the incidences of neutralizing antibody in cynomolgus monkey sera varied according to the places where the monkeys were captured. It is interesting that the cynomolgus monkeys from the Asian Continent had a rather high incidence of antibody, while those from island countries had a rather low (Indonesia, 4·5 %)

or no (Philippines) incidence. The monkeys captured in Cambodia, Vietnam and Malaya possessed neutralizing antibody in 40·5, 20·0 and 25·0% respectively. In total, cynomolgus monkeys had antibody in 19·9% (33/166).

As to Indian monkeys, 7 out of 83 bonnet monkey sera (8·4%) possessed antibody, while none of 14 rhesus monkey sera had the antibody.

The frequency of antibody reached as high as 76·4% in cercopithecus monkeys.

Table 2. *Yaba virus-neutralizing antibody in monkey sera*

(Screening test at 1/4)

Monkey species	Place captured	No. tested	No. positive	% positive
Macaca irus	Cambodia	37	15	40·5
	Vietnam	35	7	20·0
	Malaya	40	10	25·0
	Indonesia	22	1	4·5
	Philippines	32	0	0
M. radiata	India	83	7	8·4
M. rhesus	India	14	0	0
Cercopithecus aethiops	Uganda	55	42	76·4

The focus numbers in control cultures ranged from 47·8 to 185·0.

Table 3. 1211 *agent-neutralizing antibody in monkey sera*

(Screening test at 1/4)

Monkey species	Place captured	No. tested	No. positive	% positive
Macaca irus	Cambodia	15	0	0
	Vietnam	34	0	0
	Malaya	40	0	0
	Indonesia	29	0	0
	Philippines	23	0	0
M. radiata	India	72	0	0
M. rhesus	India	14	0	0
Cercopithecus aethiops	Uganda	55	3	5·5

The plaque numbers in control cultures ranged from 49·5 to 137·3.

The 1211 *agent neutralizing antibody screening*

Two hundred and twenty-seven cynomolgus, bonnet and rhesus monkey sera were tested for neutralizing antibody to 1211 agent. All the sera failed to neutralize the agent at a serum dilution of 1/4 (Table 3). Only 3 out of 55 African green monkey sera (5·5%) showed neutralization at that dilution (Table 3).

Cross neutralization test

Yaba virus monkey and 1211 agent rabbit antisera and normal cynomolgus and African green monkey sera which had neutralizing antibody to Yaba virus were assayed for both Yaba and 1211 virus antibodies. The titres of the sera against both viruses are summarized in Table 4. As shown in the table, normal and anti-Yaba monkey sera did not neutralize 1211 virus at a serum dilution of 1/4 while

their titres to homologous virus ranged from 16 to 4096. In a separate experiment 5 out of 11 Yaba virus monkey antisera, including two sera shown in Table 4, neutralized 1211 agent when tested at a dilution of 1/4. In contrast, 1211 antisera prepared in rabbits neutralized both viruses at almost identical titres.

Table 4. *Cross-neutralization test*

Serum	Code	Virus: Yaba	Virus: 1211
Yaba antisera (monkey)	8300	4096	<4
	8292	1024	<4
1211 antisera (rabbit)	A	4096	1024
	B	4096	4096
Normal cynomolgus monkey sera	6823	16	<4
	6842	16	<4
Normal African green monkey sera	G–52	4096	<4
	G–62	1024	<4

DISCUSSION

The circumstances under which Yaba virus infection was first recognized were rather interesting. In 1957, Asian monkeys developed tumours while they were kept in captivity at an African institution (Bearcroft & Jamieson, 1958) and this is the only reported case of a natural outbreak of the disease in the literature. Although Ambrus, Strandstrom & Kawinski (1969) reported a 'spontaneous' outbreak of Yaba virus infection in their rhesus monkey colony, their report implied that the monkeys acquired infection through insect vectors in their laboratory, where Yaba virus studies using monkeys had been extensively carried out. In spite of the innumerable number of monkeys used all over the world in the past decade, no Yaba virus tumour has been recognized in freshly captured Asian monkeys even though tumours induced in these animals are so conspicuous that they can hardly escape recognition during quarantine period. The monkeys which are native to Africa, where the only Yaba virus outbreak was recognized, were in general resistant to the virus (Ambrus *et al.* 1963). These peculiarities in Yaba virus epidemiology and ecology stimulated us to carry out a sero-epidemiological survey on this virus together with 1211 agent which shares common antigens with Yaba virus (Nicholas & McNulty, 1968; Crandell *et al.* 1969).

The present experiments showed that both Asian and African monkeys had neutralizing antibody to Yaba virus. The incidence of the antibody to Yaba virus in African green monkeys reached as high as 76·4 % (Table 2), and the neutralizing titre was also very high. These facts suggest that at least the African green monkey may be a natural host of the virus. Despite the high incidence of neutralizing antibody to Yaba virus, spontaneous occurrence of Yaba tumour has not been reported in this monkey species. This may partly be due to the inherent resistance of this monkey species to the virus in regard to tumourigenesis.

It is a rather surprising finding that cynomolgus monkeys had an antibody incidence of 19·9 % because no outbreak of Yaba virus infection in this monkey

species has ever been reported although the virus causes tumours in cynomolgus monkeys which can hardly be overlooked. There are three possible explanations for this. The first is that another poxvirus indigenous among Asian monkeys which has antigens common to Yaba virus may exist and this hypothetical virus causes silent infection in these monkeys. The significantly low neutralizing-antibody titre to Yaba virus in cynomolgus monkeys compared to that in cercopithecus monkeys lends support to this suggestion. The second possibility is that Yaba virus may by nature be a non-tumour producing virus even in Asian monkeys and the Yaba virus isolated in the African outbreak is a mutant virus which produces tumours efficiently in Asian monkeys. The third possibility is that Yaba virus may be spread by air among monkeys in natural conditions and the lesions caused by the virus may be restricted to the respiratory organs and escape recognition. Experimental infection of monkeys with Yaba virus by aerosol has been reported (Wolfe, Griesemer & Farrell, 1968). Tumours in their experiments, however, appeared also on the body surface of the inoculated monkeys. Either of these possibilities may explain the rare recognition of Yaba tumours in monkeys. Although we could not demonstrate neutralizing antibody in rhesus monkeys, this may be due to the small number of sera tested.

No Asian monkeys had neutralizing antibody to 1211 agent and only 5·5% of African green monkeys had the antibody. This finding suggests that at least the Asian monkey species examined are not natural hosts of 1211 agent. It could not be concluded from the results of the present experiments whether African green monkeys are, or are not, natural hosts of 1211 agent because 1211 agent shares common antigens with Yaba virus. The African green monkey sera positive for 1211 agent were also positive for Yaba virus.

Some of the Yaba virus monkey antisera had neutralizing antibody against 1211 agent while others did not. On the contrary, hyperimmune rabbit antisera against 1211 agent neutralized both homologous and heterologous viruses at almost the same efficiency. This difference of immune sera in ability to cross-react with heterologous viruses may be due to the difference of the immunized animals. A similar phenomenon was also reported in herpes simplex virus (HSV) and *Herpesvirus simiae* (B virus) (Ueda, Tagaya & Shiroki, 1968). Monkeys produced neutralizing antibodies to HSV as well as B virus upon immunization with B virus or HSV but guinea-pigs produced antibodies which specifically neutralized homologous virus when they were immunized with either HSV or B virus. The Yaba virus monkey antisera which neutralize only homologous virus may be useful for differentiation of these viruses by immunological methods.

REFERENCES

AMBRUS, J. L., FELTZ, E. T., GRACE, J. T., JR. & OWENS, G. (1963). A virus-induced tumor in primates. *National Cancer Institute Monograph* **10**, 447.

AMBRUS, J. L., STRANDSTROM, H. V. & KAWINSKI, W. (1969). 'Spontaneous' occurrence of Yaba tumor in a monkey colony. *Experientia* **25**, 64.

ANDREWES, C. H., ALLISON, A. C., ARMSTRONG, J. A., BEARCROFT, G., NIVEN, J. F. S. & PEREIRA, H. G. (1959). A virus disease of monkeys causing large superficial growths. *Acta Unionis Internationalis contra Cancrum* **15**, 760.

Bearcroft, W. G. C. & Jamieson, M. F. (1958). An outbreak of subcutaneous tumours in rhesus monkeys. *Nature, London* **182**, 195.

Casey, H. W., Woodruff, J. M. & Butcher, W. (1967). Electron microscopy of a benign epidermal pox disease of rhesus monkeys. *American Journal of Pathology* **51**, 431.

Crandell, R. A., Casey, H. W. & Brumlow, W. B. (1969). Studies of a newly recognized poxvirus of monkeys. *Journal of Infectious Diseases* **119**, 80.

Grace, J. T. Jr. & Mirand, B. A. (1963). Human susceptibility to a simian tumor virus. *Annals of the New York Academy of Sciences* **108**, 1123.

Hall, A. S. & McNulty, W. P., Jr. (1967). A contagious pox disease in monkeys. *Journal of the American Veterinary Medical Association* **151**, 833.

Hull, R. N. (1968). The simian viruses. In *Virology Monographs*, vol. 2, p. 1. Ed. S. Gard, C. Hallauer, and K. F. Meyer. Vienna: Springer-Verlag.

Kato, S., Tsuru, K. & Miyamoto, H. (1965). Autoradiographic studies on the Yaba monkey tumor virus and host cell interaction. *Biken Journal* **8**, 45.

Nicholas, A. H. & McNulty, W. P. (1968). *In vitro* characteristics of a poxvirus isolated from rhesus monkeys. *Nature, London* **217**, 745.

Niven, J. S. F., Armstrong, J. A., Andrewes, C. H., Pereira, H. G. & Valentine, R. C. (1961). Subcutaneous 'growths' in monkeys produced by a poxvirus. *Journal of Pathology and Bacteriology* **81**, 1.

Tsuchiya, Y. & Tagaya, I. (1970). Plaque assay of variola virus in a cynomolgus monkey kidney cell line. *Archiv für die gesamte Virusforschung* **32**, 73.

Tsuchiya, Y., Tagaya, I. & Tsuruhara, T. (1969). Titration and extensive serial passages of Yaba virus *in vitro*. *Japanese Journal of Microbiology* **13**, 103.

Tsuchiya, Y., Takayama, R. & Tagaya, I. (1969). Establishment of a cynomolgus monkey kidney cell line and its susceptibility to various viruses. *Japanese Journal of Medical Science and Biology* **22**, 105.

Ueda, Y., Tagaya, I. & Shiroki, K. (1968). Immunological relationship between herpes simplex virus and B virus. *Archiv für die gesamte Virusforschung* **24**, 231.

Wolfe, L. G., Griesemer, R. A. & Farrell, R. L. (1968). Experimental aerosol transmission of Yaba virus in monkeys. *Journal of the National Cancer Institute* **41**, 1175.

Woodroofe, G. M. & Fenner, F. (1962). Serological relationships within the poxvirus group: An antigen common to all members of the group. *Virology* **16**, 334.

J. Hyg., Camb. (1971), **69**, 453
Printed in Great Britain

Control at hospital level of infections by methicillin-resistant staphylococci in children

BY M. F. MICHEL AND CATHARINA C. PRIEM

Department of Bacteriology, Sophia Children's Hospital and Neonatal Unit, Academical Hospital, Medical Faculty Rotterdam, 160 *Gordelweg, Rotterdam* 4, *The Netherlands*

(*Received* 10 *March* 1971)

SUMMARY

Rapid spread of methicillin-resistant staphylococci (MeRS) in a children's hospital is described. Within 4 months of the first isolation MeRS had been isolated from infections in all clinical units. MeRS were also regularly isolated at the out-patient department. Protective isolation of one of the clinical units had no effect on the infection rate by MeRS. The use of antiseptics (Hexachlorophene and chlorhexidine) and gentamicin nose cream in children and staff members in three out of five clinical units resulted in a significant reduction of the prevalence of nose colonization by MeRS in children. In staff members a non-significant reduction of the prevalence of colonization and a significant reduction of the acquisition of MeRS was found. After a few months the infection rate decreased to zero in the units where the measures were introduced. It remained unchanged in the other units. Phage typing of two sets of strains collected at an interval of 6 months showed that the infections were mainly caused by two endemic strains of MeRS. The majority of the infections caused by MeRS was of minor importance. In 16 % of the infections a strain was isolated repeatedly and for more than 1 week. After the introduction of antiseptics a relative increase of infections by Gram-negative bacteria was observed. The significance of this phenomenon is discussed.

INTRODUCTION

Naturally occurring strains of methicillin-resistant staphylococci (MeRS) were initially described by Jevons (1961), Knox & Smith (1961) and Barber (1961). Evidence has been presented that they differ in several respects from other strains of *Staphylococcus aureus* (Knox, 1961; Sutherland & Rolinson, 1964; Barber, 1964; Annear, 1968; Sabbath, Leaf, Gerstein & Finland, 1970).

Strains of MeRS invariably show resistance to other penicillins and with few exceptions to streptomycin and tetracyclin. Resistance to methicillin is not due to an increased destruction of the antibiotic by a modified penicillinase but to intrinsic insensitivity for all penicillins. Penicillinase-negative variants of MeRS are therefore also resistant to methicillin (Dyke, Jevons & Parker, 1966).

The frequency of resistance to methicillin among staphylococcal strains investigated at the Staphylococcal Reference Laboratory in Colindale was initially

very low. It increased slowly in the period 1960–3 to 1 % and remained stationary for several years thereafter (Parker & Hewitt, 1970). During this time higher frequencies were, however, observed locally in England and abroad (Chabbert & Baudens, 1962; Cetin & Ang, 1962; Courtieu *et al.* 1964; Chabbert, Baudens, Acar & Gerbaud, 1965; Colley, NcNicol & Bracken, 1965; Chabbert & Pillet, 1967). After 1967 the frequency of methicillin-resistant strains observed in Colindale rose sharply to 5 %.

There are numerous publications on the epidemic spread and infections by MeRS in different countries. They were surveyed by Barrett, McGehee & Finland (1968). Additional reports have been published by Rountree & Beard (1968), Benner & Kayser (1968), Jessen *et al.* (1969), Mouton & van Boven (1969) and O'Toole, Drew, Dahlgren & Beaty (1970).

The present study deals with the epidemic spread of MeRS after their first isolation in a hospital for children. Emphasis is placed on the effect of several measures to restrict the colonization and infection rates on a hospital level.

MATERIAL AND METHODS

Population studied

Observations were made in a 180-bed hospital for children, in both the wards and the out-patient department. Patients are nursed in five separate units: a neonatal unit (24 beds), an infant unit (24 beds), a quarantine unit (24 beds), a medical unit (48 beds) and a surgical paediatric unit (60 beds).

Nasal swabs were taken fortnightly and later monthly from patients and staff members as indicated. Colonization rates are reported on a monthly basis.

Bacteriological methods

Conventional bacteriological methods were used for the isolation of coagulase-positive staphylococci and other bacterial species.

Sensitivities to the antibiotics mentioned in the next section were determined on Diagnostic Sensitivity Test (DST) agar (Oxoid) using paper disks (Oxoid). Readings of the tests were normally made after 24 hr. incubation at 37° C. At this time methicillin-resistant strains usually showed a narrowing of the critical zone of inhibition with or without visible growth of resistant colonies within this zone. Additional methicillin-resistant strains were detected by reincubation of the plates for 24 hr at 37° C.

Phage-typing was carried out at 1 RTD and 1000 RTD by Dr R. Scholtens, Rijksinstituut voor de Volksgezondheid, Bilthoven.

RESULTS

Primary isolation and spread of MeRS

MeRS were observed for the first time in January 1969. These and subsequently isolated strains were as a rule resistant to penicillin, streptomycin, tetracylin, erythromycin, kanamycin, methicillin and cephaloridin but sensitive to chloram-

phenicol, lincomysin, gentamicin and fucidin. The number of isolations from infections rose from seven in the second month to ten in the third month. The strains were isolated from patients of the infant and surgical units. From the fourth month onwards new infections were seen on all units, the infection rate varying between 7 and 17 per month.

Both the rapid increase and the spread of infections over all units strongly suggested the existence of an epidemic of hospital infections due to one or more strains of MeRS. Therefore from April 1969 efforts were made to reduce the number of these infections by MeRS. Measures to prevent recolonization of the infant unit previously freed of MeRS were only partially successful and are not reported here.

Use of antiseptics

When after 1 year the number of infections caused by MeRS did not show any sign of spontaneous decline an attempt was made to reduce the colonization and acquisition rates of MeRS by means of skin antiseptics. It was hoped that by this measure a secondary reduction of the infection rate could be effected. Skin disinfection was not used throughout the whole hospital but on three units where most infections were observed, i.e. the infant, neonatal and surgical unit. Starting in March 1970 the following regulations were put into operation on these units. Children were washed daily with 3 % hexachlorophene emulsion (pHisoHex). After rinsing with water the skin was treated with 1 % chlorhexidine in water. Staff members on duty used hexachlorophene bar-soap for hand washing. Carriers of MeRS were treated once or several times during 1 week with 0·3 % gentamicin nasal cream until three consecutive nasal swabs taken at weekly intervals were negative.

Prevalence and acquisition of MeRS in staff members

The influence of the measures on the prevalence and acquisition of MeRS in the nose of staff members is shown in Table 1. Both phenomena are compared during a period of 6 months preceding and following the introduction of the measures. It can be seen that the prevalence of MeRS decreased from 37 carriers in the first period to 23 in the second period. This decrease is statistically non-significant ($0{\cdot}1 < P < 0{\cdot}2$). The acquisition of MeRS on the other hand declined significantly from a total of 16 persons in the first period to 3 persons in the second period ($0{\cdot}005 < P < 0{\cdot}01$). These data indicate that the measures, although effective in preventing acquisition, were much less so in curing existing nasal colonization.

Prevalence of MeRS in patients

The prevalence of the nasal colonization in patients in each unit was compared in the same 6 months periods as above. It is shown in Table 2 that a significant reduction of the colonization occurred in the neonatal and surgical units ($P < 0{\cdot}0005$). For no obvious reason the reduction in the infant unit, although marked, was less and not significant ($0{\cdot}1 < P < 0{\cdot}2$).

Table 1. *Effect of the introduction of the use of antiseptics on the prevalence and acquisition of MeRS in the nose in staff members*

Period	n	Prevalence	n^{acq}	Acquisition
October 1969	80	7 (8·7 %)	—	—
November 1969	133	7 (5·3 %)	126	2 (1·5 %)
December 1969	149	5 (3·4 %)	144	1 (0·7 %)
January 1970	145	4 (2·8 %)	141	4 (2·8 %)
February 1970	150	6 (4·0 %)	144	5 (3·3 %)
March 1970	127	8 (6·3 %)	119	4 (3·2 %)
Total	704	37 (5·3 %)	674	16 (2·4 %)
April 1970	136	6 (4·4 %)	130	1 (0·7 %)
May 1970	133	7 (5·3 %)	126	0
June 1970	121	3 (2·5 %)	118	0
August 1970	136	4 (2·9 %)	132	0
September 1970	132	3 (2·3 %)	129	2 (1·5 %)
Total	658	23 (3·5 %)	635	3 (0·5 %)

The use of antiseptics was started in March 1970.
n, Number of nasal cultures; n^{acq}, number of nasal cultures diminished by the number of carriers.
Prevalence $\chi^2_{(1)} = 2·10$ ($0·10 < P < 0·20$); acquisition $\chi^2_{(1)} = 6·99$ ($0·005 < P < 0·01$).

Table 2. *Prevalence of MeRS in the nose in children*

Unit	Period	n	Prevalence	$\chi^2_{(1)}$
Neonatal	Before	173	48 (27·7 %)	37·58
	After	148	3 (2 %)	$P < 0·0005$
Infant	Before	206	21 (10·1 %)	1·77
	After	115	6 (5·25 %)	$0·10 < P < 0·20$
Surgical	Before	427	68 (15·9 %)	24·61
	After	314	13 (4·1 %)	$P < 0·005$

The prevalence is compared in each unit for a period of 6 months before and 6 months after the introduction of the use of antiseptics.

Infection rate

The number of new isolations of MeRS from infections on all clinical units and the out-patient department during a 12-month period (October 1969 to October 1970) is shown in Table 3. Isolations on the quarantine and medical units were evenly distributed over the whole of the observation period. Introduction of antiseptics on the infant, neonatal and surgical units in March 1970 rapidly reduced the infection rate on these units to zero ($P < 0·0005$). Although the number of positive cultures originating from the out-patient department seems remarkably high it should be remembered that they were frequently obtained in children with previous hospital contacts.

Source and types

All strains newly isolated 1 month before (53 strains) and 3 months after (10 strains) the introduction of the restrictive measures in March 1970 were phage-typed. The origin and the types of these strains are shown in Table 4.

Arranged in decreasing order of frequency the source of the strains in both periods was the following: nasal swabs, pus and sputum. One additional strain was isolated from blood and from urine in the first period.

Table 3. *Monthly isolations of MeRS from infections in clinical units and the out-patient department*

	Unit						
	Neonatal	Infant	Surgical	Quarantine	Medical	O.D.	Total
1969							
Oct.	0	1	6	0	2	0	9
Nov.	0	1	1	1	0	0	7
Dec.	5	0	2	2	1	1	11
1970							
Jan.	1	1	4	2	0	3	12
Feb.	1	2	4	3	1	5	17
Mar.	0	1	4	2	0	0	8
Apr.	0	0	0	1	0	0	3
May	0	1	0	2	0	0	4
June	0	1	0	2	0	1	5
July	1	0	0	2	2	1	6
Aug.	0	0	0	1	1	0	2
Sept.	0	0	0	2	1	0	5

In March 1970 the use of antiseptics was introduced in three out of five clinical units, i.e. the neonatal, infant and surgical unit.

O.D., Outpatients' Department.

$\chi^2_{(1)} = 12{\cdot}4$ ($P < 0{\cdot}0005$).

Table 4. *Type and source of MeRS collected during February and June* 1970

	Strains						
		Source					
Period	No.	Nose	Sputum	Pus	Blood	Urine	Phage type
February 1970	37	27	2	7	1	—	85/+
	12	8	2	2	—	—	6/47/77/84/85/+
	2	1	—	1	—	—	6/7/47/53/54/88/83A/85/+
	1	—	—	—	—	1	53/88/85
	1	1	—	—	—	—	29/52/+
June 1970	5	3	1	1	—	—	85/+
	3	—	2	1	—	—	6/42/77/84/85/+
	1	—	—	—	—	—	6/7/47/53/54/88/83A/85/+
	1	—	—	1	—	—	7/47/54/77/+

Two major (85/+, 6/47/77/84/85/+) and one minor (6/7/47/53/54/88/83A/85/+) type prevailed in both periods. Strains with type 53/88/85, 29/52/+, 7/47/54/77/+ were observed only once. The frequent isolation of two sets of multiple-resistant strains of the same type both from nasal swabs and from infections over a long period of time substantiates the endemic character of these strains.

Infections by Gram-positive and Gram-negative bacteria

The favourable effect exerted by the use of hexachlorophene on the incidence of infections in newborn infants has been well documented (Williams, Blowers, Garrod & Shooter, 1966; Baber *et al.* 1967). Recently several authors drew attention to the fact that the use of this antiseptic might involve colonization followed by a rise of infections by Gram-negative bacteria (Forfar, Gould & Maccabe, 1968; Light, Sutherland, Cockran & Sutorius, 1968; Conn, 1969). Chlorohexidine was added to the restrictive measures described here in an attempt to prevent

Table 5. *Isolations of Gram-positive and Gram-negative bacterial species from pus, blood and cerebrospinal fluid*

	Units with skin disinfection			Units without skin disinfection		
	Jan. 1970	Aug. 1970	$\chi^2_{(1)}$ P	Jan. 1970	Aug. 1970	$\chi^2_{(1)}$ P
Total no. of cultures	53	29	—	40	28	—
No. of cultures from which Gram-positive bacteria were isolated	33	8	7·68 $0{\cdot}005 < P < 0{\cdot}01$	20	5	6·00 $0{\cdot}01 < P < 0{\cdot}02$
No. of cultures from which Gram-negative bacteria were isolated	9	13	6·05 $0{\cdot}01 < P < 0{\cdot}025$	3	7	2·74 $0{\cdot}05 < P < 0{\cdot}10$

such an effect of hexachlorophene. Information about the frequency of new isolations of pathogenic Gram-positive and Gram-negative bacteria from pus, blood and cerebrospinal fluid during fixed periods before and after March 1970 in different parts of the hospital is given in Table 5. A significant decrease ($0{\cdot}005 < P < 0{\cdot}01$) of Gram-positive and increase of Gram-negative bacteria ($0{\cdot}01 < P < 0{\cdot}025$) was observed in the units where the measures were put into effect (Table 5). The same trend was observed in the other units (Table 5), although in this case the increase of Gram-negative bacteria did not attain the level of significance ($0{\cdot}05 < P < 0{\cdot}10$).

DISCUSSION

It is assumed that the close contact between children and between staff and children contributed greatly to the rapid spread followed by endemic infections by several strains of MeRS. This assumption is supported by the independent observation that similar infections occurring simultaneously in another hospital in adults hardly gave rise to colonization and rarely to cross-infections.

There are several reports (Stewart & Holt, 1963; Benner & Kayser, 1968) indicating that infections by MeRS are often seen in patients suffering from debilitating diseases. This might very well imply that the virulence of MeRS is reduced.

In our experience most infections caused by MeRS were of minor importance. Multiple isolations, mainly from sputum or pus during 1 week or longer, were observed in 12 out of 75 children in hospital in the last 12 months. One child with debilitating disease died from septicaemia. In this case *Streptococcus faecalis* and indifferent streptococci as well as MeRS were isolated from the blood.

Reversed isolation of a complete unit was only temporarily effective in the control of infections by MeRS. The high degree of contamination of the environment must undoubtedly be held responsible for this result (Shooter, 1965).

It was expected that by the use of antiseptics and gentamicin nose-cream a primary reduction of the colonization rate in staff members and children would be followed by a secondary reduction in the infection rate (Gillespie, Simpson & Tozer, 1958). Although both objectives were attained the following epidemiological details are worth mentioning. The reduction of the colonization was rapid and pronounced in children but much less so in staff members. This difference may be more apparent than real when one keeps in mind that the data of the children were favourably biased by the high turnover rate of patients. The reduction of the infection rate was further strictly confined to the units where the measures were applied. The expectation that the reduction of the colonization and infection rate would become manifest after a certain time in other units was not fulfilled.

It was established in this study that infections by endemic strains of MeRS can be successfully controlled on a hospital level. Although weakened by a similar development in other clinical units evidence was obtained that the introduction of antiseptics was followed by a relative increase of infections caused by Gram-negative bacteria. The incompatible consequences of the measures creates a dilemma which may be difficult to solve. In a given situation one may have to decide whether the suppression of staphylococcal infections justifies the risk of an increase of infections by Gram-negative bacteria. Further work on the relative importance of both kinds of infections for patients is needed before a final judgement can be expressed on the course which should be taken in this matter.

We want to thank the members of the infection committee for the constructive interest and the members of the medical and nursing staff for their help in this project. We are indebted to Professor H. A. Valkenburg, Department of Epidemiology, Medical Faculty Rotterdam, for the statistical analysis of the results.

REFERENCES

ANNEAR, D. I (1968). The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some other antibiotics. *Medical Journal of Australia* i, 444.

BABER, K. G., CORNER, B., DUNCAN, E. H. L., EADES, S. M., GILLESPIE, W. A. & WALKER, S. C. B. (1967). A prospective study of staphylococcal infection and its prevention among infants and mothers after childbirth in hospital and at home. *Journal of Hygiene* **65**, 381.

BARBER, M. (1961). Methicillin-resistant staphylococci. *Journal of Clinical Pathology* **14**, 385.

BARBER, M. (1964). Naturally occurring methicillin-resistant staphylococci. *Journal of General Microbiology* **35**, 183.

BARRETT, F. F., MCGEHEE, R. G. & FINLAND, M. (1968). Methicillin-resistant *Staphylococcus aureus* at Boston city hospital. *New England Journal of Medicine* **279**, 441.

Benner, E. J. & Kayser, F. H. (1968). Growing clinical significance of methicillin-resistant *Staphylococcus aureus*. *Lancet* ii, 742.

Cetin, E. T. & Ang, Ö. (1962). Staphylococci resistant to methicillin ('celbenin'). *British Medical Journal* ii, 51.

Chabbert, Y. A. & Baudens, J. G. (1962). Souches de staphylocoques résistant naturellement à la méthicilline et à la 5-methyl-3-phényl-9-iso-oxazolyl-penicilline (P 12). *Annales de l'Institut Pasteur* **103**, 222.

Chabbert, Y. A., Baudens, J. G., Acar, J. F. & Gerbaud, G. R. (1965). La résistance naturelle des staphylocoques à la méthicilline et l'oxacilline. *Revue française d'études cliniques et biologiques* **10**, 495.

Chabbert, Y. A. & Pillet, J. (1967). Correlation between 'methicillin resistance' and serotype in *Staphylococcus*. *Nature, London* **213**, 1137.

Colley, E. W., McNicol, M. W. & Bracken, J. M. (1965). Methicillin-resistant staphylococci in a general hospital. *Lancet* i, 595.

Conn, N. K. (1969). Coliform bacteriaemia in infants. *Scottish Medical Journal* **14**, 23.

Courtieu, A. L., Guillermet, F. N., Longeray, C., Maka, G. & Chabbert, Y. A. (1964). Fréquence des staphylocoques présentant une résistance hétèrogène à la méthicilline et l'oxacilline en milieu hospitalier. *Annales de l'Institut Pasteur* **107**, 691.

Dyke, K. G. H., Jevons, M. & Parker, M. T. (1966). Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* i, 835.

Forfar, J. O., Gould, J. C. & Maccabe, A. F. (1968). Effect of hexachlorophane on incidence of staphylococcal and Gram-negative infection in the newborn. *Lancet* ii, 177.

Gillespie, W. A., Simpson, K. & Tozer, R. C. (1958). Staphylococcal infection in a maternity hospital. *Lancet* ii, 1075.

Jessen, O., Rosendal, K., Bülow, P., Faber, V. & Eriksen, K. R. (1969). Changing staphylococci and staphylococcal infections. *New England Journal of Medicine* **281**, 627.

Jevons, M. P. (1961). 'Celbenin'-resistant staphylococci. *British Medical Journal* i, 124.

Knox, R. (1961). 'Celbenin'-resistant staphylococci. *British Medical Journal* i, 126.

Knox, R. & Smith, J. T. (1961). Nature of penicillin resistance in staphylococci. *Lancet* ii, 520.

Light, I. J., Sutherland, J. M., Cockran, M. L. & Sutorius, J. (1968). Ecologic relation between *Staphylococcus aureus* and pseudomonas in a nursery population. *New England Journal of Medicine* **278**, 1243.

Mouton, R. P. & van Boven, C. P. (1969). Letter to the editor. *Nederlands Tijdschrift voor Geneeskunde* **113**, 131.

O'Toole, R. D., Drew, W. L., Dahlgren, B. J. & Beaty, H. N. (1970). An outbreak of methicillin-resistant *Staphylococcus aureus* infection. *Journal of the American Medical Association* **213**, 257.

Parker, M. T. & Hewitt, J. H. (1970). Methicillin resistance in *Staphylococcus aureus*. *Lancet* i, 800.

Rountree, P. M. & Beard, M. A. (1968). Hospital strains of *Staphylococcus aureus*, with particular reference to methicillin-resistant strains. *Medical Journal of Australia* i, 1163.

Sabbath, L. D., Leaf, C. D., Gerstein, P. A. & Finland, M. (1970). Altered cell walls of *Staphylococcus aureus* resistant to methieillin. *Nature, London* **225**, 1074.

Shooter, R. A. (1965). In *Skin Bacteria and their Role in Infection*, ed. H. I. Maibach and G. Hildick-Smith. New York: McGraw-Hill.

Stewart, G. T. & Holt, R. J. (1963). Evolution of natural resistance to the newer penicillins. *British Medical Journal* i, 308.

Sutherland, R. & Rolinson, G. N. (1964). Characteristics of methicillin-resistant staphylococci. *Journal of Bacteriology* **87**, 887.

Williams, R. E. O., Blowers, R., Garrod, L. P. & Shooter, R. A. (1966). In *Hospital Infection, Causes and Prevention*. London: Lloyd-Luke.

J. Hyg., Camb. (1971), 69, 461
With 1 *plate*
Printed in Great Britain

The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses

III. Effects of proteolytic enzymes

BY L. HOYLE
Public Health Laboratory, General Hospital, Northampton
AND JUNE D. ALMEIDA
Virological Department, Royal Post-Graduate Medical School, Hammersmith Hospital, London

(*Received* 11 *March* 1971)

SUMMARY

The action of trypsin and pronase on the haemagglutinins and neuraminidases of eight strains of influenza virus has been examined.

The haemagglutinins of all the strains were highly susceptible to digestion by pronase but there were great variations in resistance to trypsin.

The neuraminidases of the eight strains were of three types. The neuraminidases of the A1 strains and the DSP strain of virus A were highly susceptible to destruction by both enzymes. The neuraminidases of the PR 8 and SWINE strains showed partial resistance especially to trypsin, while the A2 strains and the LEE strains of virus B possessed neuraminidases that were completely resistant to both trypsin and pronase.

Proteolytic enzymes released free neuraminidases from the A 2 and LEE viruses the morphology of which was different from that of neuraminidases released by detergent treatment.

INTRODUCTION

In previous studies (Hoyle 1969*a*, *b*) the resistance of the haemagglutinins and neuraminidases of eight strains of influenza viruses to treatment with chemical reagents acting on amino acids present in the active centres, and to reagents modifying the higher order structure of the virus proteins was described. The present work describes the results of treatment of the same strains with proteolytic enzymes.

MATERIALS AND METHODS

Proteolytic enzymes

Two enzymes have been used, trypsin and pronase.

Trypsin. A 10% solution of commercial trypsin (Difco) was prepared in distilled water adjusted to pH 7·5 by addition of disodium phosphate, centrifuged, and insoluble material discarded. The supernatant fluid was then desalted by

passage through a column of Sephadex G 25 equilibrated to distilled water of pH 7.5. Eluate samples were collected and those containing enzyme were pooled and 0·5 ml. volumes were frozen and stored at −20° C.

Pronase. Commercial pronase (BDH) was used and preparations made in the same way as with trypsin except that the distilled water was of pH 7·0.

Potency test of enzymes. Two ml. of a 1 % solution of egg albumin in distilled water was treated for 10 min. at 37° C. with 0·05 ml of enzyme at pH 7·0 in the case of pronase and at pH 7·5 with trypsin. The mixtures were then precipitated with 5 % trichloroacetic acid and the Folin colour of the supernatants measured. The trypsin preparation produced a Folin colour equivalent to 28 μg. of tyrosine, and the pronase preparation produced the equivalent of 82 μg. of tyrosine. The pronase preparation had therefore about three times the proteolytic activity of the trypsin.

Virus preparations

Virus from infected allantoic fluid was purified by two cycles of adsorption-elution from guinea-pig red cells followed by a cycle of differential centrifugation. The final deposited virus was suspended in phosphate buffered saline of pH 7·0 to a concentration of about 1% by volume. These preparations are referred to as 'virus concentrate'. Although all the preparations contained approximately the same amount of virus the haemagglutinin varied, filamentous strains giving lower titres than more highly egg-adapted spherical strains.

Electron microscopy

Virus preparations to be examined in the electron microscope were centrifuged for 1 hr. at 30,000 ***g*** and the deposit resuspended in a suitable volume of 1 % ammonium acetate. A small amount of this was mixed with an equal volume of 3 % phosphotungstic acid adjusted to pH 6·0. A drop of the mixture was then placed on a 400-mesh carbon-formvar-coated grid and excess fluid withdrawn. After drying the grid was examined immediately in a Phillips EM 300 at a plate magnification of × 60,000.

Technique of the tests

The action of the two enzyme preparations on the eight virus strains was tested under conditions of low ionic strength and for a period of 4 h. at 37 °C.

Virus concentrate (0·3 ml.) was diluted with 5·0 ml of distilled water at pH 7·0 in tests of pronase activity and at pH 7·5 with trypsin; 1·0 ml of the mixture was diluted to 3·0 ml. with phosphate buffered saline of pH 6·5 and retained as an original virus control.

To the remaining 4·3 ml. of the mixture was added 0·3 ml. of enzyme preparation and the mixture was held at 37° C. for 4 hr. It was then diluted to 12·9 ml with buffered saline pH 6·5 and centrifuged at 25,000 ***g*** for 1 hr. The supernatant fluid was removed and the deposited enzyme-treated virus resuspended in 12·9 ml. of buffer. The haemagglutinin titres and neuraminidase activities of the supernatant and resuspended deposit were then compared with those of the original virus.

Haemagglutination tests. These were done by the Salk method using 0·5% guinea-pig red cells.

Neuraminidase activity. An ovomucin substrate was used, prepared by diluting 250 ml. of egg white to 1 l. with distilled water, adjusting the fluid to pH 6·0 with acetic acid, centrifuging and extracting the precipitated ovomucin with 100 ml. of buffered saline of pH 6·5. After centrifugation to remove insoluble material the ovomucin solution was frozen and stored at – 20° C.

In the neuraminidase tests 0·4 ml. of sample was mixed with 0·4 ml. of ovomucin solution and held at 37° C. for 30 min. Released *N*-acetyl-neuraminic acid was then determined by the Aminoff (1961) modification of the Warren technique, the coloured product being extracted with 4·0 ml. of butanol-HCl and the absorptiometer readings at 549 mμ recorded.

Table 1. *Effect of trypsin treatment on influenza viruses*

Virus	Material	Haemagglutinin titre	Neuraminidase activity	Enzyme-resistant neuraminidase
A SWINE	Original virus	8,192	0·15	33%
	Trypsinized virus			
	Supernatant	Nil	0·05	
	Deposit	Nil	Nil	
A PR 8	Original virus	4,096	0·09	78%
	Trypsinized virus			
	Supernatant	32	0·06	
	Deposit	512	0·01	
A DSP	Original virus	16,384	0·20	0%
	Trypsinized virus			
	Supernatant	512	Nil	
	Deposit	25,576	Nil	
A 1 BURCH	Original virus	8,192	0·30	0%
	Trypsinized virus			
	Supernatant	32	Nil	
	Deposit	Nil	Nil	
A 1 BRATISLAVA	Original virus	8,192	0·28	0%
	Trypsinized virus			
	Supernatant	Nil	Nil	
	Deposit	128	Nil	
A 2 TAIWAN	Original virus	8,192	0·35	103%
	Trypsinized virus			
	Supernatant	Nil	0·21	
	Deposit	256	0·15	
A 2 ENGLAND 67	Original virus	2,048	0·44	141%
	Trypsinized virus			
	Supernatant	32	0·37	
	Deposit	1,024	0·25	
B LEE	Original virus	3,072	0·29	110%
	Trypsinized virus			
	Supernatant	128	0·32	
	Deposit	8,192	Nil	

RESULTS

The results of treatment of eight strains of influenza virus with trypsin and pronase are shown in Tables 1 and 2.

Table 2. *Effect of pronase treatment on influenza viruses*

Virus	Material	Haemagglutinin titre	Neuraminidase activity	Enzyme-resistant neuraminidase
A SWINE	Original virus	4,096	0·18	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A PR 8	Original virus	3,072	0·08	12%
	Pronase virus			
	Supernatant	Nil	0·01	
	Deposit	32	Nil	
A DSP	Original virus	16,384	0·27	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	128	Nil	
A 1 BURCH	Original virus	4,096	0·31	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A 1 BRATISLAVA	Original virus	4,096	0·20	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A 2 TAIWAN	Original virus	2,048	0·14	107%
	Pronase virus			
	Supernatant	Nil	0·15	
	Deposit	Nil	Nil	
A 2 ENGLAND 67	Original virus	2,048	0·42	102%
	Pronase virus			
	Supernatant	Nil	0·38	
	Deposit	Nil	0·05	
B LEE	Original virus	2,048	0·32	122%
	Pronase virus			
	Supernatant	Nil	0·37	
	Deposit	Nil	0·02	

Action of proteolytic enzymes on the haemagglutinating activity

The haemagglutinating activity of all the strains was highly susceptible to destruction by pronase, but results with the less powerful trypsin preparation were variable. The haemagglutinating activity of the SWINE and A 1 BURCH strains was destroyed, with PR 8, A 1 BRATISLAVA, and the A 2 strains there was reduction of activity, while the DSP and LEE strains showed a slight increase in titre.

The significance of these results is difficult to interpret as the haemagglutination

test does not measure the amount of haemagglutinin protein present but measures the number of particles carrying more than one haemagglutinating centre. Proteolytic enzymes remove the surface projections from the virus particle but this will only result in a decrease of haemagglutinin titre if almost the whole of the projections are removed. The following experiments illustrate the difficulty.

Electron microscopy of virus treated with trypsin and pronase. Preparations of DSP virus were treated with trypsin at pH 7·5 and with pronase at pH 7·0. After 4 hr. at 37° C. the preparations were centrifuged at 30,000 *g* for 1 hr. and the deposited virus divided into two parts, one being resuspended for measurement of haemagglutinin titre and the other being suspended in 1% ammonium acetate for electron microscopy. Electron photomicrographs of the original virus and the enzyme treated preparations are shown in Pl. 1, figs. 1–3.

The original virus exhibited the 100 Å projections characteristic of influenza-virus (Pl. 1, fig. 1). Particles treated with pronase revealed a smooth membrane with no visible projections (Pl. 1, fig. 2). The overall distinctive shape of the particles was preserved. Virus treated with trypsin showed particles with an intermediate appearance, projections being present but in much smaller numbers than in intact virus (Pl. 1, fig. 3).

The original virus suspension had a haemagglutinin titre of 16,384. No reduction in titre was produced by trypsin treatment but pronase reduced the titre to 128.

Ether treatment of trypsinized virus. Preparations of DSP virus and trypsinized DSP virus were shaken with ether, centrifuged, and the aqueous phases tested for haemagglutinin content with the following result.

	Haemagglutinin titre
Original virus	16,384
Ether-treated virus	32,768
Trypsinized virus	16,384
Ether-treated trypsinized virus	128

Ether treatment of the original virus produced a slight increase in titre. Although the trypsinized virus had an unchanged haemagglutinin titre, on ether treatment the titre was reduced to less than 1%. The result suggests that the number of haemagglutinin sub-units released from the trypsinized virus was much less than from untreated virus, so that only a few multivalent aggregates could be produced.

These experiments show that changes in haemagglutinin titre of virus after trypsin treatment do not accurately reflect the action of the enzyme on the haemagglutinin protein, and cannot be used to differentiate the haemagglutinins of different strains of virus. This difficulty does not occur in studies of the action of proteolytic enzymes on the neuraminidase activity of the viruses as neuraminidase activity is shown by both monovalent and multivalent particles.

Action of proteolytic enzymes on the virus neuraminidases

The results shown in Tables 1 and 2 indicate great differences in resistance of the virus neuraminidases to proteolytic enzymes.

With three strains, the DSP strain of virus A and the two A 1 strains, the neuraminidases are highly sensitive, being totally destroyed by both trypsin and pronase.

Two strains of virus, SWINE and PR 8, show some resistance to trypsin, and the PR 8 strain also shows some resistance to pronase. With these strains a free neuraminidase is released from the virus by trypsin treatment to appear in the supernatant fluid on centrifugation, but the activity of the released enzyme is less than that of the original virus.

The two strains of A 2 virus and the LEE strain of virus B possess neuraminidases which appear to be completely resistant to proteolytic enzymes. Both trypsin and pronase split off a free neuraminidase, pronase producing a more complete release than trypsin. In all cases the total neuraminidase activity after proteolytic enzyme treatment was greater than that of the original virus by amounts varying from 2 to 40 %. This variable increase may possibly be due to exposure of internal neuraminidase as a result of some virus disruption.

Purification and properties of the A 2 and B neuraminidases

The free neuraminidases released from A 2 and B viruses can readily be separated from residual virus and proteolytic enzyme by passage through Sephadex G 200 columns. Fig. 1 shows the result of passing 1 ml of pronase-treated A 2/ ENG/67 virus through a 20 ml column of Sephadex G 200 equilibrated to phosphate buffered saline pH 6·0. The residual virus particles were excluded by the column.

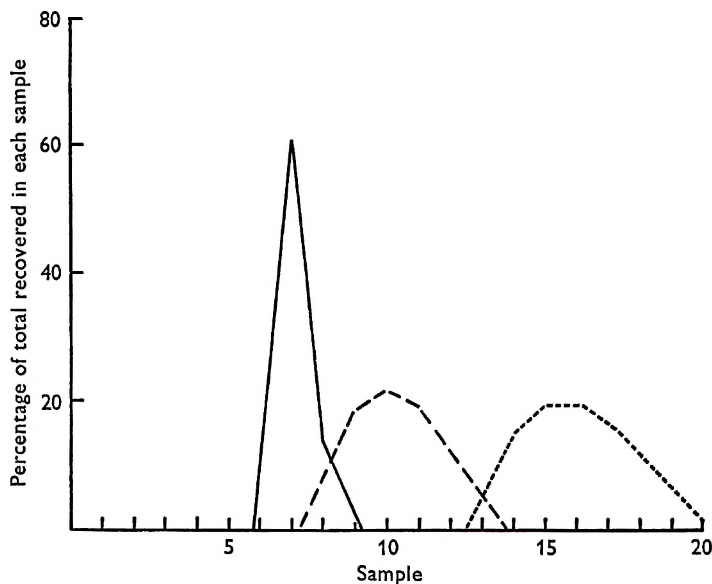


Fig. 1. Filtration of pronase-treated A 2/ENG/67 virus through Sephadex G 200. ———, Residual virus; – – – – – –, neuraminidase; - - - - - -, pronase.

Neuraminidase and pronase were retarded and well separated. Preparations purified in this way retained activity unchanged for up to 3 months when stored at 4° C., but activity was lost in preparations frozen and stored at – 20° C.

Interaction of free neuraminidase and red blood cells. The neuraminidases of both A 2 and B viruses are not readily adsorbed by red cells. Even serial adsorption in the cold with high concentrations of red cells produces only slight reduction in activity. Nevertheless, the enzymes release as much or more N-acetyl neuraminic acid from red cells as does intact virus, and enzyme treated red cells are not agglutinated by virus indicating that haemagglutinin and neuraminidase react with the same substrate.

Morphology of the free neuraminidase. Laver & Valentine (1969) showed that free neuraminidase released from A 2 virus by treatment with sodium dodecyl sulphate had a characteristic morphology, consisting of an oblong head with a thin centrally attached tail. On removal of the detergent these subunits aggregated to give a 'cartwheel' appearance, the tails being linked at the centre while the heads were peripherally distributed (Pl. 1, Fig. 6). The overall diameter of the 'cartwheel' is 330 Å, while the individual heads are approximately 70 Å. in diameter.

The neuraminidase released by proteolytic enzyme treatment has a different appearance (Pl. 1, fig. 4). No polymerized structures are seen and the grid is covered by numerous small fragments giving an appearance that can be described as 'snow'. On close examination there seemed to be two types of fragment. One was a rod-shaped structure 20 Å. in diameter and with a variable length in the range of 70–80 Å. The other form was pleomorphic with dimensions of 50–70 Å. diameter. Several of this group could be resolved as cup-shaped structures resembling the heads of the detergent-produced neuraminidase. Indeed the two structures described here, the rod and the roughly isometric particle, could possibly represent the stem and head of the sub-units forming the structure in Pl. 1, fig. 5. Alternatively it is possible that the rod-shaped structures are incompletely digested haemagglutinin sub-units, and that the neuraminidase is released from the virus particle by proteolytic digestion of the stem.

DISCUSSION

Many studies have been made of the effects of proteolytic enzymes on influenza viruses. Sugg & Cleeland (1962) attempted to relate the trypsin resistance of virus haemagglutinins to the serological character, but in the present study no definite relation was found and it seems probable that variations in resistance are due more to differences in the ease with which the surface projections are split off by trypsin than to differences in susceptibility of the haemagglutinin protein to digestion.

The release of free neuraminidase from virus particles by treatment with proteolytic enzymes has been described by Mayron, Robert, Winzler & Rafaelson (1961) and Noll, Aoyagi & Orlando (1962) using trypsin, by Wilson & Rafaelson (1963) using chymotrypsin, by Reginster (1966*a*) using caseinase C, by Seto, Drzeniek & Rott (1966) and Reginster (1966*b*) using pronase, and by Kendal, Biddle & Belyavin (1968) using nagarse protease. By these methods free neuraminidases

have been obtained from influenza A 2 strains, influenza B, some strains of influenza A, and from fowl plague virus, but not from A 1 strains. Most of these results were obtained by using minimal periods of enzyme treatment.

In the present work prolonged treatment with high concentrations of enzyme has been used and the results indicate the existence of at least two and possibly three types of neuraminidase. The neuraminidases of the A 2 strains and the LEE strain of virus B are completely resistant to the action of proteolytic enzymes. They are also resistant to the action of agents disrupting the higher order structure of protein molecules such as sodium dodecyl sulphate (Laver, 1964) and high concentrations of urea (Hoyle, 1969*b*).

The neuraminidases of the A and A 1 strains which are destroyed by SDS and urea are also sensitive to destruction by proteolytic enzymes, but appear to be divisible into two groups, the A 1 strains and the DSP strain of virus A being highly susceptible while the PR 8 and SWINE strains show some degree of resistance.

The free neuraminidases released from the A2 and LEE strains by proteolytic enzymes do not have the same morphology as those released by detergent treatment. The tails and heads appear to be separated in the enzyme released product and no aggregates of the sub-units are found. It would seem probable that proteolytic enzymes release neuraminidase by a partial attack on the tails, possibly commencing at the ends. The heads appear to be more resistant to digestion.

REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. *Biochemical Journal* **81**, 384–92.

HOYLE, L. (1969*a*). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. I. Effect of reagents reacting with aminoacids in the active centres. *Journal of Hygiene* **67**, 289–99.

HOYLE, L. (1969*b*). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. II. Effects of reagents modifying the higher order structure of the protein molecule. *Journal of Hygiene* **67**, 301–10.

KENDAL, A. P.. BIDDLE, F. & BELYAVIN, G. (1968). Influenza virus neuraminidase and the viral surface. *Biochimica et biophysica acta* **165**, 419–31.

LAVER, W. G. (1964). Structural studies on the protein subunits from three strains of influenza virus. *Journal of Molecular Biology* **9**, 100–124.

LAVER, W. G. & VALENTINE, R. C. (1969). Morphology of the isolated haemagglutinin and neuraminidase subunits of influenza virus. *Virology* **38**, 105–19.

MAYRON, L. W., ROBERT, B., WINZLER, R. J. & RAFAELSON, M. E. (1961). Studies on the neuraminidase of influenza virus. I. Separation and some properties of the enzyme from Asian and PR 8 strains. *Archives of Biochemistry* **92**, 475–83.

NOLL, H., AOYAGI, T. & ORLANDO, J. (1962). The structural relationship of sialidase to the influenza virus surface. *Virology* **18**, 154–7.

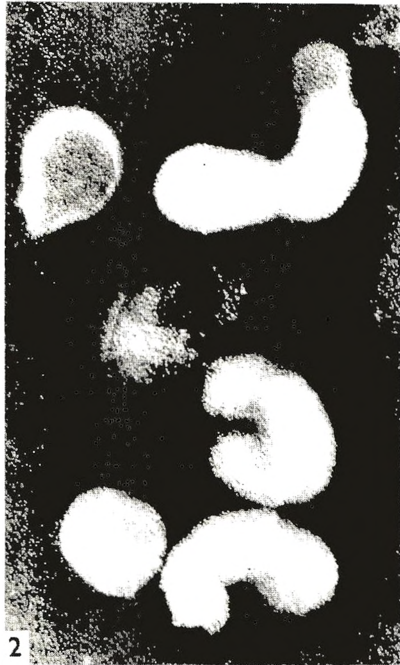
REGINSTER, M. (1966*a*). Release of influenza virus neuraminidase by caseinase C from *Streptomyces albus G. Journal of General Microbiology* **42**, 323–31.

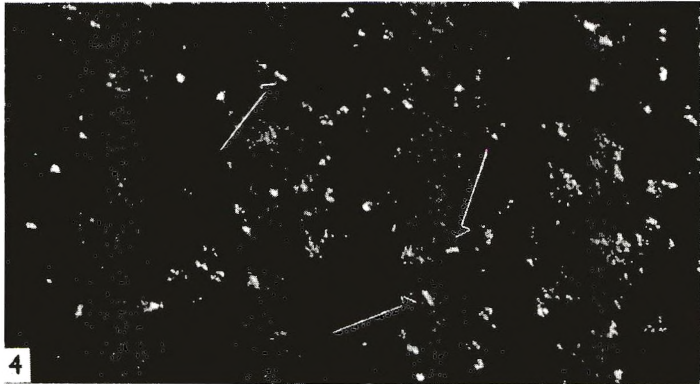
REGINSTER, M. (1966*b*). Effects of pronase on influenza virus. *Acta Virologica* **10**, 111–16.

SETO, J. C., DRZENIEK, R. & ROTT, R. (1966). Isolation of a low molecular weight sialidase (neuraminidase) from influenza virus. *Biochimica et biophysica acta* **113**, 402–4.

SUGG, J. Y. & CLEELAND R. (1962). Differences in trypsin susceptibilty among influenza viruses and relationship of the susceptibility to the antigenic type or subtype of the virus. *Journal of Immunology* **88**, 369–76.

WILSON, V. W. & RAFAELSON, M. E. (1963). Isolation of neuraminidase from influenza virus. *Biochemical Preparations* **10**, 113–17.





L. HOYLE AND JUNE D. ALMEIDA

EXPLANATION OF PLATE

Fig. 1. Untreated D.S.P. virus ×200,000. The particles are covered by projections 100 Å. in length.

Fig. 2. Pronase-treated D.S.P. virus ×200,000. The distinctive shape of the particles is still recognizable but there are no surface projections.

Fig. 3. Trypsin-treated D.S.P. virus ×200,000. The virus shows an appearance intermediate between figs 1 and 2. Projections are present on the surface but in fewer numbers than in untreated virus.

Fig. 4. Free neuraminidase from pronase-treated A 2/ENG/67 virus purified by passage through Sephadex G 200. ×300,000. Most of the particles appear pleomorphic and in the size range 50–70 Å, but a small number (arrows) have a distinct rod shape with a diameter of 20 Å. and variable length around 80 Å.

Fig. 5. Neuraminidase released from A 2/ENG/67 virus by treatment with the detergent Nonidet P 40. Here the individual units are polymerized into a wheel-shaped structure approximately 330 Å. in diameter. ×300,000.

J. Hyg., Camb. (1971), 69, 471
Printed in Great Britain

Vaccination of adults with Wistar RA 27/3 rubella vaccine

BY D. S. FREESTONE, J. PRYDIE, S. G. HAMILTON SMITH
AND G. LAURENCE

Departments of Clinical Immunology and of Virology, the Wellcome Research Laboratories, Beckenham, Kent

(*Received* 15 *March* 1971)

SUMMARY

Thirty-three adults were vaccinated subcutaneously with Wistar RA. 27/3 (live attenuated) rubella vaccine at the Wellcome Research Laboratories, Beckenham. All subjects with pre-vaccination haemagglutinating-inhibiting antibody titres of 1/20 or less and three of seven subjects with pre-vaccination titres of 1/40 showed at least fourfold rises of titre. Reactions encountered were mild and of short duration.

INTRODUCTION

It has been estimated that the extensive rubella epidemic in the United States in 1963–4 resulted in the birth of between 10,000 and 20,000 congenitally abnormal infants, in addition to many abortions and still-births (Cooper & Krugman, 1966). Thus the protection of women of child-bearing age against rubella is important, although the method by which it should be achieved remains in dispute. Vaccination of schoolgirls aged 11–13 years has been advocated but it is not yet clear that rubella vaccines will provide a 30-year duration of immunity and periodic booster doses may be required. Since there is at present no definite evidence to show that any rubella vaccine is not teratogenic if administered in early pregnancy, there is a tendency to avoid the vaccination of adult women.

This report describes the vaccination of 33 adults aged 18–65 years at the Wellcome Research Laboratories, Beckenham, with Wistar RA. 27/3 strain (live attenuated) rubella vaccine. Seronegative subjects and subjects with low antibody titres were included. The attenuation and characteristics of this vaccine have been described in detail elsewhere (Plotkin, Cornfeld & Ingalls, 1965; Plotkin, Farquhar, Katz & Ingalls, 1967). Wistar RA. 27/3 is the only rubella vaccine prepared in WI-38 human diploid cells and the only one which is regularly immunogenic when administered by the intranasal route.

METHOD

Eleven males and 22 females were vaccinated subcutaneously. All the males and 13 of the females were involved in the production and safety testing of rubella vaccine, or required entry into production and testing areas. The remaining nine female subjects, unconnected with vaccine production, took part in the clinical trial as volunteers.

Serology

Rubella haemagglutinating inhibiting (HAI) antibody titrations were carried out according to the method described by Stewart *et al.* (1967). The titration method was modified to utilize manganous chloride and heparin for the removal of non-specific inhibitors (Mann, Rossen, Lehrich & Kasel, 1967; Feldman, 1968; Plotkin, Bechtel & Sedwick, 1968) and pigeon red cells were substituted for day-old chick erythrocytes (Peetermans & Huygelen, 1967). The United States National Reference Serum, with a geometric mean HAI antibody titre of 1/100 following kaolin treatment (E. B. Seligmann, personal communication), in our hands gave a mean titre of 1/235 in 24 estimations. The difference could be accounted for by the different techniques employed to remove inhibitors. The distribution of pre-vaccination HAI antibody titres was as follows: < 1/10 (17), 1/10 (4), 1/20 (4) 1/40 (7), 1/80 (1). Fourfold increases in titre following vaccination have been reported in subjects with pre-vaccination titres up to 1/64 (Dudgeon, Marshall, Peckham & Hawkins, 1969). Since the protection afforded by low antibody concentrations appears uncertain, subjects with low antibody titres were vaccinated.

Safety precautions

A careful explanation was given of the possible reactions following vaccination, risks of vaccination during pregnancy, and upon pregnancy occurring within the following 6–8 weeks. Informed consent was obtained from all subjects. As a security measure latex and tube agglutination pregnancy tests (Prepurex, Prepuerin, Wellcome Reagents Ltd.) were carried out on urine specimens from all women of child-bearing age before vaccination. Subjects were not told that a pregnancy test was being performed unless they enquired, since it was thought undesirable for subjects to be able to place reliance on pregnancy testing if they were uncertain about pregnancy. This test was repeated 8–10 weeks after vaccination.

Vaccine

Two lots of Wistar RA. 27/3 strain live attenuated rubella vaccine were used in this study at dose titres of $10^{2\cdot94}$ and $10^{2\cdot5}$ respectively, as assayed by the indirect method in monkey kidney tissue cultures. Vaccine was produced on WI-38 cell cultures and, after stabilization, was lyophilized. Uninoculated control cells were shown to be of the normal human karyotype, susceptible to viruses and not oncogenic in hamsters. No extraneous agent was detected in the virus harvests when tested in both tissue cultures and in animals.

Reactions

All subjects were seen 3 weeks after vaccination, and if necessary as symptoms occurred. Serial weekly differential white cell and platelet counts were carried out in a group of eight subjects.

RESULTS

Development of HAI antibody

All 25 subjects with pre-vaccination HAI titres of 1/20 or less responded to vaccination with at least fourfold increases in titre and no difference could be determined in the response between subjects with no detectable antibody and those with low titres. Similarly, no differences in antibody responses were evident between subjects given either of the lots of vaccine. Serial weekly HAI antibody

Table 1. *Development of rubella HAI antibody after subcutaneous vaccination with Wistar RA*. 27/3 *rubella vaccine* (*pre-vaccination titres* < 10, 10, 20-*reciprocals*)

	Days after vaccination							
Study group	10	14	21	28	35	42	90	180
No. of subjects with some increase in antibody titre/no. examined	1/4	5/8	7/7	8/8	—	—	—	—
No. of subjects with $\geqslant$ 4-fold increase in antibody titre/no. examined	—/—	1/8	6/7	8/8	4/4	8/8	—	—
G.M.T.	—	23	126	174	269	247	247	—
Median titre	—	20	160	160	160/320	160/320	320	—
Late responder–prevaccination titre < 1/10	20	10	10	10	< 10	40	80	—
Total group								
G.M.T.	—	—	91	—	—	159	124	119
Median titre	—	—	80	—	—	160	160	80
Modal titre	—	—	80/160	—	—	160	160	80
No. of subjects in total group	—	—	14	—	—	25	17	7

G.M.T. = geometric mean titre.

titrations in a group of nine subjects (study group, Table 1) showed that antibody almost always first developed between the 10th and 21st days after vaccination, but increases in antibody titre occurred up to the 35th day after vaccination. The results of antibody development in one additional subject whose response to vaccine was markedly delayed are in complete contrast to the foregoing. This subject, who has no history of rubella or maternal contact with rubella, suffers no general medical disease and experienced no reaction to vaccination. The development of rubella specific immunoglobulins in this subject is being compared with those of other vaccinees and will form the basis of a separate report.

In three of seven subjects with pre-vaccination titres of 1/40 a fourfold increase was seen. One of these exhibited clear symptoms of arthralgia of 3 days' duration, starting on the 18th post-vaccination day, while another developed vague pain in the limbs on the 7th post-vaccination day, which persisted for 2 weeks. In one subject with a pre-vaccination titre of 1/80 no change in titre and no reactions were seen.

Reactions

Reactions after vaccination are shown in Table 2. Of the five subjects with pre-vaccination titres of 1/40 or 1/80 who did not show a four-fold increase in titres, only one reported any reaction to vaccination. This subject recorded a mild sore throat on the 3rd and 10th post-vaccination days.

Table 2. *Reactions to vaccination of* 33 *subjects with RA.* 27/3 *rubella vaccine* (*subcutaneous administration*)

Reactions	Non-immune (Total = 28)	Immune (Total = 5)
No reactions	10	4
Post-vaccination:		
Sore throat	7	1
Occurring during post-vaccination days:		
1–7	2	—
7–16	4	—
Both	1	1
Fever	2	—
Rash	2	—
Lymphadenopathy	4	—
Cough and coryza	5	—
Local discomfort at injection site	3	—
Myalgia	3	—
Joint involvement	7	—

Table 3. *Joint involvement after vaccination*

Total number of subjects = 7		Joints involved		
Subjective symptoms	Subjective and objective symptoms	Small joints of hands and feet	Knee joint	General
4	3	2	4	1

Subject/involvement	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	Days after vaccination													
A/bilaterial effusions to knees	.	×	×	×	×	×	×	×	×	×	×	×	×	.
B/pains in thighs/knees	.	.	×	×	×	.	.	.	.	.	.	.	×	.
C/generalized joint pain	.	.	×	×	.	.	.	.	.	×	×	×	×	.
D/pain in knee	.	.	.	×	×	×	×	.	.	.	.	.	.	.
E/pain in hands	.	.	.	.	.	.	.	.	.	.	×	×	.	.
F/pain in wrists	.	.	.	.	.	.	.	.	.	.	×	×	×	.
G/pain in knees	.	.	.	.	.	.	.	.	.	×	×	×	×	.

None of the 28 susceptible subjects who were vaccinated experienced a severe or incapacitating reaction. The reactions that did occur were similar in type to those reported following rubella, but were of short duration and mild in nature.

Sore throat occurred in two phases: the first centred on the 3rd post-vaccination day, and the second on the 10–16th post-vaccination days. The incidence of

lymphadenopathy in this study was low. This is not unexpected since assessments were made from subjective reports rather than frequent clinical examination of those vaccinated. Joint symptoms developed in 7 (25 %) subjects, in 3 of whom there was an effusion into a knee joint. The small joints of hands or feet were involved in 2 subjects, the knee joints in 4, while 1 subject suffered generalized joint pain. Joint symptoms were again reported in two phases between the 9th and 14th days, and between the 17th and 20th days post-vaccination. Symptoms were usually limited to a few days' duration (Table 3). Serial weekly differential

Table 4. *Report of serial platelet counts in eight subjects vaccinated with Wistar RA. 27/3 rubella vaccine subcutaneously*

	Platelet counts in 7 subjects responding serologically to vaccine			Plate count in 1 immune vaccinated subject	
	Mean	Range	change (%)	Count	change (%)
Before vaccination	180,300	150,000–234,000	—	*	*
After vaccination					
7 days	142,857	109,000–160,000	−21·1 %	188,000	—
14 days	152,286	119,000–176,000	−15·6 %	187,000	−0·5 %
21 days	190,556	119,000–305,000	+6·7 %	182,000	−2·7 %
28 days	183,143	149,000–238,000	+1·7 %	183,000	−2·6 %
35 days	177,433	127,000–205,000	−1·7 %	174,000	−7·4 %
42 days	147,571	119,000–175,000	−18·3 %	163,000	−13·3 %

* No result obtained.

white cell counts showed no abnormalities. However, certain trends are visible in the weekly platelet counts which were carried out in a small group of subjects. These data require amplification, since relatively small numbers of subjects were involved. Nevertheless, sharp falls in platelet counts occurred between the 7th and 14th days after vaccination, followed by some compensatory increase in counts in the 3rd post-vaccination week. (Table 4).

Oski & Naiman (1966) measured platelet counts for 35 days after administration of live measles vaccine, and found a maximum fall 3–7 days after vaccination, which was attributed to decreased platelet production. This finding is similar to the early fall in platelet count in our subjects. However, thrombocytopenia as a complication of natural rubella and measles is seen shortly after the appearance of the rash, and is believed to result from increased platelet destruction. We found a further fall in platelet count 8 weeks after vaccination, but this is later than might be expected, and its significance is therefore not clear.

DISCUSSION

Vaccination of adults with subcutaneously administered Wistar RA 27/3 (live attenuated) rubella vaccine is clearly effective in stimulating circulating antibody production in adult men and women. No difficulty was found in avoiding the vaccination of a pregnant woman in this small group of subjects where a

careful explanation of the hazards of vaccination in pregnancy was given and a pre-vaccination pregnancy test was also performed. Nevertheless it is clear that the potential teratogenicity of vaccine is likely to cause difficulties in vaccination of larger groups of women of child-bearing age in different communities. The administration of oral contraceptives to such women may provide an additional safeguard.

Since three of seven of our subjects with pre-vaccination HAI antibody titres of 1/40 developed fourfold increases in titre after vaccination, it is clear that vaccination of such susceptible, but seropositive subjects, might place them at risk if they were pregnant. More information is needed to determine the clinical handling of these subjects who are not immune to subcutaneously administered vaccine, but who might be immune to natural infection.

The differing techniques used in the removal of non-specific inhibitors and the varying sensitivities of the technique in different laboratories are clearly of critical importance to the handling of subjects with low titres and there is much to commend the interpretation of such titres against a national or international standard rubella reference serum.

The subjective recording of reactions to vaccination on a daily basis tends to accentuate the apparent reactivity. With the exception of joint involvement, reactions to vaccination are unlikely to be of clinical significance. Joint involvement occurred less frequently than was reported after administration of HPV-77 vaccine (Cooper *et al.* 1969), and it was usually of short duration.

We are grateful to staff of the Wellcome Research Laboratories for their co-operation in these studies, to Dr A. H. Griffith for advice and encouragement, to Dr B. A. L. Hurn for arranging the pregnancy tests, to Mr A. F. J. Fox for haematological studies, and to Miss B. Totterdell, Mrs L. Manchester, and Miss C. Barry for technical assistance in antibody titrations.

REFERENCES

COOPER, L. A. & KRUGMAN, S. (1966). Diagnosis and management in congenital rubella *Pediatrics* **37**, 335.

COOPER, L. S., ZIRING, P. R., WEISS, J., MATTERS, B. A. & KRUGMAN, S. (1969). Transient arthritis after rubella vaccination. *American Journal of Diseases of Children* **118**, 218.

DUDGEON, J. A., MARSHALL, W .C., PECKHAM, C. S. & HAWKINS, G. T. (1969). Clinical and laboratory studies with rubella vaccines in adults. *British Medical Journal* i, 271.

FELDMAN, H. A. (1968). Removal by heparin-$MnCl_2$ of non-specific rubella haemagglutinin serum inhibitor. *Proceedings of the Society for Experimental Biology and Medicine* **127**, 570.

MANN, J. J., ROSSEN, R. D., LEHRICH, J. R. & KASEL, J. A. (1967). The effect of kaolin on immunoglobulins: an improved technique to remove the non-specific serum inhibitor of reovirus haemagglutination. *Journal of Immunology* **98**, 1136.

OSKI, F. A. & NAIMAN, J. L. (1966). Effect of live measles vaccine on the platelet count. *New England Journal of Medicine* **275**, 352.

PEETERMANS, J. & HUYGELEN, C. (1967). L'emploi d'hérmaties de pigeons dans le test d'inhibition de l'hémagglutination de la rubéole. *Presse Médicale* **75**, 2177.

PLOTKIN, S. A., CORNFELD, D. & INGALLS, T. H. (1965). Studies of immunization with living rubella virus. *American Journal of Diseases of Children* **110**, 381.

PLOTKIN, S. A., FARQUHAR, J., KATZ, M. & INGALLS, T. H. (1967). A new attenuated rubella virus grown in human fibroblasts: evidence for reduced nasopharyngeal excretions. *American Journal of Epidemiology* **86**, 468.

PLOTKIN, S. A., BECHTEL, D. J. & SEDWICK, W. D. (1968). A simple method for removal of rubella haemagglutination inhibitors from serum, adaptable to finger-tip blood. *American Journal of Epidemiology* **88**, 301.

STEWART, G. L., PARKMAN, P. D., MOPPS, M. E., DOUGLAS, R. D. HAMILTON, J. P. & MEYER, H. M. (1967). Rubella virus haemagglutination inhibition test. *New England Journal of Medicine* **276**, 554.

J. Hyg., Camb. (1971), 69, 479
Printed in Great Britain

Field trials of an inactivated oil-adjuvant vaccine against louping-ill (Arbovirus group B)

By J. G. BROTHERSTON
Moredun Institute, Edinburgh

C. C. BANNATYNE, A. O. MATHIESON AND T. B. NICOLSON
Scottish Veterinary Investigation Service

(*Received* 22 *March* 1971)

SUMMARY

A single dose of inactivated louping-ill oil-adjuvant vaccine elicited a serologically detectable immune response in sheep lasting for at least 1 year. These sheep when exposed to a natural focus of louping-ill virus were completely protected from clinical disease and 1 year after vaccination were able to pass on a substantial maternal immunity to their lambs.

Twenty-nine per cent of unvaccinated sheep, exposed at the same time, died from clinical louping-ill; half of the survivors showed positive sero-conversion and became immune, while the other half remained susceptible. The incidence of fatal encephalomyelitis in sheep which were known to have circulated virus exceeded 50 % in 2 out of 3 trials conducted simultaneously in different locations in Scotland in 1969.

INTRODUCTION

Severe clinical louping-ill mainly affects 1-year-old sheep when they are returned to tick-infested pastures in the spring after wintering on tick-free ground. By this time any passive immunity which they may have acquired from their dams has waned. Young lambs born on hill areas are also at great risk, if their dams are not actively immune, because the initial protection of lambs depends entirely upon passive maternal immunity acquired from the colostrum within the first 36 hr. of birth (Brambell, 1958).

Until 1967 a formalin-inactivated louping-ill vaccine was available to protect sheep, but because of danger to laboratory staff engaged in production the manufacture was discontinued. Until an acceptable alternative was found, the main method of combating tick-borne encephalomyelitis (TBE) was no longer available to those engaged in hill farming in areas where natural foci of louping-ill virus existed in Britain.

Although in the past the vaccine had apparently successfully reduced the incidence of clinical louping-ill in hill sheep (Gordon, Brownlee, Wilson & Macleod, 1962) its mode of action presented some unique features. A single dose did not induce a serological response and did not protect the majority of animals from the circulation of virus following challenge (Williams & Thorburn, 1961). However,

when studying the persistence of antibodies to louping-ill, O'Reilly *et al.* (1968) observed that although ewes did not respond serologically within 21 days of vaccination, when subsequently challenged with virus, they produced haemagglutinin-inhibiting (HI) and neutralizing antibodies in their sera and maternal immunity was transferred to their lambs. During studies on the epidemiology of louping-ill the view was expressed by Smith *et al.* (1964) that the use of a vaccine which prevented systemic infection with louping-ill virus in ewes but did not of itself induce maternal antibody might aggravate the disease incidence in young lambs exposed to consistently high foci of infection. Smith (1969) suggested that the vaccine may have been effective because it was poorly immunogenic; it gave a measure of protection from encephalomyelitis but did not prevent a systemic infection with virus which, if experienced within the first 2 years after a sensitizing dose of vaccine, enabled ewes to acquire active immunity and thereafter transmit maternal immunity. Clearly, under these circumstances the old method of protection which had apparently acted successfully for 30 years would indeed require replacement by a very good vaccine.

A new process of preparing an inactivated antigen from tissue cultures infected with the virus of louping-ill and investigations of the immunogenic properties have recently been described by Brotherston & Boyce (1969, 1970). The antigen, when emulsified in mineral oil, constituted a potent vaccine, and one dose gave a response measurable by HI and neutralizing antibodies within 10 days of subcutaneous injection. This response was accompanied by a lasting protection against challenge with virulent louping-ill virus and sheep which had never been exposed to infection passed maternal antibodies to their lambs. This paper describes trials to study the ability of the vaccine to protect sheep introduced to tick-infected pastures and the lambs born subsequently to some of them.

MATERIALS AND METHODS

It was decided to introduce small groups of unacclimatized sheep, some of which were vaccinated with oil-adjuvant vaccine, to farms where louping-ill was reported to be endemic, rather than to use indigenous stock. Unacclimatized sheep were defined as those under 1 year old, moved to hill pastures on which they had no previous experience of the grazing limits and where they would experience all the hazards of a new environment for the first time, including tick infestation. In this way the history, progress and serological status of the test animals was well documented, adequate controls were established and there was minimum disturbance to the sheep stocks on the farms selected for the experiments. Under these circumstances the test of the capability of the vaccine was severe, but was based upon calculations obtained from trials conducted previously at Moredun Institute.

Sheep

Eighty-seven Blackfaced ewe hoggs 10 months old were purchased in March 1969 from a farm on the lower slopes of the Pentland Hills, which are tick-free; serological tests indicated that they had no previous antigenic experience of loup-

ing-ill virus. They were individually numbered with ear tags and divided into 3 groups of 28 animals, of which 16 were given louping-ill vaccine and 12 were left unvaccinated. Three vaccinated animals were retained at Moredun Institute to monitor their immunity on tick-free ground during the ensuing months. The sheep were kept at pasture until 16 April 1969, when they were distributed to a farm in Selkirkshire, one in Perthshire and one in Argyllshire, selected as likely to be infested with ticks carrying louping-ill virus. Fourteen days before this they were dipped, dosed with an anthelmentic and given a booster dose of anti-clostridial vaccine. On the farms they were introduced to hill pastures at the same time as the ewe hoggs returned from wintering. Ten Blackface rams on the Argyllshire farm considered to have acquired natural immunity were made available to observe the effect of vaccination on indigenous stock.

Louping-ill vaccine, serological tests, virus isolation and criteria used to evaluate immunity

Louping-ill oil-adjuvant vaccine, type E, batch no. SK4, was injected subcutaneously just above the point of the brisket in a quantity of 0·5 ml. A local reaction developed in about 10 % of the animals but subsided in 14 days to a stage where it was difficult to detect by palpation. The vaccine had previously been tested in sheep and elicited an HI antibody response at a serum dilution of 1/160 and a neutralization index of $\log_{10}$ 2·6, 10 days after injection, when given in a dose of 0·25 ml. (half of that used in the trial). Haemagglutinin-inhibition (HI), neutralization tests and the preparation of vaccine were conducted as described by Brotherston & Boyce (1970). Sheep with an HI antibody titre of 1/10 or greater were shown to have significant virus-neutralizing properties in the serum and positive tests are detectable for at least 2 years after successful vaccination.

The technique of virus isolation from suspected tissues has been described by Doherty (1969) and the identification of virus by plaquing techniques by Reid & Doherty (1971). The neuropathological criteria of louping-ill encephalomyelitis were those of Doherty & Reid (1971). The protection afforded by the vaccine and the incidence of natural infection were estimated from the antibody (HI) response of the vaccinated and unvaccinated animals and by recovery of virus or the presence of neuropathological evidence of infection in animals which became ill or died between March and October 1969.

Supervision of the sheep

Arrangements were made with the shepherds to look over the sheep regularly on their rounds and to report unusual events to the Veterinary Investigation Laboratory. In addition the farms were visited each month and all of the animals were blood-sampled three times between May and October, when the survivors were returned to Moredun Institute. At the time of the first blood test smears were also taken to identify those which had become infected with tick-born fever (Foggie, 1951).

When an animal was found sick it was removed to the Veterinary Investigation Laboratory to establish a diagnosis and in addition serum and brain samples were dispatched to Moredun Institute to test for evidence of infection with louping-ill

virus. Dead animals were similarly treated with all dispatch. The Veterinary Investigation Officers were not aware of the vaccine category of the individual animals until after all the diagnostic criteria had been completed and the various reports collated.

Vaccination of animals recovered from natural infection

The identity was noted of a group of ten rams exposed to tick-infested pastures in 1968 on the farm in Argyllshire and likely to have recovered from infection. They were bled to establish their serological status in May 1969, and some of them were vaccinated.

Maternal immunity from vaccinated animals

Twenty of the animals on the farm in Selkirkshire in October 1969 were left to run with the homebred stock of in-lamb ewes. Those unvaccinated at the beginning of the trial, whether they showed serological evidence of recovery from natural infection with louping-ill or not, were vaccinated with a dose of the same vaccine, batch SK4, which had been stored at 4° C. They were tested in May 1970 and the lambs were examined for evidence of maternal antibodies in their sera within 14 days of birth.

RESULTS

All the vaccinated animals had an adequate immunity, as judged by the HI test, before being transferred to the selected hill farms, and the titres of individual animals ranged from 1/10 to 1/640, with a mean of over 1/100. Three weeks after arrival at the farms most of the animals had settled to a regular grazing routine and it was established that the sheep in Selkirkshire and Argyllshire had become infected with tick-borne fever.

Selkirkshire farm

Three or four sheep consistently wandered apart from the flock because the spring of 1969 was generally late and the grazing in Selkirkshire was particularly poor, snow being on the hills until the end of April. The occurrence of clinical disease is shown in Table 1.

Three sheep were observed to become ill and two of them, both vaccinated (3K58 and 3K81), died or were killed for examination within the first 16 days. Both had severe pneumonia and septicaemia, and *Pasteurella haemolytica* type T was recovered from the lungs, livers and spleens. The third animal (4K25) was unvaccinated and found dead on the 15th day, but no definite diagnosis could be established. In all three there was no evidence of infection with louping-ill. One other vaccinated animal (4K87) died on the 22nd day, and although no cause could be defined, once again no evidence of louping-ill was detected by virus isolation procedures or neuropathology.

The first death attributable to louping-ill in unvaccinated sheep occurred in 4K17 on the 18th day. Three others died on the 19th, 22nd and 39th day and in every case virus was isolated from the brains and neuropathological lesions were

present. At the end of the trial period 4 of the 12 unvaccinated sheep had died with louping-ill and 1 from unknown causes, 3 showed serological conversion and had acquired natural immunity, while 4 remained susceptible. Thirteen of the original 16 vaccinated animals remained healthy.

Table 1. *Occurrence of clinical disease after introduction of sheep in Selkirkshire*

Sheep no.	Treat-ment	Days after intro-duction	Diagnosis: Neuro-path.	Diagnosis: Virus isolation	Cause of death
3K58	V	14	–	–	*Pasteurella* septicaemia
4K25	C	15	–	–	Not established
3K81	V	16	–	–	*Pasteurella* septicaemia
4K17	C	18	+	+	Louping-ill
4K24	C	19	+	+	Louping-ill
4K87	V	22	–	–	Not established
4K18	C	22	+	+	Louping-ill
4K11	C	39	+	+	Louping-ill

V = vaccinated.
C = unvaccinated control.
– = no lesions or virus isolation.

Perthshire farm

All 28 animals remained healthy and serum samples and smears showed no evidence of virus circulation or infection with tick-borne fever. They were all withdrawn in October, 6½ months after their introduction, except one vaccinated animal which had lost its identity tag and was recovered later. The HI tests of the vaccinated sheep, however, served as very useful controls in comparison with the serological status of vaccinated animals on the other farms, some of which had been exposed to challenge with virus.

Argyllshire farm

After the introduction of the animals the sequence of events was as follows. In contrast to central and southern Scotland, it was a mild spring in the west and the sheep quickly acclimatized. They were examined early in May and were found to be heavily infested with ticks. Blood smears indicated that the animals had been infected with tick-borne fever, but at this time there was no serological evidence in the unvaccinated animals of infection with louping-ill.

The first case of louping-ill was diagnosed in an unvaccinated sheep, which died on 23 May, 40 days after introduction. Five months later, in September, a second control animal died from louping-ill after 160 days at pasture and two others showed serological evidence of infection. The animals were returned in October and on arrival at the Institute a fifth unvaccinated animal was found to be ill. After 5 days with severe posterior paralysis it was killed and neuropathological examination of the brain and spinal cord showed extensive non-suppurative encephalomyelitis characteristic of louping-ill. The HI titre was 1/1280, and blood

values for calcium and magnesium were within the normal range, but virus was not isolated from the brain. A sixth animal, also unvaccinated, had an HI titre of 1/640 but appeared normal. Virus circulation had occurred in 6 of 12 unvaccinated sheep in this group, 3 of which died of louping-ill, 3 became naturally immune and 6 remained susceptible. The 16 vaccinated animals remained healthy throughout.

Combined results

The final assessment of the consolidated results of the trials on all three farms are shown in Tables 2 and 3. Of the 48 vaccinated sheep, three died from causes other than louping-ill and all of these were from the same farm in Selkirkshire

Table 2. *Unacclimatized BF ewe hoggs turned out between April and October in three hill areas in Scotland*

Area	Vaccinated		Unvaccinated	
	Died louping-ill	Total died	Died louping-ill	Total died
Selkirkshire	0/16	3/16*	4/12	5/12†
Perthshire	0/16	0/16	0/12	0/12
Argyllshire	0/16	0/16	3/12	3/12
	0/48	3/48*	7/36	8/36†

* Two died of Pasteurellois, one of unknown causes.
† One died of unknown causes.

Table 3. *Unvaccinated sheep exposed to foci of louping-ill virus*

	No. exposed	No. circulating virus	No. died of louping-ill	No. immune	No. susceptible
Argyllshire	12	6	3	3	6
Selkirkshire	12*	7	4	3	4
	24*	13	7	6	10

* One died of unknown causes.

where the environmental conditions were very severe. However, only the sheep on two of the farms were subsequently shown to have been exposed to natural foci of infection. A histogram of the distribution of antibody titres at the time of vaccination and 4–5 months later is shown in Fig. 1. From this it is apparent that in each group there were more animals with HI titres of 1/160 or greater at the end of 5 months, indicating that sero-conversion continued in individual animals for many weeks after vaccination. This was also the trend in the three sheep retained on tick-free pastures at Moredun Institute.

Of the 36 unvaccinated sheep, 8 died and in 7 of them louping-ill was diagnosed either by recovery of virus or by the presence of neuropathological lesions in the brain, of a severity sufficient to cause death from acute viral encephalitis. However, no deaths occurred on the Perthshire farm and as there was no indication in the

unvaccinated sheep of rise of antibody titre it is evident that only 32 vaccinated and 24 of the unvaccinated sheep in two of the groups were actually exposed to the risk of natural infection. As indicated in Table 3, 7 unvaccinated sheep died of clinical louping-ill, 6 others showed rise of antibody titre and were presumed to be naturally immune, while 10 had no HI antibodies, indicating that they had not encountered infected ticks although exposed to the same risks as their companions. Only 13 of 24 unvaccinated sheep were therefore shown to have circulated louping-ill virus after exposure to natural foci of louping-ill.

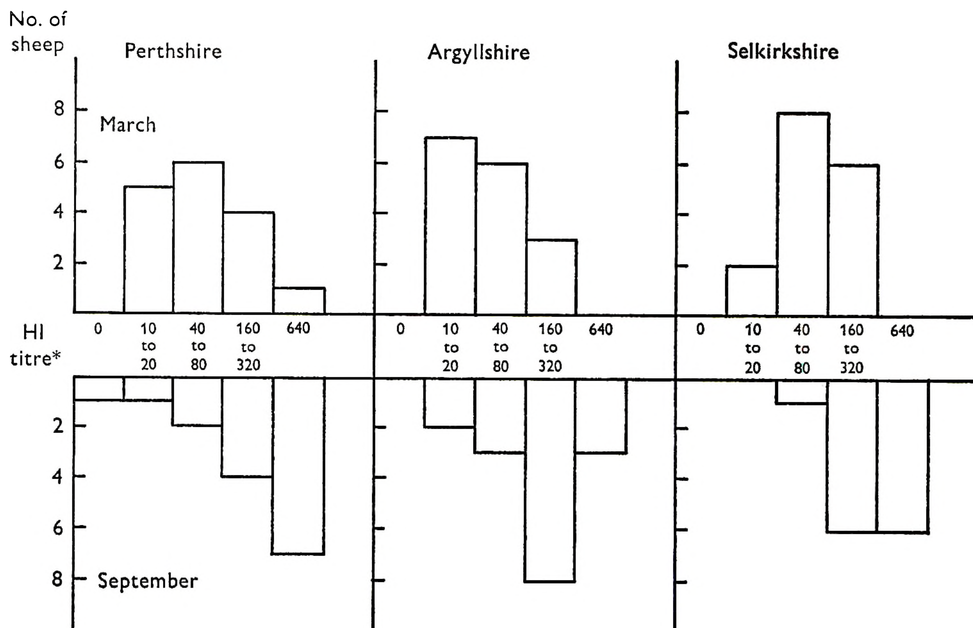


Fig. 1. Distribution of HI titres at vaccination and after 4–5 months.
* Reciprocal serum dilution.

Vaccination of animals recovered from natural infection

On the Argyllshire farm, four of the rams vaccinated in May and one which had not been vaccinated were made available for a blood test in September. In May their HI titres were 1/320, 1/320, 1/40, 1/160 and 1/160; on retest the respective titres were 1/20,480, 1/5120, 1/5120, 1/1280 and 1/160. Vaccination of naturally immune animals had apparently raised the HI titres to a remarkable degree. The last animal, which had not been vaccinated, maintained the same titre throughout the period of 5 months.

Maternal immunity transferred from ewe hoggs vaccinated in March 1969

In Table 4 are recorded the HI titres of the sheep surviving on the Selkirkshire farm and left there until they produced lambs. All of the animals vaccinated in March 1969 still had good immunity when tested 6 months later and again in May 1970 after lambing. Although the tests were conducted at an interval of 6 months

the HI titres of individual animals were in reasonable agreement except that 3K87 and 4K86 showed an 8- to 16-fold rise during the interim.

Of the seven surviving unvaccinated controls, four had no serological evidence of immunity in September and were presumed to be susceptible and three had titres of 1/40, which was judged to be the result of recovery from louping-ill. They

Table 4. *HI titres of ewes and lambs tested in September* 1969 *and May* 1970

Sheep no.	HI titre dams		Lambs, May 1970
	Sept. 1969	May 1970	
3K59	40	80	*
3K80	160	320	320
3K82	80	80	160
3K83	160	320	640
3K84	320	320	320
3K85	160	80	160
3K86	160	320	160
3K87	40	640	160
3K88	80	80	160
3K89	20	20	20
4K84	40	20	40
4K85	40	40	20
4K86	160	1280	160
	Vaccinated Oct. 1969		
4K10	0	160	640
4K12	0	80	320
4K13†	40	640	1280, 1280 ‡
4K14	0	1280	—
4K15†	40	> 2560	2560
4K16†	40	> 2560	2560
4K19	0	160	2560, 2560 ‡

* No lamb.
† Immune from natural infection.
‡ Twins.

were all vaccinated and when tested again in the following spring all had responded. Numbers 4K13, 4K15 and 4K16 had unusually high titres consistent with their previous experience of natural infection before vaccination, but 4K14 was higher than the average to be expected in an animal experiencing antigen for the first time.

Two of the 20 sheep did not produce a lamb, 2 had twins and all of the 20 lambs born had very good maternal immunity as judged by HI serum titres when tested between birth and 14 days of age (Table 4).

DISCUSSION

The results of the field trials show that a single dose of inactivated oil-adjuvant vaccine was able to elicit a serologically detectable immune response in sheep, and vaccinated animals apparently acquired complete immunity to louping-ill.

In contrast, 7 out of 24 (29%) of the unvaccinated controls known to have been exposed to a natural virus focus died with clinical and pathological evidence of louping-ill. In addition, vaccinated sheep maintained on one farm for a second season were able to pass on a very substantial maternal immunity to their lambs. Although the number of sheep was small, each trial was well controlled. Very similar results were obtained in those trials conducted simultaneously in the south and in the west of Scotland where the climatic and environmental conditions were very different and where both groups were shown to have been exposed to infection with tick-borne fever within the first few weeks of arrival. In southern Scotland the exposure to TBF and louping-ill was simultaneous, but in the west exposure to louping-ill was delayed in most of the animals until 3 months after they were introduced, and this raises some speculation about the alleged stressing effect of TBF in favouring the invasion of the central nervous system by louping-ill virus. Possibly the TBF strain was more virulent in the south, and it may be that infection with louping-ill can potentiate pre-existing infection with TBF, leading to further lowering of resistance (Macleod & Gordon, 1932; Gordon *et al.* 1962).

The serological status of the vaccinated sheep (Fig. 1) provided no evidence, from a study of individual or average antibody titres, that exposure to natural foci of virus infection between March and October was reponsible for the increased distribution of higher HI antibody titres towards the end of the period of exposure. The antibody titres of the sheep on the Perthshire farm and the three animals retained at Moredun, which were not exposed, showed a similar trend. Why sero-conversion increased in the animals in this trial for many weeks after vaccination is not immediately apparent, but it is a feature of the immunity induced by oil-adjuvant louping-ill vaccine which we have observed in earlier experiments. It may be due to the repeated stimulation of the antibody-forming mechanism by excess antigen. Brotherston & Boyce (1970) challenged ten sheep 2 months after vaccination with 10^4 ID50 of virulent virus and failed to show any increase in HI titre or neutralization index to indicate sufficient multiplication of virus to cause an anamnestic response.

The lambs born to the vaccinated sheep left on the Selkirkshire farm all showed evidence of a considerable maternal immunity and, as recorded in Table 4, all the HI titres except three were 1/160 or greater. Although it cannot be claimed that vaccination of the ewes was solely responsible for the efficient transfer of maternal antibodies after 1 year, previous experience (Brotherston & Boyce, 1970) would suggest that this was so in at least a proportion of the sheep. There was a remarkable correlation between the HI antibody detectable in the individual ewe and her lamb and this was particularly evident in 4K13, 4K15 and 4K16, where the serological responses of both were greatly enhanced.

It is recognized, however, that individual sheep vary in their immunological competence to react to an antigenic stimulus and we have found a small proportion of sheep in which HI titres reach a maximum of 1/20 after vaccination and in which the decay of antibody is faster than that of the majority which reach titres of 1/160 or greater. In Table 4 sheep 3K87 and 4K86 are recorded as showing a 16-fold and an 8-fold increase in the titre between tests conducted in September

1969 and May 1970 when compared with those of their fellows. It is suggested that in the intervening period of over a year since first vaccination the immunity in these individuals declined to a point where challenge by virus-infected ticks in the spring of 1970 was responsible for a boost to their immunity. Whether this was accompanied by virus multiplication or was an accelerated response due to previous sensitization is not known at present. Sheep 4K13, 4K15 and 4K16 also showed a greatly enhanced serological response, and this was most likely due to vaccination of animals sensitized by previous challenge rather than exposure to louping-ill virus after vaccination, although the latter cannot be ruled out entirely. However, similar results were also obtained in the immune rams vaccinated on the farm in Argyllshire and this tends to confirm the view that vaccination enhances the serological response induced by natural infection.

Epidemiological features. According to the results shown in Table 3, 13 of 24 sheep exposed to known foci of louping-ill infection circulated virus, and of these about half developed fatal encephalomyelitis and half became immune. The real incidence of clinical disease in sheep which were actually challenged was 7 out of 13, or over 50 %, but it is equally important to remember that about 40 % of the unvaccinated sheep never encountered infected ticks, so that it is reasonable to assume that this also holds good for all the animals exposed to natural foci of infection in these trials.

The idea that vaccine of low immunogenicity, particularly an inactivated one, may be used to control louping-ill by permitting systemic infection but not encephalitis is a novel one, and a similar suggestion was made by Mayer, Blaškovič, Ernek & Libíková (1969), who used a highly attenuated TBE virus before exposing sheep to contact with virulent virus circulating in nature. They suggested that the immunity conferred by immunization may be maintained or even increased by small booster doses of virus transmitted by the ticks and the concept undoubtedly worked well when all animals were subjected to repeated antigenic stimulus.

On the pastures in Argyllshire the autumn-feeding population contained the highest proportion of infected ticks because, despite a heavy spring infestation, only one sheep circulated virus compared with five which became infected 5 months later, but this situation was reversed in the trial in the south of Scotland. Clearly exposure of animals for one season to tick-infected pastures is no guarantee that all of the survivors and their offspring will be immune to infection in succeeding years or that natural foci of virus can be relied upon to regularly maintain or boost a waning immunity to louping-ill as it occurs in Britain.

More information is required about the distribution and characteristics of louping-ill virus transmitted by ticks and why some sheep develop encephalomyelitis and others become immune after systemic infection.

The use of attenuated virus as an immunizing agent poses problems and safeguards of a different nature to those encountered with an inactivated agent and the inactivated vaccine described here offers many advantages for combating infection with louping-ill virus. A single dose provides adequate protection for sheep and their lambs for at least 1 year and very probably for 2 or 3 years (Brotherston, Boyce & Renwick, unpublished) and the preparation, storage and

injection of vaccine are relatively simple. Further experience may reveal that it is possible to incorporate the antigen into other types of vaccine used by sheep farmers routinely each year. This would ensure maximum protection in breeding stock and lambs in the spring and it is likely that the residual maternal immunity would be sufficient to protect lambs from a late focus of infection occurring in autumn.

The regular use of this prophylactic measure may provide the means not only of protecting from clinical disease but also of reducing the transfer of virus from parasitized domestic animals to succeeding stages and generations of *Ixodes ricinus* ticks.

We thank Dr P. C. Doherty for the neuropathological examinations and Mr C. C. Renwick for managing and distributing the sheep flocks. We express our gratitude to Mr J. B. Boyce and Mr C. Burrells for their able assistance with the serology.

REFERENCES

Brambell, F. W. R. (1958). The passive immunity of the young mammal. *Biological Reviews* **33**, 488.

Brotherston, J. G. & Boyce, J. B. (1969). A new vaccine against louping-ill. *Veterinary Record* **84**, 514.

Brotherston, J. G. & Boyce, J. B. (1970). Development of a non-infective protective antigen against louping-ill (Arbovirus Group B). *Journal of Comparative Pathology* **80**, 377.

Doherty, P. C. (1969). Louping-ill encephalitis in C 57 black mice. *Journal of Comparative Pathology* **79**, 571.

Doherty, P. C. & Reid, H. W. (1971). Louping-ill encephalomyelitis in the sheep. II. Distribution of virus and lesions in nervous tissue. *Journal of Comparative Pathology* (in the Press).

Foggie, A. (1951). Studies on the infectious agent of tick-borne fever in sheep. *Journal of Pathology and Bacteriology* **63**, 1.

Gordon, W. S. Brownlee, A., Wilson, D. R. & Macleod, J. (1962). The epizootiology of louping-ill and tick-borne fever with observations on the control of these diseases. *Symposia of the Zoological Society of London*, no. 6, pp. 1–27.

Macleod, J. & Gordon, W. S. (1932). Studies in louping-ill (an encephalomyelitis of sheep). II. Transmission by the sheep ticks *Ixodes ricinus* L. *Journal of Comparative Pathology* **45**, 240.

Mayer, V., Blašovič, D., Ernek, E. & Libíková, J. (1969). The serological response and long-lasting resistance against infection with louping-ill virus in sheep immunized with a highly attenuated tick-borne encephalitis virus. *Journal of Hygiene* **67**, 731.

O'Reilly, K. J., Smith, C. E. G., McMahon, D. A., Bowen, E. T. W. & White, G. (1968). A comparison of measuring the persistence of neutralizing and haemagglutinin-inhibiting antibodies to louping-ill virus in experimentally infected sheep. *Journal of Hygiene* **66**, 217.

Reid, H. W. & Doherty, P. C. (1971). Louping-ill encephalomyelitis in the sheep. I. The relationship of viraemia and the antibody response to susceptibility. *Journal of Comparative Pathology* **81**, 291.

Smith, C. E. G. (1969). Arbovirus vaccines. *British Medical Bulletin* **25**, 142.

Smith, C. E. G., McMahon, D. A., O'Reilly, K. J., Wilson, A. L. & Robertson, J. M. (1964). The epidemiology of louping-ill in Ayrshire: the first year of studies in sheep. *Journal of Hygiene* **62**, 53.

Williams, H. & Thorburn, H. (1961). The serological response of sheep to louping-ill virus. *Journal of Hygiene* **59**, 437.

J. Hyg., Camb. (1971), **69**, 491
Printed in Great Britain

Salmonellosis in Botswana

I. Incidence in cattle

BY A. S. MILLER
Veterinary Diagnostic Laboratory, Gaborone, Botswana

(*Received* 23 *March* 1971)

SUMMARY

Clinical salmonellosis in cattle is infrequently recorded in Botswana, the majority of salmonella isolations being made from apparently healthy animals. A salmonella carrier rate of 6·7 % was found in testing faeces samples from 89 apparently healthy cattle. A total of 35 salmonella serotypes were isolated from specimens of bovine origin. *Salmonella dublin* was isolated infrequently, possibly in part because of prophylactic vaccination.

INTRODUCTION

The Republic of Botswana covers an area of 220,000 square miles (560,000 km^2) in central southern Africa. It has a subtropical climate, with mean summer temperatures of 25–38° C., and mean winter temperatures of 12–25° C. The average annual rainfall is in the region of 17 in. (43 mm.). The maximum rainfall occurs in the north of the country, which has an annual average of about 25 in. (65 mm.). The eastern part of Botswana enjoys from 15 to 25 in. (39–65 mm.) per year. Rainfall decreases towards the south-west of the country, which comprises part of the Kalahari desert. In all areas of the territory the annual rainfall shows marked variation.

The main industry in Botswana is livestock production. It was decided to investigate whether or not *Salmonella* is present in the country, and if so, to determine the incidence of infection, and also which serotypes are prevalent.

MATERIALS AND METHODS

Botswana's main abattoir, which is the only one dealing with export meat, is located in the south-east of the country. Animals from districts on the line of rail (which runs north–south along the eastern border) are railed direct to the abattoir. Animals from remote districts are walked to the line of rail and then railed. Animals from the west and south-west are either walked to the abattoir or transported by road.

Cattle are slaughtered between 12 and 48 hr. after arriving at the abattoir. The holding pens at the abattoir are provided with water.

There are four local abattoirs under constant supervision by the Veterinary Department, and numerous unsupervised slaughter houses. These deal with cattle within a radius of about 20 miles.

Samples

Pooled bovine bile samples

A total of 158 pooled bile samples were examined. All slaughtered cattle were examined before and after slaughter and no evidence of disease was found.

Of the total number of bile specimens examined, 60 samples were from local abattoirs. Each of these samples was from six to twelve cattle, representing about 33 % of the weekly kill.

The remaining 98 samples were from the export abattoir. In this abattoir the gall bladder from every animal is ruptured over a funnel. The bile passes along a pipe to a stainless-steel tank, where it is allowed to collect for 3–4 days. The bile is then drained through a tap at the base of the tank for concentration. After the tank has been emptied, the tank, pipe and funnel are sterilized by steam. Samples were collected from the tap at the base of the tank. The contents of the tank, when full, represent pooled bile from approximately 2000 animals. Samples of approximately 50 ml. were collected weekly, when the tank was full.

Meat and liver samples

A total of 752 meat samples from the neck, brisket, hump, the longissimus dorsi and the longus colli muscles were obtained from the country's export abattoir. Each sample consisted of about 150 g. From the same source, 117 liver samples were also collected. Carcasses and livers were random samples, and represented approximately 2 % of the daily kill. All samples were from apparently healthy animals.

Faeces samples

A total of 57 faeces samples from animals with gastro-intestinal symptoms were examined. In addition, faeces samples from 89 apparently healthy animals from the eastern part of the country were tested. Samples were collected over an 18-month period from March 1968 to September 1969.

Methods

Plates of deoxycholate citrate agar (Oxoid CM 227) and tubes of selenite broth (Oxoid CM 39) were inoculated with the samples. Meat samples were homogenized in a Waring blender before inoculation. After overnight incubation at 37° C. pale colonies were picked from the deoxycholate citrate plates into peptone water for further examination. A loopful of the selenite broth was subcultured onto a deoxycholate citrate agar plate after 18 hr. incubation, and again after 42 hr. incubation at 37 °C. Each plate was incubated overnight at 37° C. and pale colonies were picked as before.

Peptone water cultures of suspect organisms were tested for purity by plating on nutrient agar and MacConkey agar. When necessary, cultures were purified by subculture. Pure cultures were tested for urease production and lysine decarboxylase production. Cultures which gave a negative urease and a positive lysine

decarboxylase reaction were subjected to a range of biochemical tests (Cowan & Steel, 1966). Cultures giving the reactions of *Salmonella* were identified serologically, using firstly polyvalent antisera, and then single factor antisera. The subgenus of each salmonella isolated was determined according to Kauffmann (1965).

After 602 samples had been examined, strontium chloride enrichment medium (Iveson & Mackay-Scollay, 1969) was introduced, and this medium was used in parallel with the selenite broth in the remaining 571 samples. 'G.P.R.' grade strontium chloride (Hopkin and Williams Ltd.) and bacteriological peptone (Oxoid L37) were used in the preparation of this medium. A detailed comparison of the two enrichment media is in progress in this laboratory, to be published separately. The results of *Salmonella* isolations from the two media pertaining to the present study are detailed in Table 1.

Table 1. Salmonella *isolations from selenite broth and strontium chloride medium*

Samples examined ...	571
No. positive by	
Selenite alone	5
Selenite and strontium chloride	46
Strontium chloride alone	7

Table 2. Salmonella *serotypes from bovine bile from the export abattoir*

Serotype	No. of isolations	Serotype	No. of isolations
typhimurium	11	*newport*	2
anatum	11	*parow**	2
brancaster	7	*bovis morbificans*	1
tamale	7	*bradford*	1
essen	6	*derby*	1
saint-paul	6	*durbanville**	1
43:f,g,t:1,5*†	5	*heidelberg*	1
13,22:z_{39}:1,5,(7)*†	4	*newington*	1
jedburgh	3	*tinda*	1
dublin	2	*waycross*	1
hvittingfoss	2	1,4,12,27:z:1,5*†	1

* Subgenus II salmonellas.
† Hitherto undescribed serotypes.

RESULTS

Pooled bile samples

Salmonella spp. were isolated from two of the 60 samples from local abattoirs. The serotypes encountered were *Salm. typhimurium* and *Salm. fischerkietz*.

Thirty-nine of the 98 samples from the export abattoir yielded *Salmonella*, and more than one serotype was isolated from 24 of the samples. Twenty-two different serotypes were found, three of which were hitherto undescribed serotypes. The various types isolated, with the frequency of isolation, are given in Table 2.

Meat and liver samples

A total of 752 meat samples and 117 liver samples were received from the sites mentioned above. Isolations of Salmonellla from these are shown in Table 3.

Table 3. Salmonella *serotypes from meat and liver samples*

Site	No. of samples examined	Serotypes isolated
Brisket	237	—
Neck	128	windhoek* (2), offa (1)
Hump	119	—
Longissimus dorsi muscle	91	—
Longus colli muscle	177	—
Liver	117	braenderup (15), weltevreden (7), newington (3)

Figures in parentheses indicate the number of isolations.
* Subgenus II salmonella.

Faeces samples

Salmonellas were grown from 5 of the 57 faeces samples from sick animals (8·8 %) and from 6 of the 89 faeces samples from apparently healthy cattle (6·7 %). Serotypes found were *Salm. anatum*, *Salm. brancaster*, *Salm. donna*, *Salm. pomona* and *Salm. typhimurium* in the first group, and *Salm. colorado*, *Salm. enteritidis*, *Salm. goodwood*, *Salm. leopoldville*, *Salm.* $6,7{:}z{:}z_6$ and one unidentified in the second group.

DISCUSSION

Overt salmonellosis does not appear to be common in Botswana cattle. It is probable that most infected animals contract subclinical infections and continue to harbour the organisms. Clarenburg, Vink & Schuurmans (1950) demonstrated that cattle may continue to harbour *Salm. dublin* for more than 100 days after infection, and Smith & Buxton (1951) found that 0·4 % of 750 apparently healthy cattle in England were *Salmonella* excreters.

From the isolations of *Salmonella* from pooled bile samples in the abattoirs, it appears that many serotypes are able to invade the gall bladder and to survive there. A large proportion of the isolations from meat and liver samples were from the liver, which is inevitably contaminated with bile if the gall bladder is accidentally ruptured. The rest of these isolations were from neck, which can easily be contaminated by intestinal contents during the handling of the carcass. It seems therefore that salmonellas may be confined to the biliary and intestinal tracts in apparently healthy cattle, and that invasion of the tissues by these organisms, if it occurs, is extremely rare.

Although a high salmonella carrier rate of about 7 % exists in Botswana cattle, it is encouraging that out of 752 samples of export meat tested, only three were found to contain *Salmonella*, a finding which compares favourably with many other countries. For example, a Public Health Laboratory Service working-party found

Salmonella in 1·9% of 1996 meat samples from abattoirs in England and Wales (Report, 1964).

It is interesting to note that three of the serotypes from sick animals, namely *Salm. anatum*, *Salm. brancaster* and *Salm. typhimurium*, were also isolated frequently from bile, whereas not one of the serotypes isolated from healthy cattle was found there.

It is possible that the appearance of *Salmonella* in the bile is related to the pathogenicity of the strain; on the other hand, it is also possible that serotypes such as *Salm. typhimurium* and *Salm. anatum* are able to survive longer in the gall bladder than are the more uncommon types.

A feature difficult to explain is the relative infrequency of isolation of *Salm. dublin* in Botswana. This serotype is the one most frequently observed in cattle in many countries, including Britain (Sojka & Field, 1970) and Botswana's neighbour, the Republic of South Africa (Henning, 1939). Vaccine against *Salm. dublin* is readily obtainable in Botswana, and its use is widespread. Vaccination may account in part for the paucity of isolations of this serotype.

The occurrence of a high proportion of subgenus II salmonellas, and also of higher somatic group salmonellas in Botswana cattle is of interest. Of the serotypes isolated, 43% fell into one or both of these categories. Iveson, Mackay-Scollay & Bamford (1969) suggested that in Australia salmonellas of higher somatic groups were more common in remote areas, whereas lower somatic group salmonellas were mainly confined to inhabited areas. Botswana is similar to parts of Australia both in climatic conditions and in sparseness of population, and it seems that a parallel condition exists between the two countries.

Subgenus II salmonellas are found mainly in reptiles (Taylor, 1969). A limited study (to be extended) has shown that there is a high carrier rate for *Salmonella* in reptiles in Botswana. *Salmonella* serotypes were isolated from 24 out of 30 reptiles examined, and many of these were higher somatic group salmonellas. Reptiles are abundant throughout Botswana, and it is probable that cross-infection occurs between cattle and reptiles in all parts of the country.

I wish to thank Dr D. Mehlitz for much helpful advice in the preparation of this paper; Dr Joan Taylor for her help in confirming or identifying many of the serotypes isolated; Professor L. le Minor for identifying the three hitherto undescribed serotypes; and Dr J. Falconer, Director of Veterinary Services, Republic of Botswana, for permission to publish.

REFERENCES

Clarenburg, A., Vink, H. H. & Schuurmans, R. (1950). Salmonella-dragers bij runderen. *Tijdschrift voor Diergeneeskunde* **75**, 435.

Cowan, S. T. & Steel, K. J. (1966). *Manual for the Identification of Medical Bacteria.* Cambridge University Press.

Henning, M. W. (1939). The antigenic structure of salmonellas obtained from domestic animals and birds in South Africa. *Onderstepoort Journal of Veterinary Science and Animal Industry* **13**, 79.

Iveson, J. B. & Mackay-Scollay, E. M. (1969). Strontium chloride and strontium selenite enrichment broth media in the isolation of *Salmonella*. *Journal of Hygiene* **67**, 457.

IVESON, J. B., MACKAY-SCOLLAY, E. M. & BAMFORD, V. (1969). *Salmonella* and Arizona in reptiles and man in Western Australia. *Journal of Hygiene* **67**, 135.

KAUFFMANN, F. (1965). *The Bacteriology of Enterobacteriaceae*. Copenhagen: Munksgaard.

REPORT (1964). Salmonellae in abattoirs, butcher's shops and home-produced meat, and their relation to infection: Report of a Working Party of the Public Health Laboratory Service. *Journal of Hygiene* **62**, 283.

SMITH, H. W. & BUXTON, A. (1951). Isolation of salmonellae from faeces of domestic animals. *British Medical Journal* i, 1478.

SOJKA, W. J. & FIELD, H. I. (1970). Salmonellosis in England and Wales 1958–1967. *Veterinary Bulletin* **40**, 515.

TAYLOR, J. (1969). *Kauffmann–White Scheme, July 1969*. Public Health Laboratory Service.

J. Hyg., Camb. (1971), **69**, 497
Printed in Great Britain

Salmonella typhimurium contamination of processed broiler chickens after a subclinical infection

By V. A. KNIVETT

*Twyford Laboratories, Twyford Abbey Road, London, NW10 7XG**

(*Received* 24 *March* 1971)

SUMMARY

A subclinical infection of *Salmonella typhimurium* in a broiler flock was investigated and attempts were made to eradicate the infection by treatment with furazolidone. One-quarter of the chickens were still infected after they had been through the processing plant. Washing in heavily chlorinated water reduced the number of contaminated carcasses. Infected chickens were also found in four other companion flocks on the same farm.

INTRODUCTION

Now that *S. pullorum* and *S. gallinarum* infections of poultry in Britain have declined, *S. typhimurium* remains the chief cause of poultry salmonellosis (Sojka & Field, 1970; Stevens, 1971). These infections are variable, but even when they are mild clinically the pathogen may persist in the infected carcass and cause food poisoning. Anderson (1969) has shown a relationship between human food poisoning and salmonellosis in poultry by using bacteriophage typing. Phage type 14 was the strain of *S. typhimurium* most commonly isolated from poultry (74 %) and also from humans (17·5%), but this strain was not found in cattle. Phage type 20a, the next commonest strain in humans (9·15 %), was not found in poultry but did occur in cattle. A salmonella infection in a breeding flock may spread successively to the broiler flock, the processing plant and thence to the consumer, who may not appreciate the danger of handling uncooked carcasses or the importance of adequate thawing and cooking (Anon, 1969; Pennington, Brooksbank, Poole & Seymour, 1968; Semple, Turner & Lowry, 1968).

Whilst some flocks of broilers had a high incidence of salmonella infection (Griffith, 1969; Timoney, Kelly, Hannen & Reeves, 1970) other flocks had little or none (Tucker & Gordon, 1968; Patterson, 1967, 1969). *S. typhimurium* was the commonest serotype found in some poultry packing stations (Galton *et al.* 1955; Sadler, Yamamoto, Adler & Stewart, 1961; Dixon & Pooley, 1961*a*; Timoney, 1969) and also on poultry in shops (Felsenfeld, Young & Yoshimura, 1950; Galbraith, Taylor, Patton & Hagan, 1964).

Dixon & Pooley (1961*b*) recommended treatment of the spin-chiller water with 200 p.p.m. available chlorine to reduce cross-contamination. However, Barnes

* Present address: 11 Tregenna Court, 622 Harrow Road, Wembley, HA0 2EH.

(1965) pointed out that although such treatment might destroy all the bacteria washed off the carcass, it would not destroy those present in the hair follicles or in the cavity. Chlorination is of some benefit and has been shown to prolong the storage life of the chicken by reducing the number of organisms present (Nilsson & Regnér, 1963; Ranken, Clewlow, Shrimpton & Stevens, 1965; Thomson, Banwart, Sanders & Mercuri, 1967; Patterson, 1968; Wabeck *et al.* 1968).

This paper describes an outbreak of *S. typhimurium* infection in a broiler flock and the difficulty of eradicating the pathogen with furazolidone therapy and careful attention to hygiene during processing.

MATERIALS AND METHODS

Chicks

Day-old chicks were supplied by a commercial hatchery. Each intake consisted of 2000 pullets and 8000 'as hatched' chicks.

Housing

The chicks were housed in broiler houses with fresh deep litter of wood shavings, and were supplied with food and water *ad lib.* The feed was varied from starter crumbs, raising crumbs and finisher pellets as appropriate. Furazolidone was administered in the drinking water at specified times.

Broiler processing plant

The plant handled 1200 birds/hr. After evisceration the carcasses passed through two spin-chillers containing cold chlorinated water, and then were drained and packed. Vent swabs were taken at ½ hr. intervals from ten birds as they left the spin-chillers. Some carcasses were tagged and swabbed after evisceration and again after passing through the spin-chillers. The concentration of free chlorine in the spin-chillers was monitored during the day using potassium iodide and a Lovibond comparator. Fresh hypochlorite was added to maintain the concentration of 200–250 p.p.m. free chlorine.

Bacteriological examination

Samples. Livers were washed and macerated as described by Knivett & Stevens (1971) and the homogenized tissue was added to 50 ml. selenite broth. One ml. of this mixture was further diluted with 10 ml. selenite broth. Caeca were removed aseptically and small pieces were added to 50 ml. selenite broth. One ml. of this mixture was diluted with 10 ml. selenite broth. A few samples of the duodenum were taken and treated similarly.

Soiled litter was taken from several parts of the broiler house and 10 g. samples added to 100 ml. selenite broth. Dust was taken from crevices round the walls and 1 g. samples added to 10 ml. selenite broth. Samples of feed were taken from storage bins outside the broiler house and 50 g. added to 100 ml. selenite broth.

Cloacal, vent and equipment swabs were placed in 10 ml. selenite broth.

Enrichments. Selenite mannitol broth was freshly prepared and adjusted to pH 6·8. Cultures were incubated overnight at 43° C. and streaked on selective agar (Knivett & Stevens, 1971). Second enrichments were made by transferring 0·04 ml. of culture to 10 ml. of fresh selenite broth and streaking usually after 8 hr. incubation. Plates were incubated overnight at 37° C. and non-fermenting sulphide-producing colonies were checked for the absence of urease and tested with salmonella agglutinating sera.

RESULTS

An outbreak of Salmonella typhimurium *infection in a broiler house*

The events following an outbreak of *S. typhimurium* infection in a broilerhouse are described in Table 1. As soon as the Veterinary Investigation Centre diagnosed the infection, the pullets were destroyed. The remaining 'as hatched' chicks,

Table 1. *Calendar of events in house no.* 1

Age (days)	
1	Arrival of 2000 pullets and 8000 'as hatched' chicks. These were penned separately in the same house.
3	Pullets showed abnormally high mortalities and clinical symptoms of disease. Aureomycin was administered to these chicks for 5 days. Veterinary Investigation Centre subsequently diagnosed *S. typhimurium* phage type 1a.
8	Two thousand pullets destroyed and the litter removed and burnt. Furazolidone (0·04 %) administered to the remaining 'as hatched' chicks for 8 days.
18	Dead birds examined – 0/2 positive.
20	Dead birds examined – 3/4 positive.
24	Dead birds examined – positive.
32	Dead birds examined – 2/3 positive. Ten chickens examined – 6 caeca positive, all livers negative. Feed – negative. Litter and dust – positive.
47	Dead birds examined – 1/3 caeca and duodena positive, all livers negative.
66	Fifteen chickens examined – 10/15 caeca positive, 7/15 livers positive. Thirty-five cloacal swabs examined – 11 positive. Furazolidone (0·04 %) administered for 4 days.
69	Fifteen chickens examined – 9/15 caeca positive, all livers negative. Cloacal swabs examined – 4/35 positive.
74	All chickens processed. After evisceration, 10/10 vent swabs positive. After washing, 3/10 vent swabs positive. Total samples, 24/90 vent swabs positive.

separated from the pullets by a wire fence, appeared quite healthy, with no abnormal mortalities or symptoms of disease. They were kept under observation in case they might also have become infected and treated with 0·04 % furazolidone for 8 days as a precautionary measure. Subsequently samples of litter, dust, feed, dead chickens and some live chickens were taken for bacteriological examination, and showed that in spite of furazolidone treatment, some of the chickens were infected. A second treatment with 0·04 % furazolidone for 4 days was given 8 days before they went to the packing station. To assess the efficacy of furazolidone treatment, cloacal swabs were taken at random from 35 of the chickens, which were then tagged and penned separately (Table 2). Another 15 chickens were taken to the laboratory for examination. After the furazolidone treatment was over, those

chickens which had positive cloacal swabs were identified and taken to the laboratory for examination. No salmonellas were isolated from the livers after furazolidone treatment. There were fewer positive cloacal swabs after treatment than before and the infection still remained in many caeca.

Table 2. *Isolation of* Salmonella typhimurium *from chickens before and after furazolidone treatment*

Samples	Before treatment	After treatment
Randomly selected chickens		
Cloacal swabs	11/35* positive	4/35 positive
Livers	7/15 positive	0/5 positive
Caeca	10/15 positive	1/5 positive
Infected chickens†		
Cloacal swabs	10/10 positive	Caeca 8/10 positive Livers 0/10 positive

* These chickens were tagged and penned separately from the others.

† Chickens whose cloacal swabs were positive before treatment were examined in the laboratory after treatment for the presence of salmonellas in the liver and caeca.

As a precaution, the other flocks on the farm were treated with 0·02 % furazolidone for 8 days, although they had shown no clinical signs of disease, adverse weight gains or abnormal mortalities to suggest that they might be infected. A few tests were made to see whether infection had spread to these flocks (Table 3). Eventually the infection was detected in the litter from the five houses from which it was examined, and as a further precaution all flocks were given 0·04 % furazolidone treatment for 4 days, 8 days before they were due to go to the packing station.

Bacteriological tests at the packing station

Salmonella isolations from processed chickens. The equipment was thoroughly cleaned after the day's operations, and the next morning swabs were taken from several surfaces before the new intake was started. These were all negative on each occasion. Vent swabs were taken from ten successive chickens emerging from the second spin-chiller at ½ hr. intervals (Table 4). There was little evidence for any build-up of infection during the working day. Flocks II and III and the combined total had slightly more positive results during the second half of the day than during the first half, but this was not so in every case. The water in the first spin-chiller was changed at mid-day (except for flock V), but this had no significant effect on the number of isolations subsequently. All bacteriological samples taken from either spin-chiller were negative.

Effect of chlorination. Approximately 200–250 p.p.m. free chlorine was maintained in the spin-chillers. Considerably fewer positive results were obtained after chickens had been through the spin-chillers compared with the same samples examined before washing (Table 5). There was no evidence for cross-contamination in the spin-chillers, except for one sample which was negative before entering the bath and was positive on leaving.

Table 3. Salmonella typhimurium *infection in other flocks on the farm*

	Flock no. and date of birth					
	I	II	III	IV	V	VI
Age (days)	13 Mar.	24 Mar.	31 Mar.	7 Apr.	14 Apr.	31 Mar.
3	M+	.	.	.	.	.
7	.	.	.	L− D−	.	0/2M+
14	.	.	L+ F+†	.	L− D− 0/5M+	.
18	0/2M+	.	.	.	.	.
20	3/4M+	.	.	.	.	.
21	.	L+	.	.	.	.
23	.	.	.	L− D− 0/5M+	.	.
32	L+ D+ F− 2/3M+ 6/10C+	.	0/2M+	.	.	.
35	.	0/4M+	.	.	.	.
46	.	.	.	.	L+ 1/5CS+ D+	.
47	1/3M+	.	.	.	.	.
53	.	.	.	D− 0/5CS+ L+	.	.
66	10/15C+ 11/35CS+	.	.	.	.	.
69	9/15C+ 4/35CS+	.	.	.	.	D−
Pro.*	24/90VS+	40/92VS+	17/90VS+	20/90VS+	21/80VS+	0/89VS+

C = chickens; CS = cloacal swabs; D = dust; F = feed; L = litter; M = dead chickens; VS = vent swabs.

Flock no. I received 0·04 % furazolidone for 8 days at 8 days of age, and the other flocks received 0·02 % furazolidone for 4 days. All flocks received 0·04 % furazolidone for 4 days, 8 days before processing.

* Pro. = processed chickens. † Unidentified salmonella.

Possible spread of contamination from carcass to carcass. There were 122 positive results from 442 samples (flock VI omitted), i.e. about one in four positives. However, these were not randomly scattered, and several positives occurred in succession. There was one group of seven positives in succession, one of six, and one of five all from flock II during the latter part of the day. There was one group of five in succession, two groups of four, seven groups of three, and seven pairs together. The remaining 56 isolations were all singles. Thus twenty groups of between

two and seven successive chickens made up over half of the total salmonella isolations. Not all of these chickens may have been positive when they entered the packing station.

Table 4. Salmonella typhimurium *contaminated carcasses after washing (no. of positive swabs/batch of* 10 *samples)*

Batch no.	Flock no.						Total
	I	II	III	VI	IV	V	
1	3	0	0	0	1	4	8
2	6	2	1	0	4	2	15
3	1	5	1	0	5	1	13
4	3	3	1	0	0	3	10
5	4	6	1	0	2	5	18
6	1	6	4	0	4	1	16
7	1	4	5	0	1	3	14
8	3	7	4	0	1	2	17
9	2	6	0	0*	2	—	10
10	—	1†	—	—	—	—	1
Total	24/90	40/92	17/90	0/89	20/90	21/80	122
%	26·7	43·5	18·9	0	22·2	26·2	

Combined total (omitting flock no. VI): 122/442 (27·6 %).

Batches of ten successive samples were taken at ½ hr. intervals (with a ½ hr. mid-day break with flocks nos. I, III and VI). The water in the first spin-chiller tank was changed at mid-day (except flock no. V).

* Nine samples. † Two samples.

Table 5. *Effect of washing* Salmonella typhimurium *contaminated carcasses in chlorinated water (no. of positive swabs/*10 *samples)*

	Flock no.				
	I	III	VI	IV	V
Before washing	10	10	0	9	10
After washing	3	4	0	4*	1

Vent swabs were taken from ten tagged carcasses after evisceration and before they entered the first spin-chiller. These carcasses were again swabbed after leaving the second spin-chiller.

* One swab was negative before washing and positive afterwards.

DISCUSSION

Experiments at the Houghton Poultry Research Station (Knivett & Tucker, 1971) have shown that 2 months after a challenge with *S. typhimurium*, half of the chickens still had infected alimentary tracts, irrespective of whether they had received furazolidone treatment or not. They also showed that the caeca were the most frequently infected portions of the alimentary tract (86 % of infected chickens), whereas liver and spleen were the most frequently infected tissues (about 25 %). Livers are of interest because they are often eaten without adequate cooking and may cause food poisoning.

They also showed that, except during the early stages of an infection, cloacal swabs were inefficient, and on average only 1 out of 5 infected chickens gave a positive cloacal swab and sometimes this proportion was as low as 1 in 12, or even 1 in 20. Brobst, Greenberg & Gezon (1958) swabbed 265 chickens entering a poultry-processing plant and later they swabbed the abdominal cavities of these chickens. Every cloacal swab was negative whilst 43 % of the cavity swabs were positive. Although persistent carriers may remain after a mild infection, it is difficult to discover which chickens remain carriers or how many might be present in the flock. The examination of litter samples indicated whether the flock was infected or not and this was later confirmed by tests on the processed chickens.

The infection spread from house to house, but did not reach the furthest one (no. VI), and no infected chickens were found in this flock. Thus some flocks may be heavily infected and others completely free.

The plant was thoroughly sterilized after each day's work and there was no carry over of infection from previous occasions. Test swabs taken from equipment before the day's work commenced all proved negative. Some workers (Galton *et al.* 1955; Glezen *et al.* 1966) allege that chickens were rarely infected when they entered a processing plant and a few contaminated chickens spread infection to many others. The possible extent of such cross-infection was shown in an interesting experiment by Stewart (1965), who deliberately infected a single bird with a tracer organism (a red pigmented *Serratia*) and followed the distribution of this organism to other birds and the operative's hands. The following 42 birds on the line were all heavily infected with the tracer organism and infection was still detectable on the 150th bird. The organisms were also detectable on the hands of all but one of the operatives on the eviscerating line. He estimated that perhaps one-third of contaminated birds had become infected during processing.

Washing in chlorinated water effectively reduced the number of contaminated chickens. Although almost all the freshly eviscerated chickens were contaminated, it was not possible to tell whether they had become contaminated during evisceration or whether they might have already been infected on the farm or both.

The farmer suffered a considerable economic less in house no. I through the abnormally high death-rate, poorer food conversion, increased cost of medication, as well as the loss of the condemned pullets. There would have been no indication that *S. typhimurium* was present in the other houses had not bacteriological samples been taken. The existing methods of combating salmonellosis in poultry have not been adequate.

I am most grateful to the Manager of the Packing Station and his staff, who assisted in the investigation. I wish to thank Mr W. K. Stevens, who assisted with the field work, and Miss J. A. Bishopp, Messrs K. R. Comber and J. E. Thurgood for technical assistance.

REFERENCES

Anderson, E. S. (1969). The use of bacteriophage typing in the investigation of outbreaks of salmonella food poisoning. In *Bacterial Food Poisoning*, p. 47. Ed. J. Taylor. London: The Royal Society of Health.

Anon. (1969). Wanted: instructions on how to thaw. *Farmer and Stockbreeder*, 12 August.

Barnes, E. M. (1965). The effect of chlorinating chill tanks on the bacteriological condition of processed chickens. *Bulletin of the International Institute of Refrigeration*, annexe 1, p. 219. International Institute of Refrigeration Congress, Karlsruhe.

Brobst, D., Greenberg, J. & Gezon, H. M. (1958). Salmonellosis in poultry and poultry processing plants in Western Pennsylvania. *Journal of the American Veterinary Medical Association* **33**, 435.

Dixon, J. M. S. & Pooley, F. E. (1961*a*). Salmonellae in a poultry processing plant. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **20**, 30.

Dixon, J. M. S. & Pooley, F. E. (1961*b*). The effect of chlorination on chicken carcasses infected with salmonellae. *Journal of Hygiene* **59**, 343.

Felsenfeld, O., Young, V. M. & Yoshimura, T. (1950). A survey of salmonella organisms in market meat, eggs and milk. *Journal of the American Veterinary Medical Association* **116**, 17.

Galbraith, N. S., Taylor, C. E. D., Patton, J. L. & Hagan, J. G. (1964). Salmonella infection in poultry. *Medical Officer* **111**, 354.

Galton, M. M., Mackel, D. C., Lewis, A. L., Haire, W. C. & Hardy, A. V. (1955). Salmonellosis in poultry and poultry processing plants in Florida. *American Journal of Veterinary Research* **16**, 132.

Glezen, W. P., Hines, M. P., Kerbaugh, M., Green, M. E. & Koomen, J. (1966). Salmonella in two poultry processing plants. *Journal of the American Veterinary Medical Association* **148**, 550.

Griffith, W. (1969). An investigation of the incidence of *Salmonella virchow* in New York dressed chickens. *Environmental Health* **77**, 234.

Knivett, V. A. & Stevens, W. K. (1971). The evaluation of a live Salmonella vaccine in mice and chickens. *Journal of Hygiene* **69**, 233.

Knivett, V. A. & Tucker, J. F. (1971). Comparison of oral vaccination or furazolidone prophylaxis for *Salmonella typhimurium* infections in chicks. *British Veterinary Journal* (in the Press).

Nilsson, T. & Regnér, B. (1963). The effect of chlorine in the chilling water on salmonella in dressed chicken. *Acta veterinaria scandinavica* **4**, 307.

Patterson, J. T. (1967). The incidence of enteropathogenic *E. coli* and salmonellae in processed broilers and in New York dressed poultry. *Record of Agricultural Research* **16**, 151.

Patterson, J. T. (1968). Bacterial flora of chicken carcasses treated with high concentrations of chlorine. *Journal of Applied Bacteriology* **31**, 544.

Patterson, J. T. (1969). Salmonellae in meat and poultry, poultry plant cooling waters and effluents and animal feeding stuffs. *Journal of Applied Bacteriology* **32**, 329.

Pennington, J. H., Brooksbank, N. H., Poole, P. M. & Seymour, F. (1968). *Salmonella virchow* in a chicken-packing station and associated rearing units. *British Medical Journal* iv, 804.

Ranken, M. D., Clewlow, G., Shrimpton, D. H. & Stevens, B. J. H. (1965). Chlorination in poultry processing. *Poultry Science* **6**, 331.

Sadler, W. W., Yamamoto, R., Adler, H. E. & Stewart, G. F. (1961). Survey of market poultry for *Salmonella* infection. *Applied Microbiology* **9**, 72.

Semple, A. B., Turner, G. C. & Lowry, D. M. O. (1968). Outbreak of food poisoning caused by *Salmonella virchow* in spit-roasted chicken. *British Medical Journal* iv, 801.

Sojka, W. J. & Field, H. I. (1970). Salmonellosis in England and Wales 1958–1967. *Veterinary Bulletin* **40**, 515.

Stevens, A. J. (1971). The economics of disease. (British Egg Marketing Board Poultry Symposium) *In Poultry Disease and World Economy*, p. 25. Ed. R. F. Gordon and B. M. Freeman. Edinburgh: British Poultry Science Ltd.

Stewart, D. J. (1965). The occurrence of enteropathogenic *E. coli* and salmonellae in processed broilers. *Proceedings of the First International Congress of Food Science and Technology*, vol. II, p. 485.

THOMSON, J. E., BANWART, G. J., SANDERS, D. H. & MERCURI, A. J. (1967). Effect of chlorine, antibiotics, β-propiolactone, acids and washing on *S. typhimurium* on eviscerated fryer chickens. *Poultry Science* **46**, 146.

TIMONEY, J. (1969). The sources of salmonellae in poultry carcasses. *Veterinary Record* **87**, 158.

TIMONEY, J., KELLY, W. R., HANNEN, J. & REEVES, D. (1970). A study of salmonella contamination in some Dublin poultry processing plants. *Veterinary Record* **87**, 158.

TUCKER, J. F. & GORDON, R. S. (1968). The incidence of salmonellae in poultry packing stations. *British Veterinary Journal* **124**, 102.

WABECK, C. J., SCHWALL, D. V., EVANCHO, G. M., HECK, J. G. & ROGERS, A. B. (1968). Salmonella and total count reduction in poultry treated with sodium hypochlorite solutions. *Poultry Science* **47**, 1090.

THE QUARTERLY JOURNAL OF MEDICINE

(NEW SERIES)

EDITED BY

D. A. K. BLACK P. HUGH-JONES E. M. McGIRR
W. B. MATTHEWS J. D. N. NABARRO A. G. W. WHITFIELD

CONTENTS

NEW SERIES VOL. 40 No. 159 July 1971

(Vol. 64 of the continuous series)

The Effects of Chemotherapy on Iron, Folate, and Vitamin B_{12} Metabolism in Tuberculosis. *By Daphne H. Line, B. Seitanidis, J. O. Morgan, and A. V. Hoffbrand*

Endemic Typhoid Fever. *By A. C. B. Wicks, G. S. Holmes, and Lindsay Davidson*

The Treatment of Cystinuria with *N*-acetyl-*D*-penicillamine, a Comparison with the Results of *D*-penicillamine Treatment. *By A. D. Stephens and R. W. E. Watts*

Granulomatous Hepatitis of Obscure Aetiology. *By Muiris X. Fitzgerald, Oliver Fitzgerald, and Robert P. Towers*

Immunohistology in Renal Disease. *By Rawle M. McIntosh, Binger Tinglof, Donald Kaufman, Leslie Dornfeld, Harvey Gonick, Fred G. Smith, and Robert L. Vernier*

Atlanto-Axial Subluxation and Cervical Myelopathy in Rheumatoid Arthritis. *By J. C. Stevens, N. E. F. Cartlidge, M. Saunders, A. Appleby, M. Hall, and D. A. Shaw*

A Follow-up Study of Patients with Post-cricoid Webs. *By Morag Chisholm, G. M. Ardran, Sheila T. Callender, and Ralph Wright*

Iron Deficiency and Autoimmunity in Post-cricoid Webs. *By Morag Chisholm, G. M. Ardran, Sheila T. Callender, and Ralph Wright*

Polyneuritis Associated with Cytomegalovirus Infections. *By J. C. Leonard and J. O'H. Tobin*

Medullary Carcinoma of the Thyroid Gland. *By W. Milo Keynes and A. S. Till*

Price £1.50 Subscription price £4.25 per annum

OXFORD UNIVERSITY PRESS

Ely House, 37 Dover Street, London, W1K 4AH

6700 current abstracts a year on
PUBLIC HEALTH, SOCIAL MEDICINE AND HYGIENE
in 12 fully indexed monthly issues

With the annual volume of medical literature published throughout the world increasing so rapidly, it becomes ever more difficult for the individual specialist to keep abreast of the latest developments in his particular field. For this reason Excerpta Medica has been publishing, since 1955, in English, a journal exclusively devoted to Public Health, Social Medicine and Hygiene.
Some 3000 of the world's most important journals are screened consistently to form the basis for each of the 38 Excerpta Medica abstract journals.
Public Health, Social Medicine and Hygiene currently contains 6700 abstracts per year.

Each issue contains a subject and author index which is annually cumulated and published in a separate issue. This, as well as a detailed classification allow for rapid location of abstracts.
The references in the subject index (computer-collated) are based on both the title and contents of the article.

- *Quotations on back volumes will be provided on request*
- *Specimen copies will be sent on request*
- *Annual subscription rate: US $50.00/ £20.95 Stg./Dfl. 180,00*

EXCERPTA MEDICA

119–123 Herengracht—Amsterdam—The Netherlands

N.Y. Acad. of Med. Bldg.—2 East 103rd Street—New York, N.Y. 10029

Chandos House—2 Queen Anne Street—London W1M 9LE

The Journal of General Microbiology

Edited by B. A. FRY, S. W. GLOVER, J. R. POSTGATE and M. F. MADELIN

The Journal of General Microbiology publishes original work on algae, bacteria, microfungi, protozoa and other micro-organisms with particular emphasis on general studies of these forms and their activities. The journal is published by Cambridge University Press for the Society for General Microbiology and the Proceedings of the Society are published in appropriate issues.

The Journal of General Microbiology is published fifteen times a year. Annual subscription £30 net. Please write for a specimen copy.

CAMBRIDGE UNIVERSITY PRESS

P.O. Box 92, London, NW1 2DB

32 East 57th Street, New York, N.Y. 10022

The Journal of Hygiene Volume 69, No. 3 September 1971

CONTENTS

PAGE

IVESON, J. B. Strontium chloride B and E.E. enrichment broth media for the isolation of *Edwardsiella*, *Salmonella* and *Arizona* species from tiger snakes . . . 323

SOBEY, W. R. AND CONNOLLY, DOROTHY. Myxomatosis: the introduction of the European rabbit flea *Spilopsyllus cuniculi* (Dale) into wild rabbit populations in Australia . . . 331

ROSS, P. W. Beta-haemolytic streptococci in saliva . . . 347

ROSS, P. W. Bacteriological monitoring in penicillin treatment of streptococcal sore throat . . . 355

ROBERTS, D. H. Interaction of porcine mycoplasmas with fresh animal serum . 361

HILLARY, IRENE, B. Persistence of antibody after subcutaneous vaccination with Wistar RA 27/3 rubella vaccine. . . . 369

STOCKS, PERCY. Multiple sclerosis distribution in England and Wales and parts of Europe . . . 373

GOODWIN, R. F. W. AND WHITTLESTONE, P. The comparative susceptibility of hysterectomy-produced, colostrum-deprived pigs and naturally born, enzootic-pneumonia-free pigs to enzootic pneumonia . . . 391

PEREIRA, MARGUERITE S. AND CANDEIAS, J. A. N. The association of viruses with clinical pertussis . . . 399

WAAIJ, D. VAN DER, BERGHUIS-DE VRIES, J. M. AND LEKKERKERK-VAN DER WEES, J. E. C. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice . . . 405

WENSINCK, F. AND RUSELER-VAN EMBDEN, J. G. H. The intestinal flora of colonization-resistant mice . . . 413

HAMMOND, B. J. AND TYRRELL, D. A. J. A mathematical model of common-cold epidemics on Tristan da Cunha . . . 423

CLOONAN, M. J., HAWKES, R. A. AND STEVENS, L. H. Postnatal decline of maternally acquired viral antibodies of different specificities . . . 435

TSUCHIYA, Y. AND TAGAYA, I. Sero-epidemiological survey on Yaba and 1211 virus infections among several species of monkeys . . . 445

MICHEL, M. F. AND PRIEM, CATHARINA C. Control at hospital level of infections by methicillin-resistant staphylococci in children . . . 453

HOYLE, L. AND ALMEIDA, JUNE D. The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. III. Effects of proteolytic enzymes . . . 461

FREESTONE, D. S., PRYDIE, J., SMITH, S. G. HAMILTON AND LAURENCE, G. Vaccination of adults with Wistar RA 27/3 rubella vaccine . . . 471

BROTHERSTON, J. G., BANNATYNE, C. C., MATHIESON, A. O. AND NICOLSON, T. B. Field trials of an inactivated oil-adjuvant vaccine against louping-ill (Arbovirus group B) . . . 479

MILLER, A. S. Salmonellosis in Botswana. I. Incidence in cattle . . . 491

KNIVETT, V. A. *Salmonella typhimurium* contamination of processed broiler chickens after a subclinical infection . . . 497

Printed in Great Britain at the University Printing House, Cambridge