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Winter vomiting disease in Florida students

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SUMMARY

On 14 November 1967 an investigation was begun at the University of South Florida to determine the cause of an increased number of gastrointestinal illnesses on campus. An estimated 300 students suddenly became ill with nausea, vomiting, abdominal pain, and diarrhoea. While at first food poisoning was suspected, no common food, water, or toxic exposure could be found. The data collected were most consistent with Winter Vomiting Disease. This entity is characterized by acute gastrointestinal illnesses which may occur in epidemic form in residential schools between September and March. Most investigators suspect that a viral agent is responsible for the illnesses, though attempts to isolate a virus have been unsuccessful. The incubation period in the University outbreak was estimated at 28 hr., and contact was the most likely mode of transmission. The outbreak illustrated that Winter Vomiting Disease in residential institutions may be an accentuation of the gastrointestinal illnesses occurring simultaneously in the community at large.

INTRODUCTION

Winter Vomiting Disease was first described by Zahorsky (1929). Subsequently, several large autumn and winter outbreaks of acute gastrointestinal illness have been reported, in which viral agents were suspected but could not be isolated (Reimann, Hodges & Price, 1945; Reimann, Price & Hodges, 1945; Gordon, Ingraham & Korns, 1947; Kuhns & Wetherbee, 1950; Webster, 1953; Haworth, Tyrrell & Whitehead, 1956; Walker *et al.* 1960; McLean, McNaughton & Wyllie, 1961; Pollock & Clayton, 1964). The incubation period and mode of spread of this disease are not established. The following report describes an outbreak of Winter Vomiting Disease in which the data collected support previous theories implicating contact as the most likely mode of spread, and permit an estimate of the incubation period. In addition, Winter Vomiting Disease in the residential school studied appeared to be only an accentuation of the gastrointestinal illnesses occurring simultaneously in the adjacent community.

DESCRIPTION OF THE OUTBREAK

On Tuesday 14 November 1967, the health department in Hillsborough County, Florida, was notified of a suspected 'food poisoning' epidemic at the University of

South Florida. An estimated 300 students had suddenly become ill with symptoms of nausea, vomiting, abdominal pain, and diarrhoea. Thirteen had been admitted to the infirmary. All thirteen were resident students who ate at the same cafeteria. The university officials suspected food poisoning related to that cafeteria.

MATERIALS AND METHODS

The University of South Florida has 11,500 students including 2750 resident students and 8750 commuters. The resident students live in 12 dormitories which are divided into two complexes: complex A includes the suspect cafeteria and three surrounding dormitories, and complex B includes the 'other' cafeteria and nine surrounding dormitories. Both cafeterias have the same commercial source of raw food products, but they prepare foods independently. According to infirmary records, the average number of students reporting to the infirmary with gastrointestinal symptoms, in previous winters, was eight per week.

Permission was granted to sample one of the three dormitories of complex A. Complex A contained the suspect cafeteria and included a total of 1300 students. Between 15 and 17 November, questionnaires were completed by 360 of 412 students in Alpha dormitory. Alpha dormitory was chosen by a random method from the three dormitories in the complex, and turned out to be a male dormitory. The questionnaire asked if the student had become ill in the last few days, asked for the date and time of onset of illness, and for the symptoms (nausea, vomiting, diarrhoea, chills, fever, stomach pain, other). The student was then asked where he had eaten his meals on Sunday 12 November and Monday 13 November, and a list was given of the foods served at the cafeteria on those days which was to be checked yes, no, or don't know, for each food.

For comparison purposes, similar questionnaires were completed by a dormitory in complex B. Complex B was thought, by the university authorities, to be relatively free from gastrointestinal illnesses. The questionnaires were completed by 245 of 246 students in the comparison dormitory, which had been chosen by a random method from the nine dormitories in the B complex. The comparison dormitory turned out to be a female dormitory.

Interviews were held with each of the ill students in the Alpha and comparison dormitories. To be counted as ill, a student had to indicate on the questionnaire that he had become ill in the last few days and check at least one of the following symptoms: nausea, vomiting, abdominal pain, diarrhoea.

Bacteriological and virological examinations were performed on a representative group of human and environmental specimens. Specimens from patients were obtained within 12 hr. of onset of symptoms.

Rectal swabs, vomitus specimens, and the left-over food were inoculated on to *Salmonella–Shigella* (S.S.) agar plates; Wilson Blair (W.B.) agar plates; tetrathionate broth and brilliant green (B.G.) agar plates. After 24 hr., suspect colonies were subcultured on triple-sugar-iron (T.S.I.) agar slants for identification. Anaerobic cultures were not done.

In addition, the vomitus specimens, swabs of skin lesions, and the left-over food

were inoculated on blood agar plates, eosin–methylene-blue (E.M.B.) plates and tellurite agar plates.

The water specimens were examined by the Millipore membrane filter technique (American Public Health Association, 1965).

Rectal swabs and throat washings (collected in 0·5 % lactalbumin hydrolysate) were inoculated on primary monkey kidney, HEp-2 (continuous human epithelial) and RU-1 (diploid human embryonic lung fibroblast) cells at 37° C. and observations made for cytopathic effect. A second blind passage was made in the HEp-2 and RU-1 cell cultures. The throat washings were also inoculated on primary monkey kidney cells at 32° C. Newborn mice were inoculated with the original specimens and observed for 14 days.

The monkey kidney tissue cultures were tested for haemadsorption with 0·4 % guinea-pig red cells seven days after inoculation of throat and stool specimens. Tissue culture fluids were harvested at nine days from the monkey kidney cultures and tested for hemagglutination with both guinea-pig and human-O red cells. On the ninth day of incubation, the medium was poured off and the monkey kidney tissue culture cells were challenged with 1000 TCID 50 of ECHO-11 virus. After incubation for three more days, the tests were read for the presence of an interfering agent.

The acute and convalescent sera were tested against the following antigens: influenza A, influenza B, parainfluenza 1, parainfluenza 3, adenovirus, and *Mycoplasma pneumoniae*.

During the course of the investigation, it was learned that illnesses similar to those at the University were occurring in the community at large. Local physicians were seeing many patients with characteristic acute gastrointestinal illnesses in which repetitive vomiting was a prominent feature. Therefore, a telephone survey was conducted on 20 December to determine the incidence of acute gastrointestinal illnesses in the Temple Terrace community, which surrounds the University. The Temple Terrace population is approximately 6750. A systematic sample, using a random start, was selected, and 500 phone calls were completed. Those called were read the following statement: 'Were you or other members of your immediate family ill since November 1st with symptoms of vomiting, diarrhoea, or abdominal pain lasting about 24 hours.' If the answer was yes, the respondent was asked how many were ill, whether the illness(es) occurred before or after Thanksgiving, and the approximate date. If the respondent himself had been ill, he was asked whether he had had nausea, vomiting, diarrhoea, or abdominal pain, with or without fever.

Statistical significance of differences in attack rates was tested by standard chi-square analysis.

RESULTS

Clinical illnesses

Eighty of the 360 students in Alpha dormitory reported that they had become ill between 10 November and 17 November, and checked at least one of the four symptoms of gastroenteritis listed. Four additional Alpha students reported that they had become ill within the last few days but couldn't remember the date, or

gave it as before 8 November. Of the 84 ill students, 57 (67 %) indicated that they had nausea and 63 (75 %) reported abdominal pain. Twenty-nine (35 %) reported vomiting, which was usually repetitive and extended over a period of 4–6 hr. Forty-eight students (57 %) reported diarrhoea, which usually followed the vomiting and consisted of two to three non-bloody, loose stools. Thirty (36 %) thought they had had fever and 27 (32 %) reported accompanying chills. The illnesses were self-limited and nearly all who were affected felt well within 24 hr. of the onset of symptoms.

The epidemic curve for the 84 ill Alpha students is shown in Fig. 1.

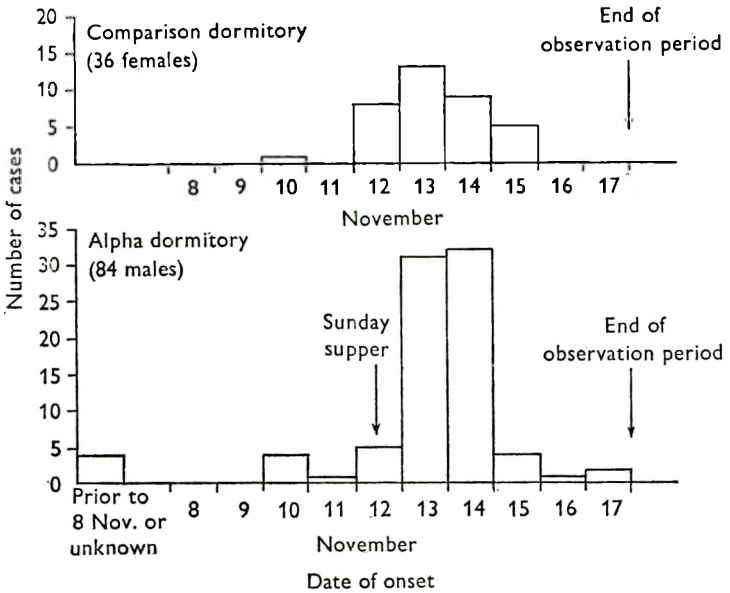

Fig. 1. Gastrointestinal illness by date of onset in resident U.S.F. students—Tampa, Florida, November 1967.

Analysis of the questionnaires obtained in the comparison dormitory revealed that gastrointestinal illnesses had occurred in complex B as well as in complex A. Thirty-six of the 245 (16 %) complex B respondents (females) reported illness between 10 and 17 November and checked at least one of the four symptoms of gastroenteritis listed. Nausea (58 %) and abdominal pain (77 %) were again the most commonly reported symptoms. Seven of the complex B students (20 %) reported vomiting, 11 (31 %) reported diarrhoea, 11 (31 %) reported chills but only two (5·5 %) thought they had had fever.

The epidemic curve for the 36 ill students in the comparison dormitory is also shown in Fig. 1. It was noted to be similar to the Alpha epidemic curve, despite the fact that the Alpha students and the students in the comparison dormitory eat in separate cafeterias. Also, additional cases continued to appear despite the fact that all left-over food had been discarded at the beginning of the investigation.

Laboratory studies

Six acutely ill infirmary patients, including five from complex A and one from complex B, had laboratory examinations of rectal swabs, throat washings, and acute and convalescent sera:

The rectal swabs were found negative for *Salmonella* and *Shigella.*

No viral isolate was made from the rectal swabs or throat washings in the culture systems used. The newborn mice likewise remained well throughout the observation period.

No haemagglutination or haemadsorption was noted, and no interfering agents were demonstrated.

No rise in titre was found in the acute and convalescent sera against the viral respiratory antigens tested, though constant titres to one or more antigens were found.

Two vomitus specimens from infirmary patients were found negative for *Salmonella*, *Shigella* and staphylococci.

In addition, rectal swabs on 8 female and 12 male outpatients and on all 30 employees in the suspect cafeteria were negative for *Salmonella* and *Shigella*. Three of the suspect cafeteria employees had small skin lesions. These lesions were cultured and all were positive for coagulase positive-staphylococci. Combination salad was the only food left over from meals served on Sunday and Monday, 12 and 13 November. It was negative for *Salmonella*, *Shigella* and staphylococci.

The University has an independent water supply consisting of five wells interconnected into one system with a one-half million gallon storage tank. Water samples taken, during the week of 13 November, from the suspect and 'other' cafeterias, the Alpha and control residence halls, and from the storage tank were all considered negative since they contained one or fewer coliform organisms per 100 ml.

Epidemiologic associations

No common food, water, or toxic exposure could be found to explain the illnesses in the Alpha dormitory. Attempts to associate illness with several small social events that had taken place at the University over the weekend were unsuccessful.

However, analysis of the 360 Alpha questionnaires for associations between illnesses and places and times of eating revealed one statistically significant ($p < 0{\cdot}001$) association (Table 1). Among Alpha students, eating supper on campus on Sunday and illness were associated. For those who ate in the suspect cafeteria, the attack rate was 32·5 %. For those 40 Alpha students who ate at the 'other' cafeteria, the attack rate was 27·5 %. Both attack rates were significantly different from the 10·3 % attack rate among those who ate that meal off campus.

Food history analysis was done for each food served at the suspect cafeteria on Monday 13 November and for each food served for supper on Sunday 12 November. The only statistically significant difference ($P < 0{\cdot}05$) in attack rates between eaters and non-eaters was for tossed salad served for Sunday supper. The food specific attack rates for that meal are shown in Table 2. Of 70 Alpha students who

ate tossed salad, 30 (42·8 %) became ill. Twenty-five of 99 (25·2 %) non-eaters of tossed salad became ill.

Table 1. *Associations in* 360 *Alpha (male) students between illness and eating place for supper, Sunday,* 12 *November,* 1967, *University of South Florida, Tampa, Florida*

Location of eating place	No. of students with or without acute G.I. illness: Ill	Not ill	Total no. students	Attack rates
Suspect cafeteria	54	112	166	32·5
Other cafeteria	11	29	40	27·5
Elsewhere on campus (infirmary, dormitory, coffee shop)	4	4	8	(50·0)
Off campus	15	131	146	10·3
	84	276	360	23·3

Table 2. *Food history analysis for suspect cafeteria, U.S.F., Sunday supper,* 12 *November*

Food or beverage	Persons who ate specified food: Ill	Not ill	Total	Attack rate (%)	Persons who did not eat specified food: Ill	Not ill	Total	Attack rate (%)
Ham	16	29	45	35·6	39	85	124	31·5
Chicken	15	42	57	26·3	40	72	112	36·7
Veal	17	37	54	31·5	38	77	115	33·0
Potatoes	32	68	100	32·0	23	46	69	33·3
Combination salad	9	26	35	25·7	46	88	134	34·3
Tossed salad	30	40	70	42·8	25	74	99	25·2
Carrots	9	18	27	33·3	46	96	142	32·3
Cabbage	10	13	23	43·5	45	101	146	30·8
Corn	20	40	60	33·3	35	74	109	32·1
Cake	19	59	78	24·4	36	55	91	39·6
Pie	14	25	39	35·9	41	89	130	31·5
Pudding	5	12	17	29·4	50	102	152	32·9
Jello	21	43	64	32·8	34	71	105	32·3
Coffee	2	9	11	18·1	53	105	158	33·5
Tea	16	47	63	25·3	39	67	106	36·8
Milk	19	32	51	37·3	36	82	118	30·5
Fruitade	19	30	49	38·8	36	84	120	30·0

In the comparison group no association could be found between illnesses and eating place or between illnesses and foods eaten. Food history analysis done for each food served at the corresponding meals at the 'other' cafeteria on Sunday and Monday, 12 and 13 November, revealed no statistically significant difference ($P < 0{\cdot}05$) in attack rates between eaters and non-eaters for any food. Individual interviews held with the 36 ill students in the comparison dormitory revealed, however, that 10 of the 36 (28 %) were room-mates. Twenty of the 36 (56 %) were suite-mates, and 26 of the 36 (72 %) were suite-mates or had had more than casual

contact with an ill person within 48 hr. of becoming ill herself. Two of the 36 ill students in the comparison group had been off campus for the weekend, returned to an ill room-mate on Sunday night, and became ill an average of 26 hr. after return.

Table 3. *Incidence of acute gastrointestinal illness in Temple Terrace, Florida, 1 November, 1967—20 December, 1967**

Population group	Time interval	Number	No. ill	Incidence (%)
Total sample	1–23. xi. 67	1802	199	11·1
Total sample	1. xi.–20. xii. 67	1802	374	20·8
1 and 2 member households	1. xi.–20. xii. 67	276	31	11·2
3 and 4 member households	1. xi.–20. xii. 67	791	175	22·1
5 and more member households	1. xi.–20. xii. 67	735	168	22·9

* Via telephone survey of 500 households, 20 December 1967.

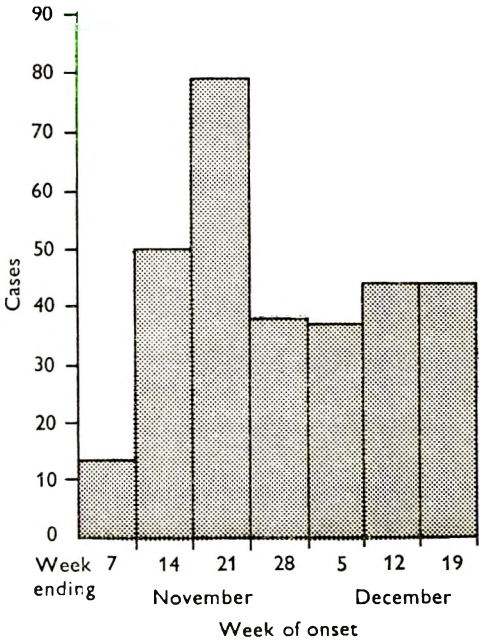

Fig. 2. Gastrointestinal illness by week of onset, Temple Terrace, Florida, 1 November to 20 December 1967.

The results of the community survey are shown in Table 3. One hundred and ninety-nine of 1802 persons questioned (11·1 %) reported gastrointestinal symptoms between 1 and 23 November (Thanksgiving). During the 7-week period of 1 November to 20 December, 374 persons (20·8 %) reported such symptoms. The attack rates were higher in the larger families. Ninety-three respondents had illnesses themselves. Of these, 55 % reported nausea, 63 % abdominal pain, 46 % vomiting, 71 % reported diarrhoea, and 41 % reported fever. Of the 374 ill persons an approximate date of the onset of symptoms could be obtained in 305. The

epidemic curve for these 305 persons is shown in Fig. 2. The peak of the curve occurred at the same time that the outbreak occurred at the University.

DISCUSSION

The data collected at the University of South Florida were inconsistent with the diagnosis of food poisoning. The inability to culture a typical pathogen from food or ill students, the finding of a significant number of ill students who had not had a common meal exposure, and the continued appearance of cases, all argued against food poisoning as the explanation for the outbreak. Actually two observations had suggested food poisoning: the staphylococcal isolations from the hands of the three suspect cafeteria employees, and the statistical association of tossed salad with illnesses. However, two of the three employees were not involved in food handling, and the third, a salad maker, was off duty on Sunday 12 November, when the above-mentioned salad was prepared and served. Of the 70 in Alpha who ate tossed salad, 30 became ill. The latter number is insufficient to explain the 84 illnesses in Alpha. Also, the tossed salad, prepared fresh for each meal and kept on ice until served, was an unlikely source of bacterial contamination with human pathogens. Finally, the combination salad, prepared by the same person who prepared the tossed salad, was negative on culture for *Salmonella*, *Shigella* and staphylococci. When the Alpha data were added to the data from the comparison group and both were added to the information obtained from the community at large, no common food, water, social, or toxic exposure could be found to explain the characteristic illnesses which had occurred.

The illnesses seen at the University were most consistent with Winter Vomiting Disease. This syndrome is characterized by acute gastrointestinal illnesses, which may occur in epidemic form in residential schools or institutions. The outbreaks have nearly always taken place between September and March. Numerous reports (Zahorsky, 1929; Miller & Raven, 1936; Gray, 1939; Bradley, 1943; Reimann *et al.* 1945*a*; Gordon *et al.* 1947; Hargreaves, 1947; Kuhns & Wetherbee, 1950; Ingalls & Britten, 1951; Webster, 1953; Simpson, 1954; Haworth *et al.* 1956, Pollock & Clayton, 1964; Cumming & McEvedy, 1969) in the British and American literature over the past three decades have described epidemics of Winter Vomiting Disease (synonyms are epidemic vomiting, epidemic gastroenteritis, epidemic nausea and vomiting, and epidemic diarrhoea and vomiting), and the subject has been reviewed in recent years (Editorial, 1969; Webb & Wallace, 1966). Many of the outbreaks were initially thought to be food poisoning.

The etiology of Winter Vomiting Disease has never been established, though most investigators suspect that a viral agent is responsible. Filtrable agents including the enteroviruses (Cheever, 1967) have been associated with viral dysentery, the term Reimann (1963) uses to include Winter Vomiting Disease and its synonyms, but attempts to isolate and identify a virus in several large-scale autumn and winter school and institutional outbreaks have been unsuccessful (Webster, 1953; Haworth *et al.* 1956; Pollock & Clayton, 1964).

The mode of transmission of Winter Vomiting Disease has not been definitely

established. Hargreaves (1947), discussing an epidemic which occurred in a Cornwall institution, and which affected certain wards but spared others, concluded that the 'infection is airborne'. Ingalls & Britten (1951), describing an epidemic in a school for boys, felt that their data supported dissemination through personal contact. Gordon (1955) agreed that the usual mode of spread was by person to person contact. Cumming & McEvedy (1969), after investigating a recent outbreak at a Reading University residence hall, thought that a food-borne agent was the most likely explanation for Winter Vomiting Disease, but spread by contact was also mentioned as a possibility. Our data suggest contact* as the most likely mode of transmission.

The evidence for contact as the mode of transmission in this University outbreak includes:

(1) The higher attack rate among those Alpha students congregating on campus for supper on Sunday compared with those who ate off campus. Congregating in either cafeteria appeared equally significant.

(2) The high degree of contact among the 36 ill comparison students.

(3) The two comparison students who were off campus for the weekend but who became ill following contact with an ill room-mate.

(4) The higher attack rates in the larger families in the community, characteristic of diseases spread by contact.

Spread by contact, probably via droplet spread in the suspect and 'other' cafeterias on Sunday, would best explain the majority of the Alpha illnesses. Contact would also appear to be the most likely mode of spread in the comparison group.

Clearly not all of the 84 ill Alpha students became infected at the Sunday supper. Fifteen of the 84 were not on campus for that meal. Several of the Alpha students had their onset of symptoms within a few hours of that meal and were most likely infected elsewhere. Yet there was a strong statistical association between illness in Alpha students and eating at the suspect cafeteria for supper on Sunday, which could not be explained by infection from a common food source. It would seem most likely that this association occurred because of the contact between the Alpha students that occurred at that meal. Many students return to the campus for Sunday supper after being off campus for part or all of the weekend. Assuming that contact (direct, indirect, or droplet spread) at that meal produced the association with illness, a rough estimate of the incubation period of Winter Vomiting Disease at the University can be made as follows: There were 54 ill Alpha students who were at that meal; the median incubation period among those who became ill after the meal was 28 hr. The latter figure corresponded to the 26 hr. estimated incubation period in the two control students who were away for the weekend, and to the experimental incubation period of 27 hr. which Jordan, Gordon & Dorrance (1953) found in studies of epidemic non-bacterial gastroenteritis in Cleveland.

Reimann *et al.* (1945*b*) were able to transmit epidemic gastroenteritis to volunteers by both inhalation and ingestion of bacteria-free faecal filtrates. Gordon *et al.*

* Contact as defined by the 10th edition of the *A.P.H.A. Manual, Control of Communicable Diseases in Man*, includes direct contact, indirect contact, and droplet spread.

(1947) were able to transmit the disease from a New York institutional outbreak by feeding bacteria-free stool filtrates to a group of volunteers. Jordan *et al.* (1953), in a series of experiments in a group of Cleveland families, found that they too were able to transmit the disease to volunteers. They concluded that there are at least two different types of acute, infectious, non-bacterial gastroenteritis; an afebrile type and a febrile type.

The afebrile type, produced by the Marcy agent obtained in New York (Gordon *et al.* 1947), had an experimental incubation period of 60 hr. It was characterized by profuse, watery diarrhoea and no fever. The illnesses lasted as long as a week.

The febrile type, caused by the (FS) agent obtained in Cleveland, was characterized by fever, abdominal pain, and constitutional symptoms. Vomiting was a prominent feature. The (FS) symptoms were of short duration, with complete recovery usually occurring within 24 hr. of onset. The University of South Florida outbreak and the descriptions of Zahorsky correspond best to Jordan's (FS) type illness. It was the (FS) type illness that Jordan was able to transmit to volunteers with an experimental incubation period of 27 hr. Our estimate in a natural epidemic of an incubation period of 28 hr. would tend to support Jordan's work.

The results of the community survey suggest that Winter Vomiting Disease in the University population was merely an accentuation, probably because of close contact, of the gastrointestinal illnesses which were occurring simultaneously in the community at large. Webb & Wallace (1966) state in their review that 'epidemic gastroenteritis, presumably viral in etiology, is probably the most prevalent gastrointestinal disorder in this country after one year of age'. Thus it is not surprising that the attack rates at the University and in the community were so high.

A complete understanding of Winter Vomiting Disease will probably not be attained until the etiologic agent (or agents) is isolated and identified. Vigorous efforts towards such isolation are advocated by Cheever (1967) in his recent review of the subject of viral agents in gastrointestinal disease.

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REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION (1965). *Standard Methods for the Examination of Water and Waste Water*, 12th ed. p. 610, New York.

BRADLEY, W. H. (1943). Epidemic nausea and vomiting. *British Medical Journal* i, 309–12.

CHEEVER, F. S. (1967). Viral agents in gastrointestinal disease. *Medical Clinics of North America* **51**, 637–41.

CUMMING, J. D. & MCEVEDY, C. P. (1969). An outbreak of 'Winter Vomiting Disease' in a university hall of residence. *Journal of Hygiene*, **67**, 147–52.

EDITORIAL, EPIDEMIC VOMITING (1969). *British Medical Journal* i. 327.

GORDON, I., INGRAHAM, H. S. & KORNS, R. F. (1947). Transmission of epidemic gastroenteritis to human volunteers by oral administration of fecal filtrates. *Journal of Experimental Medicine* **86**, 409–22.

GORDON, I. (1955). The nonamebic nonbacillary diarrheal disorders. *American Journal of Tropical Medicine and Hygiene* **4**, 739–55.

GRAY, J. D. (1939). Epidemic nausea and vomiting. *British Medical Journal* i, 209–11.

HARGREAVES, E. R. (1947). Epidemic diarrhoea and vomiting. *British Medical Journal* i, 720–2.

HAWORTH, J. C., TYRRELL, D. A. J. & WHITEHEAD, J. E. M. (1956). Winter vomiting disease with meningeal involvement. *Lancet* ii, 1152–4.

INGALLS, T. H. & BRITTEN, S. A. (1951). Epidemic diarrhea in a school for boys. *Journal of the American Medical Association*. **146**, 710–12.

JORDAN, W. S., GORDON, I. & DORRANCE, W. R. (1953). A study of illness in a group of Cleveland families. VII. Transmission of acute non-bacterial gastroenteritis to volunteers: Evidence for two different etiologic agents. *Journal of Experimental Medicine* **98**, 461–75.

KUHNS, D. M. & WETHERBEE, D. G. (1950). An epidemic of vomiting and diarrhea. *United States Armed Forces Medical Journal* **1**, 861–5.

MCLEAN, D. M., MCNAUGHTON, G. A. & WYLLIE, J. C. (1961). Infantile gastroenteritis: Further viral investigations. *Canadian Medical Association Journal* **85**, 496–7.

MILLER, R. & RAVEN, D. M. (1936). Epidemic nausea and vomiting. *British Medical Journal* i, 1242–4.

POLLOCK, G. T. & CLAYTON, T. M. (1964). Epidemic collapse: A mysterious outbreak in three Coventry schools. *British Medical Journal* ii, 1625–7.

REIMANN, H. A., HODGES, J. H. & PRICE, A. H. (1945). Epidemic diarrhea, nausea, and vomiting of unknown cause. *Journal of the American Medical Association* **127**, 1–6.

REIMANN, H. A., PRICE, A. H. & HODGES, J. H. (1945). The cause of epidemic diarrhea, nausea and vomiting. *Proceedings of the Society for Experimental Biology and Medicine* **59**, 8–9.

REIMANN, H. A. (1963). Viral dysentery. *American Journal of the Medical Sciences* **246**, 404–9.

SIMPSON, A. S. (1954). Epidemic or seasonal vomiting. *Medical Officer* **91**, 175–6.

WALKER, S. J., MCLEAN, D. M., ROY, T. E., MCNAUGHTON, G. A. & TIBBLES, J. A. R. (1960). Infantile gastroenteritis: A search for viral pathogens. *Canadian Medical Association Journal* **83**, 1266–7.

WEBB, C. H. & WALLACE, W. M. (1966). Epidemic gastroenteritis presumably viral. *Pediatrics* **38**, 494–8.

WEBSTER, R. C. (1953). A large outbreak of epidemic vomiting. *Medical Officer*, **90**, 39–40.

ZAHORSKY, J. (1929). Hyperemesis hiemis or the Winter Vomiting Disease. *Archives of Pediatrics* **46**, 391–5.

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Antistreptolysin O titres amongst children in a rural area of Ceylon

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SUMMARY

Antistreptolysin O titres in the sera of children in a rural population of Ceylon were determined, to establish the upper limit of the normal ASO titres in non-rheumatic children, and to compare these ASO titres with results of surveys done in other countries.

Swabs were taken from throats and ulcers of the children examined and cultured specifically for group A haemolytic streptoccoci. Of a total of 257 children, 29·5 % had ASO titres of over 166 Todd units. The greatest number of children showed ASO values between 100 and 166 units. The percentage of children showing values over 166 units increased with age until a maximum of 54 % was reached between 9 and 10 years. Group A haemolytic streptococci were isolated from five throat swabs, but there were no isolations from ulcers. The values obtained from this survey have been compared with those from a few other developing countries.

INTRODUCTION

Group A haemolytic streptoccoci, which may be associated with rheumatic fever, produce a toxin, streptolysin O, which evokes the production of streptolysin O antibodies in the host (Todd, 1932). The antistreptolysin O (ASO) titre in the blood can be accurately measured with reliable and reproducible results (Hollinger, 1953) and high values indicate past infection with group A haemolytic streptococci.

An increase in the incidence of rheumatic fever and chronic rheumatic heart disease has been observed in Ceylon during the last few years (Reports of the Epidemiologist, Ceylon, release no. 23). Of 8094 blood samples sent from suspected cases of rheumatic fever from various parts of the island for the determination of antistreptolysin O titres, 66 % showed values of over 300 Todd units. No survey has been carried out in Ceylon to determine the ASO titres in healthy (non-rheumatic) children. It was therefore decided that the determination of the ASO titres in the sera of healthy children of various age groups would be helpful in obtaining evidence of the incidence of past streptococcal infections, and also in establishing the upper limit of the 'normal' ASO titre in non-rheumatic children.

MATERIALS AND METHODS

A rural area (Udahamulla) close to Colombo was chosen for the purpose of this survey. This village has a population of *ca.* 2800 in an area of about 1 square mile. Children with any history of swelling of joints, swelling or puffiness of the face and feet, or any evidence of genito-urinary disease were excluded from the survey after information was obtained from the parents. Those with any cardiac murmurs were also excluded. Blood samples were collected at the infant clinic, preschool clinic, milk feeding centre and in the junior school of the area. Throat swabs were taken on dry, sterile, cotton-wool swabs from all the children at the time of blood sampling. Swabs were also taken from any ulcers found on the legs or hands. A note was also made of any healed, multiple ulcers on the body. Sera from the blood samples were frozen on the day of collection and stored at $-20°$ C. The throat swabs were plated on blood-agar within a few hours. β-haemolytic streptococcal colonies were picked up after overnight aerobic incubation of the plates at 37° C. and tested for sensitivity to Bacitracin using 2 μg. strength Bacitracin disks on blood-agar plates.

The antistreptolysin O titre determinations were carried out using 'Wellcome' brand of reduced streptolysin O (dried) by the method based on that of Rantz & Randall (1945). Sheep cells were used as indicator. A control containing a known strength of antistreptolysin O ('Wellcome' brand) was used in parallel with every batch of sera tested.

RESULTS

The results of the antistreptolysin titre determinations are shown in Table 1 and Fig. 1. It is seen that 29·5 % of a total number of 257 children tested showed ASO titres of over 166 Todd units. Only 7 % showed values higher than 250 units. There were none over 333. The greatest number of children (40 %) were between the levels 100– < 166 units. None of the infants below 1 year showed a titre of over

Table 1. *Results of the antistreptolysin O survey*

		ASO titres, Todd units/ml.						
Age in years	Total no. examined	Below 50	50– < 100	100– < 166	166– < 250	250– < 333	⩾ 333	Percentage ⩾ 166
0– < 1	16	11	3	2	0	0	0	0
1– < 2	22	11	3	5	2	1	0	14
2– < 3	22	12	2	7	0	1	0	5
3– < 4	24	4	2	11	5	2	0	29
4– < 5	17	3	4	8	2	0	0	12
5– < 6	23	3	1	15	4	0	0	17
6– < 7	22	5	1	9	5	2	0	32
7– < 8	23	2	2	11	5	3	0	35
8– < 9	20	3	1	6	10	0	0	50
9– < 10	26	0	1	11	9	5	0	54
10– < 11	22	2	0	10	5	5	0	45
11– < 12	20	0	3	7	10	0	0	50
Total	257	56	23	102	57	19	0	29·5
%	—	22	9	40	22	7	—	—

166 units. In fact most of these showed values below 50 units. The percentage of children showing values over 166 units increased with age until a maximum (54 %) was reached between 9 and 10 years. (See Fig. 2). After an erratic beginning the graph showed a steady rise after the 5 year age group.

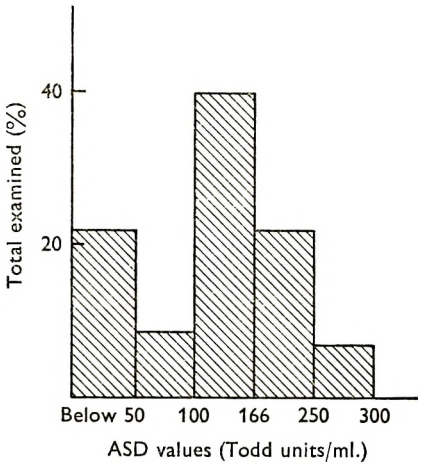

Fig. 1. Percentage of children showing different ASO values.

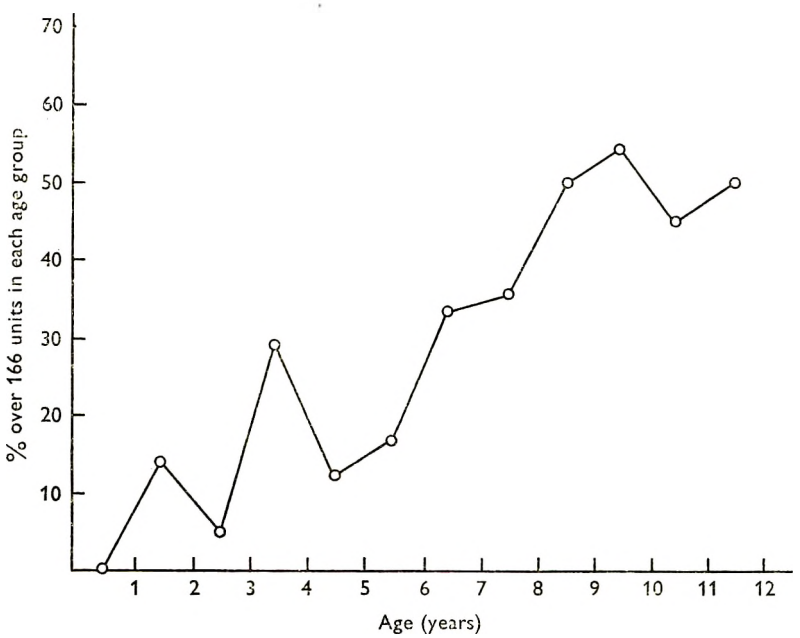

Fig. 2. Percentage of children in each age group showing ASO values of over 166 units/ml.

Group A haemolytic streptococci were isolated from five throat swabs taken from the children. The ASO titres of these five children were, however, below 166 units. There were no streptococci isolated from five swabs taken from ulcers.

DISCUSSION

Following an acute streptococcal infection, the ASO titre rises to a maximum within 2–4 weeks. The return to normal is, however, much slower and may take 6–12 months (Stollerman, Lewis, Schultz & Taranta, 1956). The sera of most healthy adults contain a certain amount of antistreptolysin O due to past infection. Most workers are agreed that the upper limit of normality in adults is about 200 Todd units (Williams, 1958). Different writers have, however, quoted widely different values as being normal. Coburn & Pauli (1935) who carried out a survey among adults in a New York hospital found a 'natural human level' of *ca.* 50 units. Rantz, Di Caprio & Randall (1952) working in San Francisco found a mean value of 150 units in healthy children between the ages of 5 and 7 years. The mean ASO titre in children between 8 and 12 years was 184. The results of the present survey are almost in complete agreement with those of Rantz *et al.* (1952). Thus the mean ASO value amongst the children in the 5–7 age group is 166 units, while those between 8 and 12 years show a mean value of 207 units. The highest mean value appears in late childhood (9–10 years of age). This is also in agreement with the findings of Rantz *et al.*

An analyis of the cases showing an antistreptolysin titre of over 166 units in each age group is shown in Table 1. None of the children below the age of 1 year showed ASO titres of over 166 units. Being a γ-G globulin, antistreptolysin O would pass the placental barrier and one would expect at least a few infants in the neo-natal period to show a raised titre in their blood. Unfortunately the cases included under 1 year did not have any infants below 6 months of age. The steady rise in the graph after 5 years may be due to the fact that children in Ceylon start school at 5 years, and that the crowded schools provide a suitable environment for the spread of infection. The highest value of 54 % is found in children between 9 and 10 years. Although there is a slight drop after 10 years, the values in adults were not measured to determine whether this high titre is maintained throughout adult life.

These figures may be compared with surveys done in a few other developing countries. Rotta *et al.* (W. H. O. Surveillance Reports, CES/SR/66·4) found that in an urban area of Pakistan, 13 % of children under 10 years showed ASO values of over 199 units. The same workers found the rate amongst a rural population in Thailand to be 17 %, whilst a rate of 56 % was found in eastern Nigeria in children of the same age group. The present survey shows that 26 % of the children below 10 years have ASO values of over 166 units. Dunbar & Erwa (1967) in a recent study in Khartoum (Sudan) found that 48 % of children between 7 and 11 years of age had values of over 166 units. The present survey where 46 % of the children in the corresponding age group show values of over 166 units, is in agreement with their findings.

The haemolytic streptococci from the swabs taken from the children were isolated from blood-agar plates grown aerobically at 37° C., and only the group A streptococci were selected by the bacitracin test. β-haemolysis itself is a variable character and anaerobic culture of the plates might have yielded more haemolytic

colonies (Wilson & Miles, 1966). Variant type 12 of group A which produce haemolysis at 22° C. only, isolated by Coburn & Pauli (1941) from an epidemic of respiratory infection, produce O streptolysin. These were reported to have a higher degree of infectivity than the normal haemolytic streptococci. More positive cultures of streptococci might have been obtained if primary cultures were done in an enrichment medium (Williams, 1958). Apart from group A streptococci, for which a specific search was made, there may have been other groups which produce streptolysin O. Group C and G streptococci which are known to infect human beings produce streptolysin O. Mallen, Evans & Balcazar (1957) report that they isolated group G haemolytic streptococci from 11 % of people examined in a tropical climate.

The problem of high ASO titres in populations with a low rate of isolation of haemolytic streptococci has been observed by other workers. Dunbar & Erwa (1967) found that lesst han 1 % of children amongst whom 48 % had ASO titres of over 166 units had haemolytic streptococci in their throats. Mallen *et al.* (1957) could isolate group A haemolytic streptococci from throat swabs of only three out of 102 people whereas 55 % showed high ASO values.

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REFERENCES

Coburn, A. F. & Pauli, R. H. (1935). Studies on the immune response of the rheumatic subject and its relationship to activity of the rheumatic process. *Journal of Experimental Medicine* **62**, 129.

Coburn, A. F. & Pauli, R. H. (1941). The interaction of host and bacterium in the development of communicability by *Streptococcus haemolyticus*. *Journal of Experimental Medicine* 73, 551.

Dunbar, J. M. & Erwa, H. H. (1967). Antistreptolysin titres in school children in Khartoum. *Bulletin of the World Health Organization* **37**, 492.

Hollinger, N. F. (1953). Antistreptolysin-O serum levels. *American Journal of Public Health* **43**, 561.

Mallen, M. S., Evans, M. & Balcazar, J. (1957). Further studies in rheumatic fever epidemiology. *American Heart Journal* **53**, 767.

Rantz, L. A., Di Caprio, J. M. & Randall, E. (1952). Antistreptolysin O and antihyaluronidase titres in health and in various diseases. *American Journal of the Medical Sciences* **224**, 194.

Rantz, L. A. & Randall, E. (1945). A modification of the technic for determination of the antistreptolysin titre. *Proceedings of the Society for Experimental Biology and Medicine* **59**, 22.

Stollerman, G. H., Lewis, A. J., Schultz, I. & Taranta, A. (1956). Relationship of immune response to group A streptococci to the cause of acute, chronic and recurrent rheumatic fever. *American Journal of Medicine*, **20**, 163.

Todd, E. W. (1932). Antihaemolysin titres in haemolytic streptococcal infection and their significance in rheumatic fever. *British Journal of Experimental Pathology* **13**, 248.

Wilson, G. S. & Miles, A. A. (1966). *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th ed. p. 699. London: Arnold.

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

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The role of normal skin in the spread of streptococcal pyoderma

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SUMMARY

The primary body site of acquisition of group A streptococci was examined prospectively in a population with endemic streptococcal pyoderma. Weekly cultures were obtained during the skin infection season from apparently normal upper respiratory and cutaneous sites (and from skin lesions when present) in 44 children and adults living on the Red Lake Indian Reservation.

During the 9-week period of the study 705 of a total of 2305 cultures were positive for group A streptococci. The percentage of positive cultures from the various sites were: throat (20%); nose (24%); wrist (32%); ankle (35%); back (22%); and skin lesions (81%). Group A streptococci were also isolated from fingernail dirt, clothing and bedding as well as from a few household pets and insects.

Analysis of serial cultures obtained from the same individuals at weekly intervals suggested that the strains isolated from skin lesions first appeared on normal skin in the 2 weeks preceding the lesion. Spread to the nose and throat followed skin acquisition and/or skin lesions.

The high prevalence of group A streptococci on normal skin in the absence as well as the presence of pyoderma, and their appearance on normal skin before recovery from either skin lesions or the upper respiratory tract are consistent with the view that skin acquisition was a primary predisposing factor to pyoderma. Since the literature indicates that group A streptococci are rarely part of the normal skin flora, these findings raise the possibility of unique biological properties of these and perhaps other pyoderma strains, as distinct from other group A streptococci.

INTRODUCTION

In streptococcal infections of the upper respiratory tract, considerable epidemiological evidence points to the importance of human reservoirs, particularly nasal carriers, in the dissemination and transmission of infection (Hamburger, Green & Hamburger, 1945; Hamburger & Green, 1946; Wannamaker, 1954). In streptococcal pyoderma, the source and the primary site of infection are less certain and their determination is complicated by the observation that individuals with skin

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infection may also harbour the infecting strain in the upper respiratory tract (Anthony, Perlman & Wannamaker, 1967*a*; Futcher, 1940). During a recent outbreak of post-streptococcal nephritis on the Red Lake Indian Reservation in northern Minnesota, U.S.A. (Anthony *et al.* 1967*b*), the epidemic strain first became prevalent in skin lesions and subsequently in the upper respiratory tracts of the population involved. Although this study suggested that the upper respiratory tract was an unlikely source of the streptococci causing pyoderma, it left unclear the origin of streptococci recovered initially in skin lesions. Despite the prevailing opinion in the literature that group A streptococci are rarely found on normal skin (Williams, 1965; Kligman, 1965) normal skin still seemed to be a possible site for acquisition of streptococci on the body before their appearance in skin lesions. In the summer of 1968 this hypothesis was tested during a prospective study of streptococcal pyoderma on the Red Lake Indian Reservation.

MATERIALS AND METHODS

Forty-four individuals from five families participated in the study. Beginning 1 July, weekly visits were made to each household for a 9-week period, and each participant had cultures taken of his anterior nares, throat, three normal skin sites (volar surface of one wrist, the medial aspect of one ankle and the middle of the back) and skin lesions if present. Cultures of fingernail dirt, personal clothing, bedding, throat cultures of household pets as well as cultures of a variety of insects trapped in or around each house were also obtained. Normal skin cultures and cultures of bedding and clothing were taken by moistening a cotton swab in Todd–Hewitt broth, rubbing a small, approximately 2-in. square, area of the skin or clothing surface firmly for several seconds and then immediately inoculating the swab on a sheep blood agar plate containing 0·1 % crystal violet. Fingernail dirt from beneath one or two nails was collected in sterile screw cap vials and later suspended in 2 ml. of Todd–Hewitt broth. After overnight incubation at 37° C., a loopful of the broth culture was streaked on blood agar plates containing crystal violet. Insects were collected and processed intact in a similar manner. All other cultures were processed and beta-hemolytic streptococci classified serologically* as previously described (Anthony *et al.* 1967*b*). Sub-cultures of three individual colonies were made from each primary plate and were identified serologically.

RESULTS

During the 9 weeks of this study a total of 2305 cultures were obtained, and of these 705 (30·6 %) were positive for group A streptococci. Between 230 and 275 cultures were obtained each week from 44 persons and their environment. The results of T-agglutination patterns and M-precipitation reactions indicated that 96·3 % of all group A streptococci isolated belonged to two different strains.

* Grouping sera as well as anti-T and anti-M sera were obtained from the Communicable Disease Center, Atlanta, Georgia. Additional anti-T sera were generously supplied by Dr M. T. Parker and Mr W. R. Maxted, Central Public Health Laboratory, Colindale, London, England.

Although these strains are not completely characterized they do possess sufficiently distinct markers to permit epidemiologic analysis (Table 1). The first of these two pyoderma strains, designated 'satellite strain', is readily distinguished by its failure to grow aerobically on routine sheep blood agar* except when the organisms appear as satellites around a variety of other bacterial species. Studies of the growth characteristics and nutritional requirements of this strain will be the subject of a separate report (S. S. Chapman, unpublished observations). Shortly

Table 1. *Characteristics of the two pyoderma strains*

	Percentage of total group A streptococci recovered	T-agglutination pattern	M-precipitation reaction
'Satellite strain'	62·7	3/B3264	43
'Other pyoderma strain'	33·6	14/3/B3264	41, 43

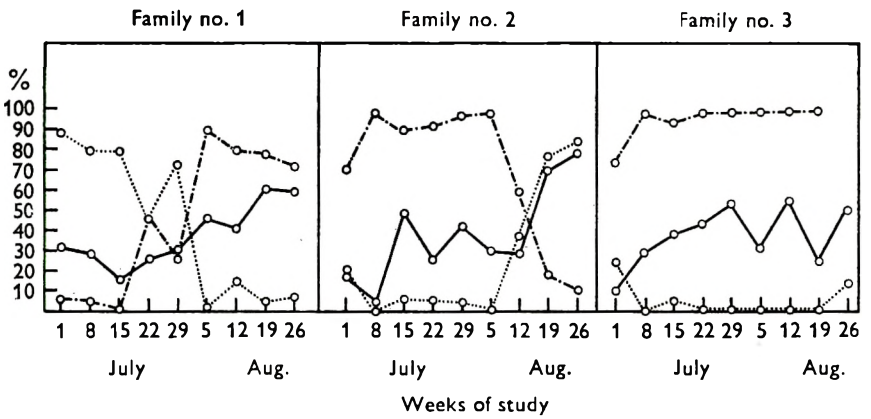

Fig. 1. Weekly recovery of group A streptococci in three families showing the percentage of total cultures positive for group A streptococci and the percentage of group A strains belonging to the 'satellite' and 'other pyoderma' strains. Weeks of study are indicated by the first day of the work week (7/1 = July 1, etc.). —, Percentage positive for group A streptococci; —·—, percentage positive for group A, satellite strain; . . . . . , percentage of group A, other pyoderma strain.

after this strain was encountered during the first week of the study, trypticase soy sheep blood agar was modified by the addition of 1% Todd–Hewitt broth which permitted good aerobic growth. The 'satellite strain' gave a T-agglutination pattern of 3/B3264 and an M-precipitation reaction with type 43 M antisera. Further tests, including indirect bactericidal tests, will be required to determine whether the 'satellite strain' is an M type 43. The second strain, referred to as the 'other pyoderma strain', grew well on blood agar, showed a T-agglutination

* Routine sheep blood agar at the time of initiation of these studies was prepared from commercial trypticase soy blood agar base (Baltimore Biological Laboratories) plus 6% sheep blood.

pattern of 14/3/B3264 and gave precipitation reactions with both types 41 and 43 M antisera.

The isolation patterns of these two strains from three of the families studied are shown in Fig. 1. For each family the patterns are distinctly different. In Family No. 1 the 'other pyoderma strain' predominated at the outset but was replaced by the 'satellite strain' during the latter half of the summer. In Family No. 2 the converse was true. In Family No. 3 the 'satellite strain' predominated throughout the summer, accounting for 75–100 % of all group A streptococci isolated each week.

Table 2. *Percentage of cultures positive for group A streptococci at various sites*

Culture site	All subjects (368 culture visits)	Without streptococcal pyoderma (297 culture visits)	With streptococcal pyoderma (71 culture visits)
Throat	20·8	20·7	19·7
Nose	24·4	22·3	33·8
Wrist	32·8	27·6	54·9
Ankle	35·5	30·9	54·9
Back	21·8	19·5	33·8

One or both pyoderma strains were isolated at some time from 43 of the 44 persons who participated in the study. Of these, 34 were children under 15 years of age. Thirty-three percent of all cultures taken from this age group were positive. Ten teenagers and adults participated in the study and considerably fewer of their total cultures were positive (17·5 %).

Table 2 shows the percentage of cultures positive for group A streptococci at individual culture sites. The term 'culture visit' refers to one complete set of cultures obtained from one person during one weekly visit. When all subjects were considered, the highest percentage of positive cultures came from the wrist, 32·8 %, and ankle, 35·5 %. The data were also analysed to permit separate consideration of those culture visits associated with pyoderma and those not associated with streptococcal pyoderma in the individual. The percentages of positive cultures from the nose and from all three normal skin sites are definitely higher when streptococcal pyoderma is present. This suggests that endogenous shedding (Williams, 1965) may be one factor resulting in positive normal skin cultures. However, in the absence of pyoderma, there is still a significantly higher prevalence of group A streptococci on normal skin (wrist and ankle) than in the upper respiratory tract based on observed and expected frequencies of positive cultures at all sites ($P = < 0{\cdot}01$).

Of 368 culture visits, 233 (62·2 %) were associated with recovery of group A streptococci from one or more apparently normal body sites. When consideration is limited to these 233 positive culture visits (Table 3), the greatest percentage (77·3 %) was associated with recovery of group A streptococci from normal skin at one or more sites whereas fewer of such visits were associated with positive

cultures from the upper respiratory tract and skin lesions. Furthermore, over one-third of the positive normal skin cultures occurred at a time when streptococci were *not* recovered from any other site (Table 3, footnote). The two pyoderma strains accounted for virtually all of the group A isolations from normal skin sites and skin lesions.

Table 3. *Frequency of recovery of organisms at various sites when present at some site*

Site of recovery	Group A streptococci (233 'culture visits')	Two pyoderma strains (216 'culture visits')
Upper respiratory tract (nose and/or throat)	125 (53·6 %)	104 (48·1 %)
Normal skin (one or more of three sites)	180* (77·3 %)	176 (81·5 %)
Skin lesions	71 (30·5 %)	70 (32·4 %)

* 66/180 associated with recovery of organisms from normal skin only.

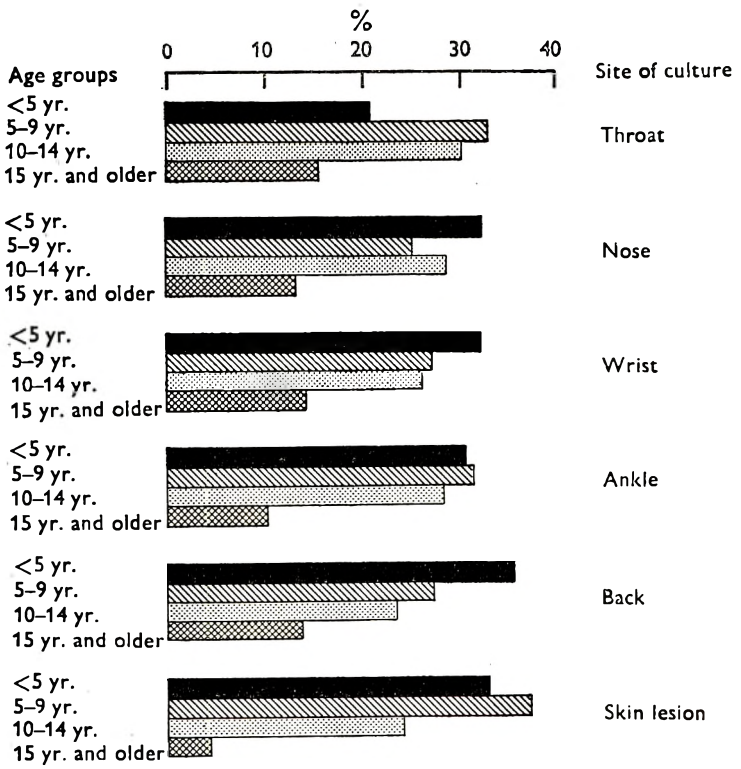

Fig. 2. Percentage distribution of positive cultures for group A streptococci among various age groups by culture sites.

Counting of beta-haemolytic streptococcal colonies present on each culture plate was difficult in some instances because of the nature of the growth of the predominant strain in a satellite pattern. In those instances where it was possible to make some estimation of the number of colonies, approximately two-thirds of the

wrist and ankle cultures contained 1–10 colonies whereas over 80 % of the back cultures contained this number. Both wrist and ankle sites yielded similar percentages of cultures containing 10–50 colonies (17 and 18 % respectively) and 50–500 colonies (14 and 17 % respectively).

Fig. 2 shows the percentage distribution of positive cultures for group A streptococci by age for each culture site. The 44 persons were divided into four age groups of approximately equal numbers: less than 5 years (11 children); 5–9 years (10 children); 10–14 years (13 children); and 15 years and older (10 adolescents and adults). The oldest age group accounted for the lowest percentage of positive cultures at all sites but especially with respect to skin lesions, where only 4·4 % of positive cultures occurred in this age group. The youngest age group had the greatest percentage of positive cultures from both the back and the wrist as well as the nose.

Table 4. *Environmental cultures*

Site cultured	Number of cultures	Percent of cultures positive: Group A streptococci	Percent of cultures positive: Two pyoderma strains
Fingernail dirt	196	29·5	29·5
Personal clothing	43	80·4	76·8
Bedding	33	60·6	60·6
Insects	54	5·5	5·5
Household pets	38	5·5	5·5

The results of environmental cultures obtained during the study are shown in Table 4. The percentage of fingernail dirt cultures positive for group A streptococci was similar to that obtained for both the wrist and ankle. At the time of the initial recovery of either of the two pyoderma strains from nail dirt, 80 % of the normal skin cultures were positive for these strains at one or more sites and 40 % of the individuals had pyoderma. High percentages of clothing (80·4 %) and bedding (60·6 %) cultures were positive for group A streptococci. In each instance the majority of individuals wearing the clothing or sleeping in the bed had the same strain recovered from normal skin or skin lesions at the same time. Only an occasional household pet or insect culture was found to have group A streptococci. The number of environmental cultures taken varied considerably from week to week. Because of this variation, no attempt was made to correlate in detail the results of these cultures with those obtained from the upper respiratory tract, normal skin and skin lesions.

The finding that normal skin yielded such a high percentage of positive cultures for the two group A pyoderma strains recovered during this study suggested that the presence of these streptococci on normal skin could well be a significant factor in the spread of streptococcal pyoderma. In order to examine this possibility the sequence of appearance of specific pyoderma strains at various body sites was determined. The results of culture visits in the 2 weeks preceding the first appearance of a strain at a specified body site were considered. Each site of appearance and each individual were analysed separately and the combined results are shown

in Table 5. While the majority of the preceding weeks' cultures were negative before the appearance of a strain at any of the four sites studied, significantly more of the culture visits were associated with negative cultures before the initial appearance of a strain on normal skin (86·6%) compared with other sites ($P = < 0{\cdot}001$). This suggests that normal skin was the primary site of acquisition of a strain on the body and this is further supported by the results of culture visits associated with positive cultures. Relatively small percentages of cultures were positive at other sites during the 2 weeks preceding the appearance of either pyoderma strain on normal skin. In particular the finding that only 6·1% of preceding culture visits were associated with pyoderma is perhaps the most compelling evidence suggesting that shedding of organisms from skin lesions of the

Table 5. *Results of cultures preceding appearance at a specific site*

Specific sites of appearance studied	Culture results at other sites 1–2 weeks previously				
	Percent negative at all sites	Percent positive for group A streptococci			
		Normal skin	Lesions	Nose	Throat
Normal skin	86·6	—	6·1	3·6	6·1
Skin lesions	56·8	36·4	—	15·9	6·8
Nose	59·4	30·4	18·8	—	4·3
Throat	51·3	30·7	23·7	20·5	—

same individual was not the only factor resulting in the high prevalence of positive cultures from normal skin. During the 2 weeks before initial recovery of pyoderma strains in skin lesions, 36·4% of normal skin cultures were positive for the same strain whereas significantly small percentages of nose, 15·9% ($P = > 0{\cdot}02 < 0{\cdot}05$) and throat 6·8% ($P = < 0{\cdot}001$) cultures were positive for the same strain during the preceding 2 weeks. Before appearance in the nose the highest percentage of preceding positive cultures was again from the normal skin (30·4%) but 18·8% of the culture visits were associated with pyoderma. This suggests that both of these sites contributed to acquisition of strains by the nose ($P = > 0{\cdot}10, < 0{\cdot}20$). Finally previous to the appearance of strains in the throat, the percentages of positive cultures from normal skin, skin lesions and the nose did not differ significantly. This is consistent with the view that all of these sites contributed to throat acquisition ($P = > 0{\cdot}2, < 0{\cdot}3$).

DISCUSSION

The finding that only two easily differentiated strains accounted for 96% of all group A streptococci was fortunate. The epidemiologic findings with respect to the appearance of either strain in families and more specifically at various body sites were probably clearer than would have been the case had multiple less well-defined strains been encountered. Both the 'satellite strain' and the 'other pyoderma strain' belong to the T-antigen complex 3/13/B3264, a pattern known to be associated with streptococcal pyoderma (Parker & Williams, 1961; Dillon, 1967). The unique biologic property of the 'satellite strain' (i.e. the inability to grow aero-

bically on routine sheep blood agar) permitted separate analysis of these two strains. The finding that the 'other pyoderma strain' showed precipitation reactions with both types 41 and 43 M antisera is of interest in view of the recent report by Wiley & Bruno (1968) which describes precipitin and bactericidal cross-reactions between types 33, 41, 43, 52 and Ross. This strain apparently has many of the characteristics of the Ross strain, but the exact nature of the precipitation reactions and possible interrelationships of these two strains will await more detailed studies.

Earlier studies have indicated the rarity with which beta-haemolytic streptococci may be recovered from cultures of normal skin (Colebrook & Maxted, 1933; Colebrook, Maxted & Johns, 1935; Williams & Miles, 1949). Indeed, the investigations by Colebrook & Maxted (1933) also showed that these organisms do not survive when artificially inoculated on the surface of human skin. The experimental work of Ricketts, Squire & Topley (1951) demonstrated that human skin lipids, in particular unsaturated fatty acids of sebum, are highly effective in killing beta-haemolytic streptococci both *in vitro* and *in vivo*. Their study provided a biologic basis for the earlier observations and since that time the possibility of finding beta-haemolytic streptococci on normal skin has seemingly been dismissed by most investigators. An exception is the study of Markham & Stenhouse (1959) in which 13% of normal skin cultures from healthy inhabitants of Rarotonga in the Cook Islands yielded group A streptococci. In this same population 81% of pyoderma lesions were positive for group A organisms.

The results of the present study indicate an even higher prevalence of group A beta-haemolytic streptococci on normal skin among the individuals studied and in fact suggest that skin was the best normal body site for isolation of the two pyoderma strains prevalent at the time of the study. It is of interest that, although the pyoderma strains were isolated from normal skin in both adults and children, those children less than 5 years old provided the best source for normal skin isolations. Whether this reflects biological differences in skin properties among different age groups or simply greater contamination is unknown.

Analysis of the sequence of initial appearance of these pyoderma strains suggests that in the individuals studied the body site where the organisms were most likely to appear initially was normal skin, and, further, that appearance of streptococci on normal skin was a primary factor in the development of streptococcal pyoderma. Following skin acquisition it is possible that some type of trauma such as an insect bite, a laceration or an excoriation permitted the organism to penetrate the skin and produce pyoderma. From pyoderma lesions, as well as from normal skin, the strains appeared to spread to the upper respiratory tract. Whether this pattern of spread is characteristic of all pyoderma strains or certain selected ones remains to be determined. Based on these observations it is not possible or necessary to postulate that pyoderma strains parasitize or multiply on normal skin. However, these findings do support the possibility of survival of these streptococci on normal skin without loss of infectivity, which may have been an important factor in the development of the streptococcal pyoderma reported in this study.

Although the ability to culture these pyoderma strains readily from clothing,

bedding and an occasional pet or insect may reflect nothing more than environmental contamination, the possible role of these sources as secondary reservoirs has yet to be explored under these circumstances. The viability of group A streptococci in blankets and dust has been well-documented (Loosli, Lemon, Wise & Robertson, 1948) but later studies of the transmission of these organisms in Army barracks suggested that there was no increased risk of streptococcal respiratory infections among men who were issued with blankets naturally contaminated with streptococci or who inhaled contaminated dust (Perry *et al.* 1957*a*, *b*). However, despite many basic similarities, strains of group A streptococci that predominantly cause pyoderma may differ in their serologic characteristics (Parker & Williams, 1961) and perhaps in other respects from those strains primarily associated with respiratory disease. Thus what applies epidemiologically in one situation may not in the other.

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REFERENCES

Anthony, B. F., Perlman, L. V. & Wannamaker, L. W. (1967*a*) Skin infections and acute nephritis in American Indian children. *Pediatrics, Springfield* **39**, 263.

Anthony, B. F., Kaplan, E. L., Chapman, S. S., Quie, P. G. & Wannamaker, L. W. (1967*b*). Epidemic acute nephritis with reappearance of type-49 streptococcus. *Lancet* ii, 787.

Colebrook, L. & Maxted, W. R. (1933). Antisepsis in midwifery. *Journal of Obstetrics and Gynaecology of the British Empire* **40**, 966.

Colebrook, L., Maxted, W. R. & Johns, A. M. (1935). The presence of haemolytic and other streptococci on human skin. *Journal of Pathology and Bacteriology* **41**, 521.

Dillon, H. C. Jr (1967). Pyoderma and nephritis. *Annual Review of Medicine* **18**, 207.

Futcher, P. H. (1940). Glomerular nephritis following infections of the skin. *Archives of Internal Medicine* **65**, 1192.

Hamburger, M., Jr & Green, M. J. (1946). The problem of the 'dangerous carrier' of hemolytic streptococci. IV. Observations on the role of the hands, of blowing the nose, of sneezing and of coughing in the dispersal of these micro-organisms. *Journal of Infectious Diseases* **79**, 33.

Hamburger, M., Jr, Green, M. J. & Hamburger, V. G. (1945). The problem of the 'dangerous carrier' of hemolytic streptococci. II. The spread of infection by individuals with strongly positive nose cultures who expelled large numbers of hemolytic streptococci. *Journal of Infectious Diseases* **77**, 96.

Kligman, A. M. (1965). The bacteriology of normal skin. In *Skin Bacteria and their Role in Infection*. Ed. I. Maiback and G. Hildick-Smith. p. 13. New York: McGraw-Hill Co.

LOOSLI, G. G., LEMON, H. M., WISE, H. & ROBERTSON, O. H. (1948). Studies on the transmission and control of respiratory disease within army barracks. I. Hemolytic streptococcal contamination of the environment. *Journal of Infectious Diseases* **82**, 59.

MARKHAM, N. P. & STENHOUSE, A. C. (1959). A bacteriological investigation of wound infections in Raratonga, Cook Islands. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **53**, 404.

PARKER, M. T. & WILLIAMS, R. E. O. (1961). Further observations on the bacteriology of impetigo and pemphigus neonatorum. *Acta paediatrica, Stockholm* **50**, 101.

PERRY, W. D., SIEGEL, A. C., RAMMELKAMP, C. H., Jr., WANNAMAKER, L. W. & MARPLE, E. C. (1957*a*). Transmission of group A streptococci. I. The role of contaminated bedding. *American Journal of Hygiene* **66**, 85.

PERRY, W. D., SIEGEL, A. C. & RAMMELKAMP, C. H., Jr (1957*b*). Transmission of group A streptococci. II. The role of contaminated dust. *American Journal of Hygiene* **66**, 96.

RICKETTS, C. R., SQUIRE, J. R. & TOPLEY, E. (1951). Human skin lipids with particular reference to self-sterilizing power of skin. *Clinical Science* **10**, 89.

WANNAMAKER, L. W. (1954). The epidemiology of streptococcal infections. In *Streptococcal Infections*, p. 157, Ed. M. McCarty. New York: Columbia University Press.

WILEY, G. G. & BRUNO, P. N. (1968). Cross-reactions among group A streptococci. I. Precipitin and bactericidal cross-reactions among types 33, 41, 43, 52 and Ross. *Journal of Experimental Medicine* **128**, 959.

WILLIAMS, R. E. O. (1965). Pathogenic bacteria on the skin. In *Skin Bacteria and their Role in Infection*. Ed. I. Maiback and G. Hildick-Smith. p. 49, New York, McGraw-Hill Co.

WILLIAMS, R. E. O. & MILES, A. A. (1949). Infections and sepsis in industrial wounds of the hand. *Medical Research Council, Special Report Series*, No. 266.

J. Hyg., Camb. (1970), **68**, 29
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Effect of suspending media on freeze-drying and preservation of vaccinia virus

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SUMMARY

Unpurified and purified smallpox vaccines were prepared from calf dermal pulp, or chorioallantoic membrane (CAM) of hen eggs infected with vaccinia virus, and freeze-dried. The protective effect of various suspending media was investigated both in the course of the freeze-drying and in the period of subsequent storage of the dried product at different temperatures, including 100° C.

Single media consisting of either sodium glutamate or peptone were effective in the preservation of both unpurified and purified vaccines prepared from calf dermal pulp or CAM. It was shown that there was an optimal concentration of sodium glutamate for the preservation of the vaccine preparations, especially of the purified vaccine.

Combined media, consisting of soluble starch, polyvinylpyrrolidone or sodium carboxymethyl cellulose with sodium glutamate, were effective with the purified vaccine when the concentration of sodium glutamate exceeded the optimum necessary for preservation.

INTRODUCTION

It is now generally accepted that in order to prevent the reduction of biological activity during both the freeze-drying process and the preservation of biological products, the addition of some suspending medium to the product is very important (Harris, 1954).

With regard to viral products, Collier (1955) reported that peptone acted protectively in the freeze-drying and the preservation of smallpox vaccine. However, with peptone there is a possibility of an unfavourable side reaction. On the other hand, sodium glutamate is a pure crystalline compound and exerts a powerful protective influence on the viability of dried BCG vaccine (Cho & Obayashi, 1956; Obayashi & Cho, 1957). No sufficient work, however, has been undertaken on the effect of sodium glutamate on viral activity during and after the freeze-drying.

The present paper deals with the effect of the suspending media on vaccinia virus preparations both in the freeze-drying and in subsequent preservation.

MATERIALS AND METHODS

Virus vaccine source

Calf dermal pulp and chorioallantoic membrane (CAM) of developing hen eggs infected with vaccinia virus (strain IKEDA) were used.

Unpurified calf dermal pulp smallpox vaccine

Calf dermal pulp was suspended in the proportion of 1 g. to 3 ml. of 0·004 M McIlvaine buffer (pH 7·3), and homogenized in a Waring blender. The virus suspension was filtered through 80 mesh wire screen. After centrifugation at 2,000 rev./min. for 20 min., three volumes of the supernatant obtained were added to one volume of the suspending medium.

Unpurified CAM smallpox vaccine

The infected CAM was suspended in the proportion of 1 g. to 0·5 ml. of 0·004 M McIlvaine buffer (pH 7·3), and homogenized in a Waring blender. The following procedure was the same as in the preparation of the unpurified calf dermal pulp vaccine.

Purified calf dermal pulp smallpox vaccine

The calf dermal pulp was purified with 33 % fluorocarbon (trichlorotrifluoroethane) buffer mixture by the method of Epstein (1958), and the suspending medium was added to an equal volume of the vaccine.

Purified CAM smallpox vaccine

The vaccine, that is, the elementary body suspension, was prepared from the infected CAM by the 10 % extract method of Collier (1955) using fluorocarbon. The suspending medium was added to an equal volume of the vaccine.

Suspending media

In the first group of media a number of simple substances, such as sodium glutamate or peptone, were used alone dissolved in 0·004 M McIlvaine buffer (pH 7·3). In the second group, sodium glutamate was used in combination with soluble starch, polyvinylpyrrolidone (PVP) or sodium carboxymethyl cellulose (SCMC). According to Obayashi, Ota & Arai (1961) the first group of substances exert a sublimation-retarding effect, whereas the second group of substances, such as PVP and SCMC, rather have a sublimation-promoting property.

Freeze-drying

The liquid vaccine suspension was pipetted in 0·5 ml. volumes into ampoules which were frozen in a chamber of the drying apparatus at below $-30°$ C. for about 2 hr., and dried for 8 to 11 hr. An example (batch no. 115) of the freeze-drying process in the present experiments is shown in Fig. 1. The maximum temperature of the vaccine in the final drying stage was controlled at about 20° C. After the drying, each ampoule was sealed under vacuum.

Residual moisture content

Residual moisture content was measured by Abderhalden's method. The dried products were heated at 60° C. for 3 hr. under a pressure of 0·1 mm Hg in an apparatus specially made for this purpose, in order to evaporate the residual moisture still contained. Residual moisture content of dried products was calculated by the following equation:

$$\text{Residual moisture content } (\%) = \frac{A-B}{A} \times 100,$$

where A is the initial weight of the dried product and B is the weight after 3 hr. desiccation. Statistical significance level ($P < 0{\cdot}05$) between the residual moisture contents obtained in a preliminary test was 1·19 %.

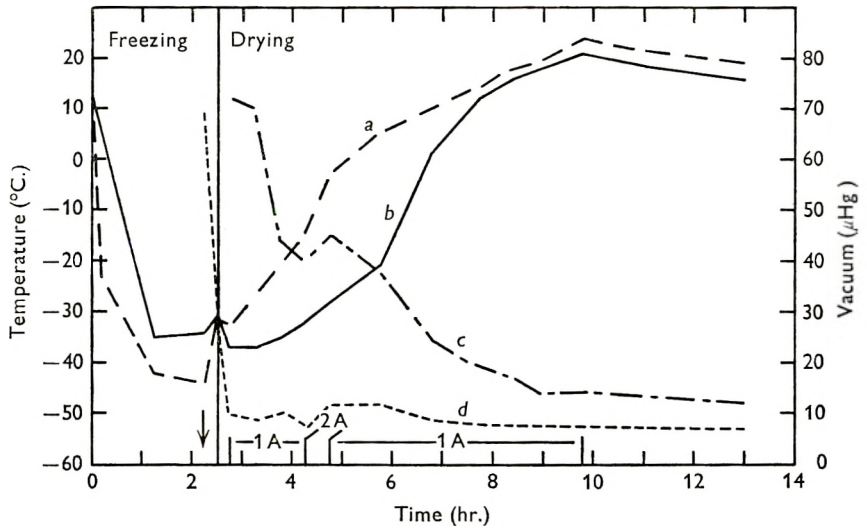

Fig. 1. An example of the freeze-drying process in the present experiments (batch no. 115). (*a*) Temperature of shelf; (*b*) temperature of vaccine; (*c*) Pirani gauge reading; (*d*) temperature of condenser. 1A and 2A, Heater current (amp.). ↓, Changing point of cooling solvent flow from chamber to condenser. Freeze-drying apparatus: Kyowa RL-500S type, made by Kyowa Vacuum Engineering Co. Ltd., Tokyo, Japan.

Virus titration

After the reconstitution of dried vaccine with 0·5 ml. phosphate buffered saline (PBS, pH 7·4) serial tenfold dilutions were made, and the virus titre was measured by the method of Westwood, Phipps & Boulter (1957) using CAM of developing hen eggs. The titre was expressed as log pock-forming units per millilitre (log PFU/ml.) of the reconstituted virus suspension. Statistical significance level ($P < 0{\cdot}05$) between the titres obtained in a preliminary test was 0·66 log PFU/ml.

Boiling test

In order to observe the effect of the suspending media on the stability of virus titre, the dried vaccine was heated in a boiling water bath for a certain period of time.

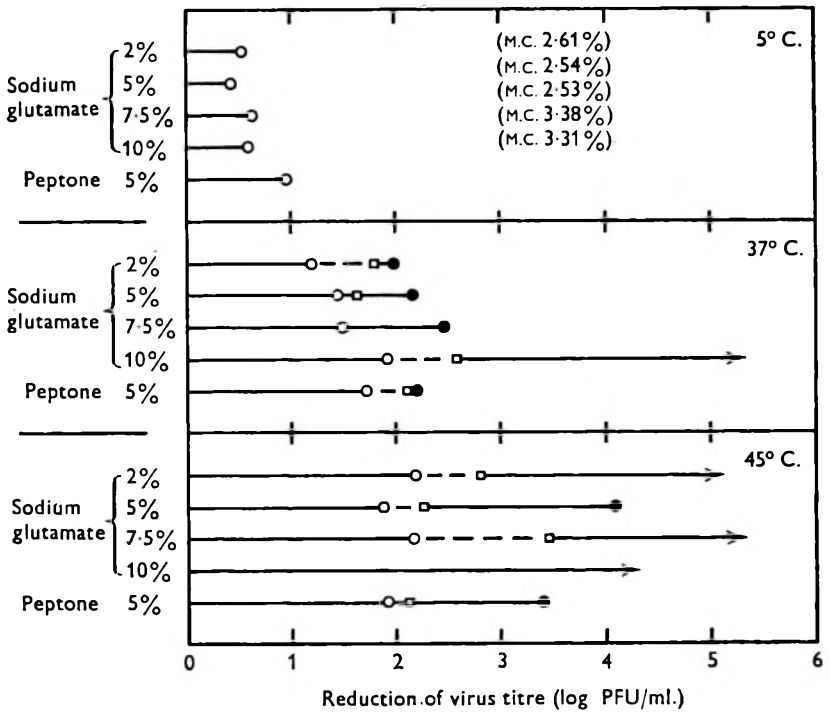

Fig. 2. Effect of single suspending media on preservation of dried unpurified calf dermal pulp vaccine (batch no. 109). ○, Preserved at 5° C. for 43 months; preserved at 37° C. for 12 months; preserved at 45° C. for 6 months. □, Preserved at 37° C. for 36 months; preserved at 45° C. for 21 months. ●, Preserved at 37° C. for 48 months; preserved at 45° C. for 36 months. →, Larger reduction of virus titre than the given point. M.C. Residual moisture content of dried vaccine obtained immediately after freeze-drying.

Preservation test

In this test the dried vaccines were preserved at 5°, 37° and 45° C., to evaluate the stability of the dried vaccine.

RESULTS

Experiment 1. *Unpurified smallpox vaccine*

Effect of single suspending media on unpurified calf dermal pulp vaccine (batch no. 109)

Using 2, 5, 7·5 and 10% sodium glutamate, and 5% peptone as single media, their effect was investigated in freeze-drying and in preservation at 5°, 37° and 45° C.

Virus titre of the dried vaccine after freeze-drying was between 8·09 and 8·31

log PFU/ml., and the reduction of virus titre by the freeze-drying was between 0·22 and 0·56 log PFU/ml.

During 43 months preservation at 5° C., only a slight difference was observed in the reduction of virus titre among the different suspending media mentioned (Fig. 2). After storage at 45° C. for 36 months, the best results were obtained with 5 % peptone and 5 % sodium glutamate.

These results indicated that peptone and sodium glutamate were effective for the preservation of the unpurified calf dermal pulp vaccine. However, the use of sodium glutamate in concentrations as high as 7·5 % and 10 % clearly resulted in a considerable reduction in virus titre, particularly after long preservation at high temperature.

This experiment was chiefly made from a practical viewpoint in order to observe the stability of dried smallpox vaccine at high temperatures for several years, and in the following study comparison of the effect of different kinds and concentrations of suspending media on stability of dried products was made after preservation for several months.

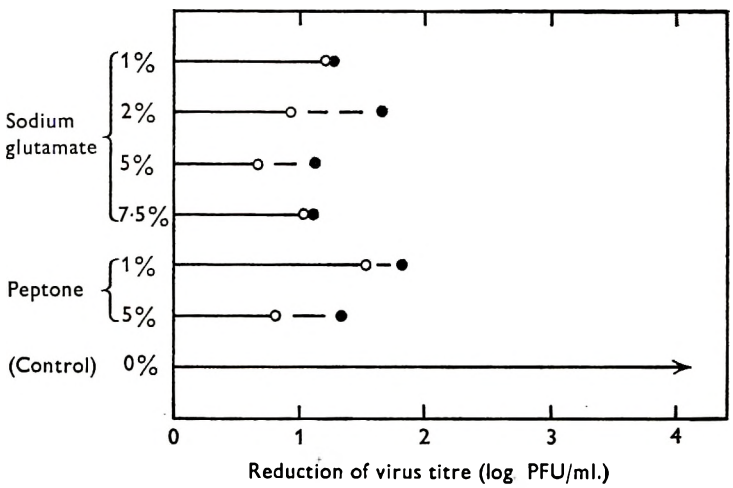

Fig. 3. Effect of single suspending media on preservation of dried unpurified CAM vaccine (batch no. C-1). ○, Preserved at 45° C. for 2 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

Effect of single suspending media on unpurified CAM vaccine (batch no. C-1)

Unpurified CAM vaccine was freeze-dried with 1, 2, 5 and 7·5 % sodium glutamate and 1 and 5 % peptone as single media.

Virus titre immediately after freeze-drying was between 6·99 and 7·44 log PFU/ml., and the reduction of virus titre by the freeze-drying was below the significance level (0·66 log PFU/ml.).

The reduction of virus titre after the preservation of the resulting vaccines is shown in Fig. 3. After preservation for 2 and 5 months at 45° C., all of these vaccines were far more stable than the control without any suspending medium, and no significant difference in survival was observed among different concentrations of sodium glutamate and peptone.

Effect of combined suspending media on unpurified calf dermal pulp vaccine (batch no. 114)

These combined media consisted of 5 and 7·5 % sodium glutamate as the first group, with various concentrations of soluble starch, PVP or SCMC as the second group.

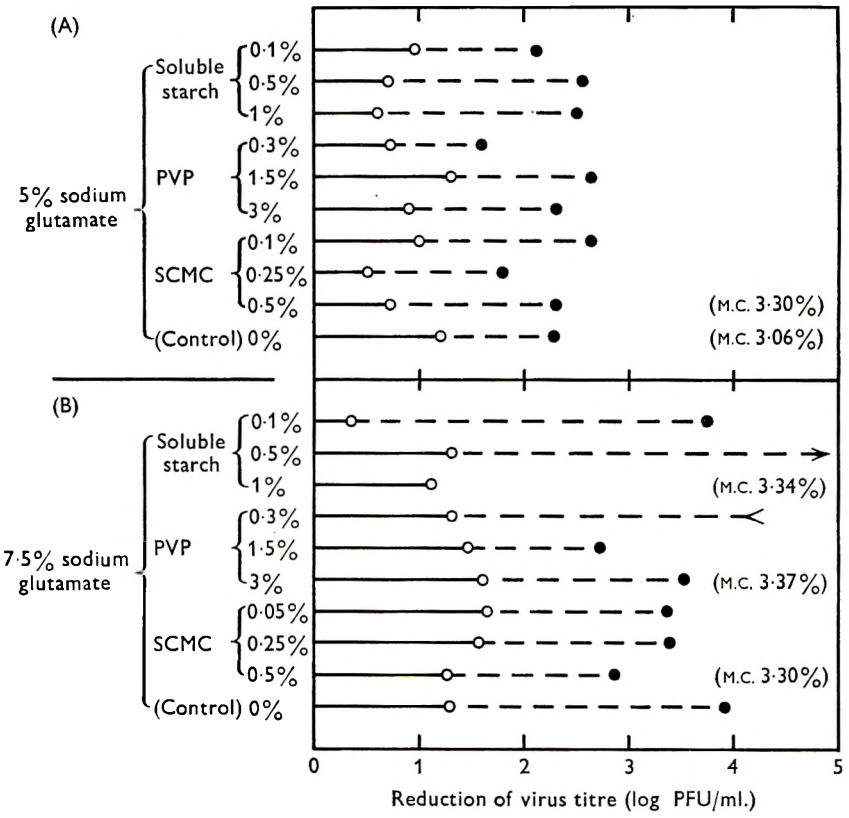

Fig. 4. Effect of combined suspending media on preservation of dried unpurified calf dermal pulp vaccine (batch no. 114). ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 9 months. ≺, Smaller reduction of virus titre than the given point. →, Larger reduction of virus titre than the given point. M.C. Residual moisture content of dried vaccine obtained immediately after freeze-drying.

Virus titre of the dried vaccines after freeze-drying was between 7·45 and 8·09 log PFU/ml. with batch no. 114 (A), and between 7·66 and 8·32 log PFU/ml. with batch no. 114 (B).

The virus titre of the dried vaccine was tested after preservation at 45° C. for 1 and 9 months as shown in Fig. 4. The addition of the media of the second group showed no stability-enhancing effect, the degree of reduction of virus titre being almost similar to that of the control vaccines dried with sodium glutamate alone.

Experiment 2. Purified smallpox vaccine

Effect of single suspending media on purified calf dermal pulp vaccine (batch no. 115)

The protective effects on the purified vaccine of sodium glutamate and peptone were compared. In order to clarify the effect of the concentration of sodium glutamate, a wide concentration range from 0·1 to 7·5 % was tested.

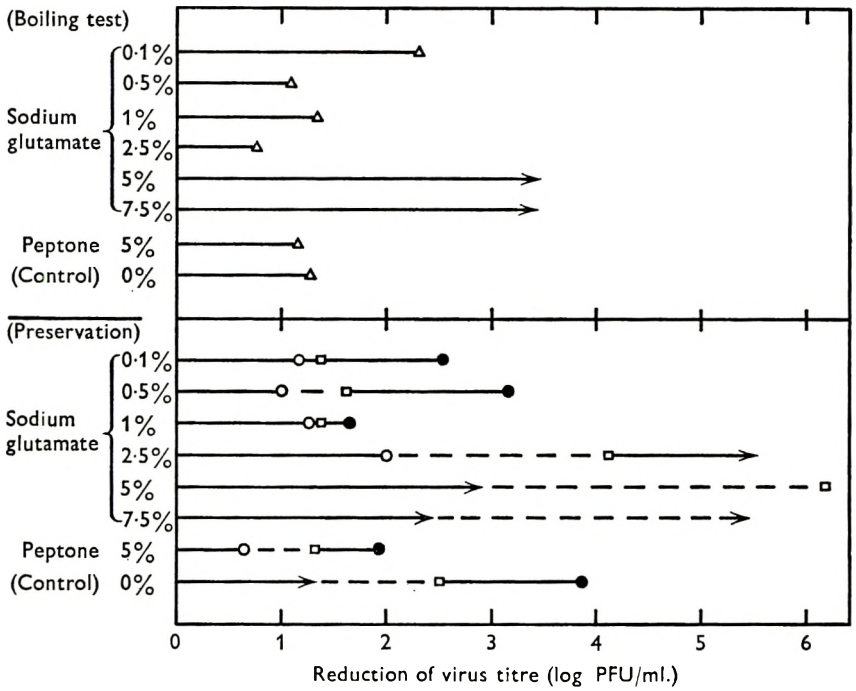

Fig. 5. Effect of single suspending media on preservation of dried purified calf dermal pulp vaccine (batch no. 115). △, Heated in a boiling bath for 10 min. ○, Preserved at 45° C. for 1 month. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

Virus titre of the dried vaccine after the freeze-drying was between 6·30 and 7·56 log PFU/ml., and the degree of reduction of virus titre by the freeze-drying was rather slight except with 0·1 % sodium glutamate, and in the control.

In the boiling test no particular stability-enhancing effect was observed either in peptone or in sodium glutamate (Fig. 5). The reduction of virus titre was rather more marked with 5 % and 7·5 % of sodium glutamate. However, in the preservation at 45° C. the reduction of the virus titre was lowest in 1 % sodium glutamate and in 5 % peptone, higher concentrations of sodium glutamate resulting in a larger reduction of titre than the control.

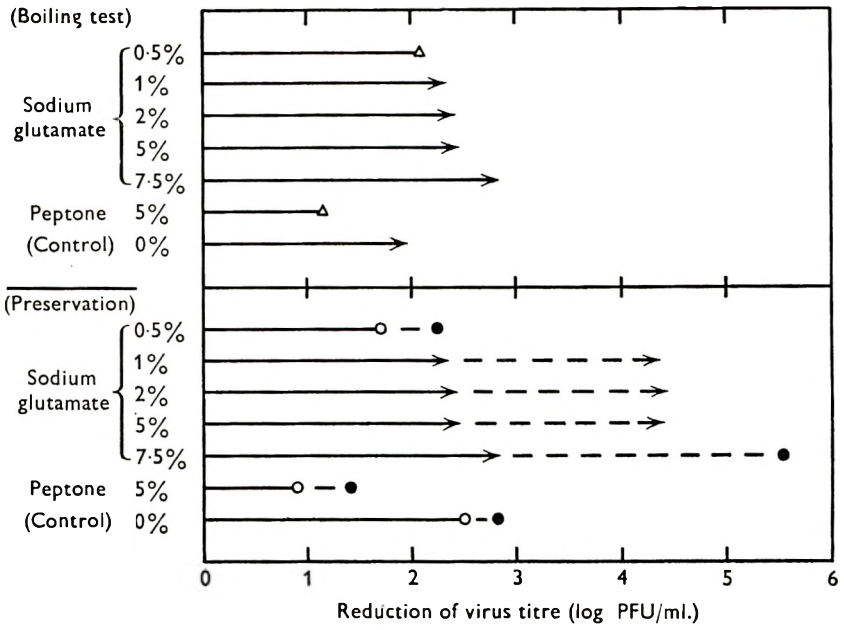

Fig. 6. Effect of single suspending media on preservation of dried purified CAM vaccine (batch no. C-2). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 2 months. →, Larger reduction of virus titre than the given point.

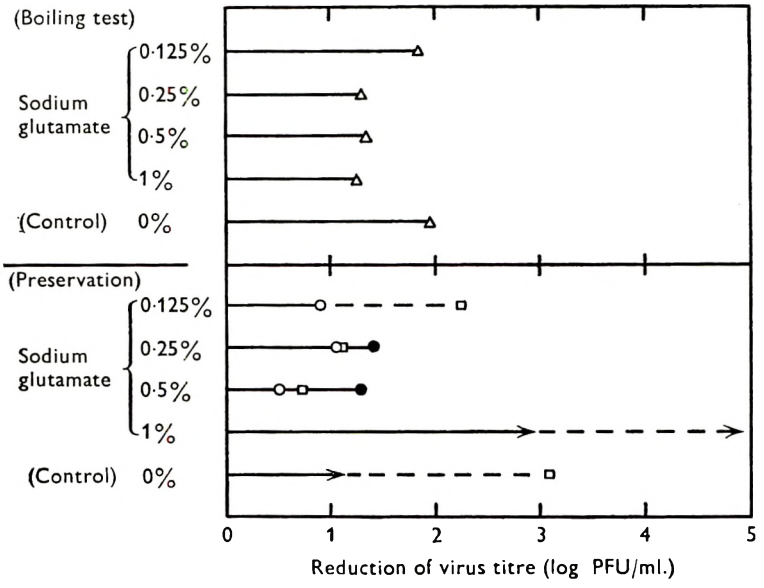

Fig. 7. Effect of single suspending media on preservation of dried purified CAM vaccine (batch no. C-5). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

Effect of single suspending media on purified CAM vaccine (batch no. C-2 and C-5)

In Figs. 6 and 7 the effect of different concentrations of sodium glutamate ranging from 0·5 to 7·5 % (batch no. C-2) and from 0·125 to 1 % (batch no. C-5) upon the stability of dried purified CAM vaccine was examined.

Virus titre of the dried vaccine after the freeze-drying was between 7·32 and 7·80 log PFU/ml. except in the control in batch no. C-2, and 6·38 and 6·91 log PFU/ml. except in the control in batch no. C-5. The control vaccine without medium decreased to a virus titre of 5·88 log PFU/ml. after the drying in the former batch, and 5·08 log PFU/ml. in the latter.

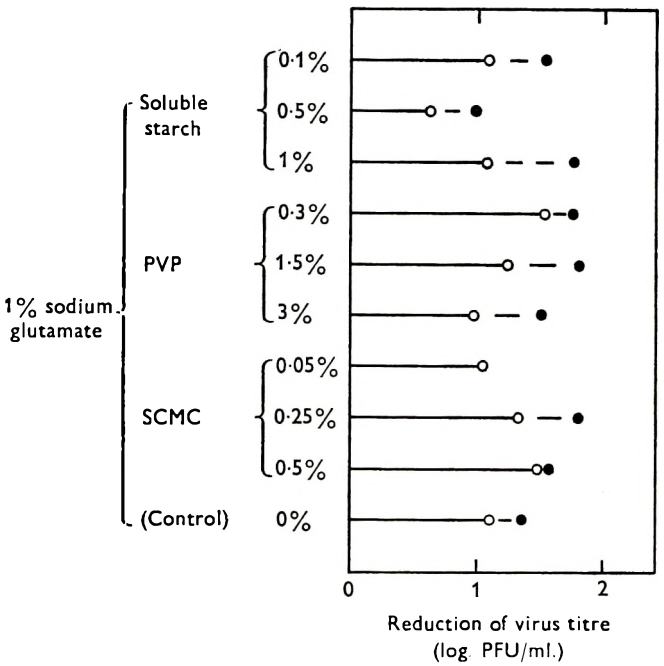

Fig. 8. Effect of combined suspending media on preservation of dried purified calf dermal pulp vaccine (1) (batch no. 118). ○, Preserved at 45° C. for 2 months. ●, Preserved at 45° C. for 5 months.

Reduction of virus titre by the freeze-drying ranged from 0·18 to 0·50 log PFU/ml. in batch no. C-2, and from 0·14 to 0·36 log PFU/ml. in batch no. C-5 except in the two controls.

In the preservation test, the best result was obtained with 0·5 % sodium glutamate followed by 0·25 %, and 5 % peptone also revealed a good protective property.

Effect of combined suspending media on purified calf dermal pulp vaccine (batch no. 118 and 119)

Since the addition of the medium of the second group to sodium glutamate exerted no beneficial effect in the case of unpurified calf dermal pulp vaccine, this point was further examined by using purified vaccine. As shown in Fig. 8, soluble

starch, PVP or SCMC was added to 1 % sodium glutamate which was confirmed as the optimum concentration when used alone (Fig. 5).

Dried vaccines from 6·51 to 7·20 log PFU/ml. in batch no. 118, and from 7·15 to 7·42 log PFU/ml. in batch no. 119 were preserved.

The reduction of virus titre by the freeze-drying, and after 5 months preservation at 45° C., was similar to the reduction in the controls containing sodium glutamate alone. Thus when sodium glutamate was used in its optimal concentration, the addition of the medium of the second group exerted no added protection.

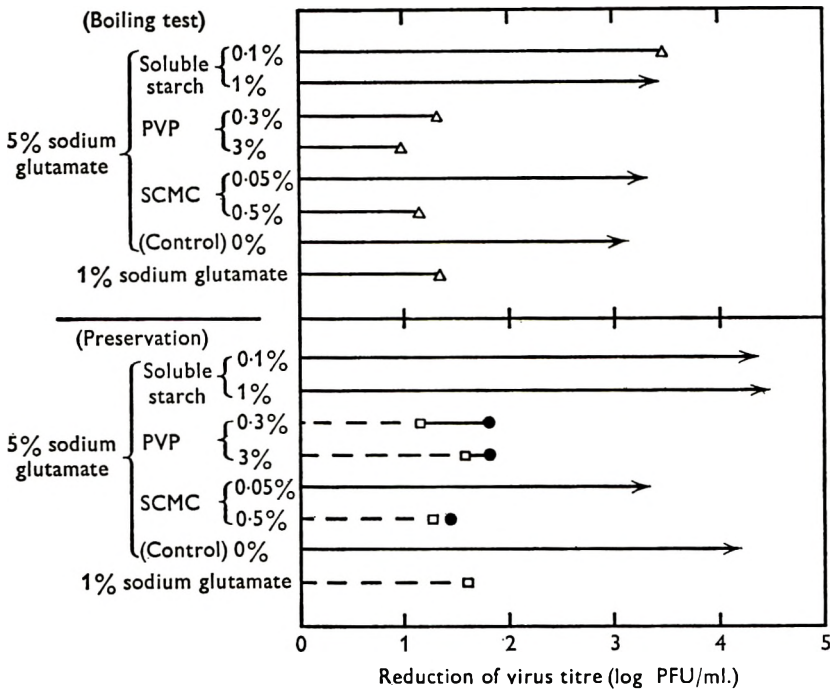

Fig. 9. Effect of combined suspending media on preservation of dried purified calf dermal pulp vaccine (2) (batch no. 119). △, Heated in a boiling bath for 10 min. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

However, when the concentration of sodium glutamate was increased to 5 %, the addition of PVP and a high concentration of SCMC effected an increase of protection up to the level of 1 % sodium glutamate alone (Fig. 9). But soluble starch and a lower concentration of SCMC did not show such protective effect. It thus became clear that the addition of PVP and SCMC produced added protection only when sodium glutamate was used in higher concentration than the optimum one.

Effect of combined suspending media on purified CAM vaccine (batch no. C-4)

The effect of the addition of media of the second group in the purified CAM vaccine using 2 % sodium glutamate was investigated.

Dried vaccines from 6·34 to 7·14 log PFU/ml. were tested, and the reduction of virus titre by the freeze-drying was not great.

As shown in Fig. 10, the addition of PVP and a high concentration of soluble starch and SCMC to sodium glutamate again showed a certain protective effect.

DISCUSSION

The role of suspending media is concerned firstly with the protection of biological products from the damaging effect of freeze-drying. In this respect, however, the degree of the reduction of virus titre by the freeze-drying is relatively

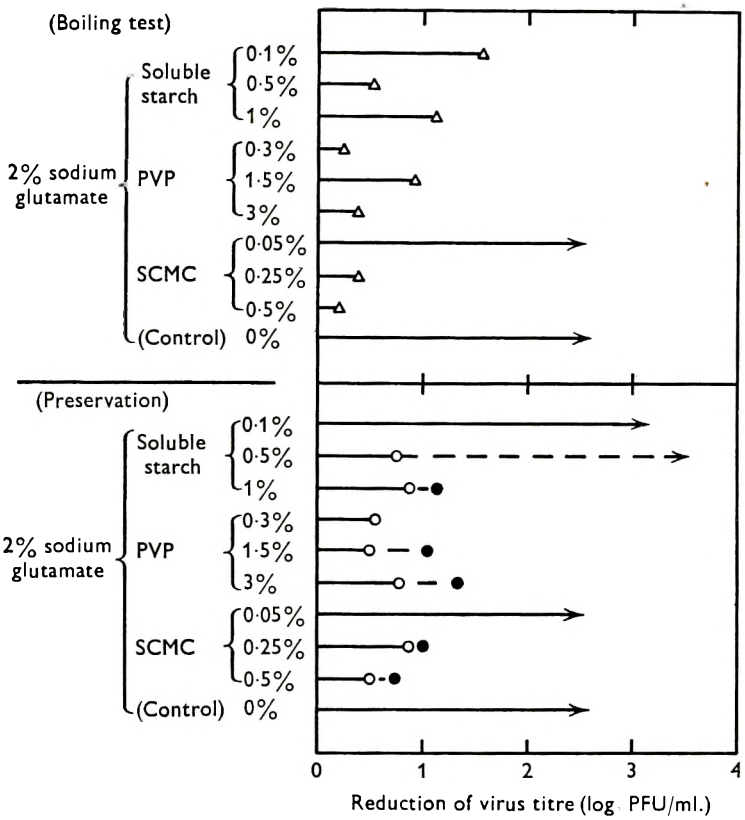

Fig. 10. Effect of combined suspending media on preservation of dried purified CAM vaccine (batch no. C-4). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

smaller in the case of vaccinia virus as compared with that of poliovirus (Kraft & Pollard, 1954) or Coxsakie virus (Tyrrel & Ridgewell, 1965). Therefore, in the present study the effect of suspending media was chiefly examined with regard to the preservation process.

For the preservation of dried vaccinia virus, sodium glutamate and peptone were effective in both the unpurified and purified vaccines, while the existence of an optimal concentration of sodium glutamate was observed clearly only in the purified vaccines owing to the relative absence of the extraneous non-viral sub-

stances which were present in the unpurified vaccine. The existence of an optimal concentration of sodium glutamate was essentially similar to that seen in BCG and *Lactobacillus bifidus* (Cho & Obayashi, 1956; Obayashi & Cho, 1957; Ota, 1959; Obayashi *et al.* 1961). Obayashi *et al.* (1961) suggested that the concentration of sodium glutamate is related to the residual moisture content of the final product, and that for the preservation of the product there existed an optimum residual moisture content. They observed that the increase in the concentration of sodium glutamate tended to depress the sublimation during the drying in contrast to soluble starch and PVP, in which no such diminution of sublimation occurred with increase of the concentration.

Also in the present study, soluble starch, PVP and SCMC in the combined media raised the protective activity up to that attained by using the optimal concentration of sodium glutamate alone. In this case therefore the role of the suspending media of the second group seems to lie in their sublimation-promoting effect when too high a concentration of sodium glutamate retards the sublimation.

Greiff & Rightsel (1968) observed a certain degree of correlation between the residual moisture content of dried influenza virus and its stability at high temperatures. So far in our experiment with unpurified calf dermal pulp vaccines dried in either sodium glutamate or peptone, no correlation was found between the residual moisture content below about 4 % and the reduction of virus titre after preservation or by the boiling test (Suzuki, 1969). However, this point should be further examined with the purified smallpox vaccines, since the presence of an extraneous non-viral substance might obscure the relation between these two components.

The major role of suspending medium for dried bacterial products, such as protection of living bacilli during both the freeze-drying process and subsequent period of storage, was observed also in the case of vaccinia virus when sodium glutamate and peptone were used as suspending medium. As to the mechanism of this protection, the sublimation-retarding activity of sodium glutamate and sublimation-promoting activity of the media of the second group, such as soluble starch, PVP and SCMC, was considered to play some part. As to this point further studies are required, especially with purified virus. However, for practical purposes the present study may provide some useful information for those engaged in the freeze-drying of virus vaccines.

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REFERENCES

CHO, C. & OBAYASHI, Y. (1956). Effect of adjuvant on preservability of dried B.C.G. vaccine at 37° C. *Bulletin of the World Health Organization* **14**, 657.

COLLIER, L. H. (1955). The development of a stable smallpox vaccine. *Journal of Hygiene* **53**, 76.

EPSTEIN, M. A. (1958). An investigation into the purifying effect of a fluorocarbon on vaccinia virus. *British Journal of Experimental Pathology* **39**, 436.

GREIFF, D. & RIGHTSEL, W. A. (1968). Stability of suspensions of influenza virus dried to different contents of residual moisture by sublimation in vacuo. *Applied Microbiology* **16**, 835.

HARRIS, R. J. C. (1954). The preservation of viruses. In *Biological Applications of Freezing and Drying*. P. 436. Ed. R. J. C. Harris. New York: Academic Press.

KRAFT, L. M. & POLLARD, E. C. (1954). Lyophilization of poliomyelitis virus. Heat inactivation of dry MEF 1 virus. *Proceedings of the Society for Experimental Biology and Medicine* **86**, 306.

OBAYASHI, Y. & CHO, C. (1957). Further studies on the adjuvant for dried B.C.G. vaccine. *Bulletin of the World Health Organization* **17**, 255.

OBAYASHI, Y., OTA, S. & ARAI, S. (1961). Some factors affecting preservability of freeze-dried bacteria. *Journal of Hygiene* **59**, 77.

OTA, S. (1959). Studies on freezing and freeze-drying of *Lactobacillus bifidus* (4). *Ochanomizu Igaku Zassi* **7**, 2968.

TYRREL, D. A. J. & RIDGEWELL, B. (1965). Freeze-drying of certain viruses. *Nature* **206**, 115.

SUZUKI, M. (1969). Relation between reduction of vaccinia virus titer and residual moisture content. In *Freezing and Drying of Microorganisms*. P. 111. Ed. T. Nei. Tokyo: University of Tokyo Press, and Baltimore & Manchester: University Park Press.

WESTWOOD, J. C. N., PHIPPS, P. H. & BOULTER, E. A. (1957). The titration of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. *Journal of Hygiene* **55**, 123.

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Staphylococcal epidemiology in Antarctica

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SUMMARY

An investigation of staphylococcal epidemiology was undertaken at an Australian National Antarctic Research Expedition station during 1965–1966. It concerned the carriage of staphylococci by the men and their dogs, and the occurrence of staphylococci in the station environment. The year-long study indicated that coagulase-negative strains survived better in the Antarctic environment than coagulase-positive strains. It was demonstrated that naturally acquired coagulase-positive strains could not maintain colonization on forearm skin under the usual cold exposure experienced at Mawson station, though coagulase-negative skin strains appeared to thrive during the winter. *Staphylococcus albus* and *S. aureus* were able to persist in the anterior nares, despite the sometimes lower temperatures recorded in this micro-climate, probably because of the greater humidity and denser populations found there. The majority of the nasal carriers of *S. aureus* were persistent carriers, only two men in 27 being found to be occasional carriers of nasal strains, which was consistent with the observation that transfer of this pathogen from man to man is not common under Antarctic conditions. Half of the 27 sledge dogs at the station were found to carry coagulase-positive staphylococci but this did not appear to be of pathological significance to their human handlers. The local inanimate environment, including mess hut, sleeping huts and sleeping bags used on expeditions, was searched for contamination by *S. aureus* but none was detected.

INTRODUCTION

A study in staphylococcal epidemiology was undertaken at the Australian National Antarctic Research Expedition (ANARE) station, Mawson, during 1965 concurrently with a previously reported virology programme (Cameron & Moore, 1968). Changes in the staphylococcal carrier rate have been noted in men who move to a cold climate. McLean (1919) found that *Staphylococcus aureus* (identified by morphology and colony colour) was not carried in the upper respiratory tract of his subjects after 3 months of exposure to Antarctic conditions; nor did it reappear over the following 6 months of continual cold weather. He noted that *S. aureus* was not recovered from the dust in the living hut though he recorded an increase in the numbers of *S. albus*, bacilli and moulds.

Christie (1958) studied the nasopharyngeal and forearm flora of a small party on expedition in Greenland for three months. Bacterial strains were again identified

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by microscopic appearance and colonial features. Christie found a decrease in *S. aureus* carriage in the naso-pharynx and perhaps on the skin, while the *S. albus* isolation rate was unaltered.

Sladen (1965) furthered this work on nasal carriage of staphylococci but found that *S. aureus* and *S. albus* persisted in the noses of men in his Antarctic study groups, though he noted that the numbers of intermittent and occasional carriers of *S. aureus* decreased. Sladen's identification of staphylococcal strains was aided by coagulase testing and phage typing. Few examples of transfer of strains from man to man were detected though there appeared to be ample opportunity for it to occur.

Conditions of cold climate living are difficult to reproduce in temperate climates but Hicks, Poe & Dooley (1964) report a study using a small group of volunteers engaged in acclimatization experiments in a cold chamber. A tendency for an increase in the rate of carriage of *S. aureus* was noted and some previous non-carriers or intermittent carriers became persistent carriers during the cold phase of the experiment, which lasted 6 weeks.

Hare & Thomas (1956) demonstrated that staphylococci were spread from the anterior nares of a carrier to his skin and clothing and thence to the surroundings; therefore the methods used in the present study included repeated nose and skin swabbing, air sampling in huts and monitoring of sleeping bags used on sledging expeditions. Nose swabs were also collected from the sledge dogs at the station, for some men had repeated close contact with them and dogs have been shown capable of carrying pathogenic human staphylococci (Pagano *et al.* 1960; Live & Nichols, 1961, 1965).

METHODS AND MATERIALS

The subjects and their environment

The party under study consisted of 27 healthy Caucasian men. They arrived by sea at Mawson station (Latitude, 67° 36′ S; Longitude, 62° 53′ E) in January 1965. This party occupied the station for 13 months before returning to Australia in March 1966. The only visitors during their sojourn were 11 Russian airmen who stayed at the station for one day just before the final relief.

The climate at Mawson is relatively mild compared with inland stations in Antarctica. The mean air temperature during 1965 was +10·1° F. with extremes of −30·2° F. and +47·8° F. The annual mean wind speed was 20·1 miles per hour and a maximum gust of 134 miles per hour was recorded. Drifting snow was noted on 106 days, usually accompanied by gale-force winds. The relative humidity averaged 59% but dropped to between 20 and 30% inside the sleeping huts and 15% in most of the work huts. The huts were heated by bricquette or oil stoves which kept inside temperatures around 65° F. throughout the year. All but two of the station buildings were separated from each other, thus all men necessarily experienced some cold exposure during the normal routine of station life (Budd, 1964). Cold exposure was increased considerably on the frequent sledging and tractor-train expeditions mounted to adjacent coastal and inland features. All members of the party were engaged in some field work during the year.

Collection techniques

(i) An area approximately 1·5 cm. square was sampled half-way along the midline of the anterior aspect of the forearm. A cotton-tipped applicator stick moistened with sterile nutrient broth was firmly rubbed and rotated over this area for 30 strokes. This site was used in preference to the dorsum of the wrist, a site used in previous surveys of skin flora; for rhinorrhoea is common in the cold and it is tempting for a man to brush his nose with the back of his hand.

(ii) Both anterior nares were sampled using a similar moistened swab.

(iii) When throat swabs were taken for the virus survey, duplicate swabs were obtained for bacteriology. They were immediately streaked on blood agar plates for identification of potential pathogens.

(iv) Air sampling in the mess, recreation room, surgery and sleeping huts was undertaken using Millipore field monitors (MHBG 037 00). Air was sampled at the rate of 10 l./min. at sites which gave representative samples of the circulating air in the huts, both when occupied and when vacant.

(v) Sleeping bags were sampled following the return of expeditions from the field. Satisfactory samples of the bacterial flora of these bags were obtained by swabbing several sites on each bag with cotton-tipped applicator sticks moistened with saline.

(vi) Techniques for collection and processing of nasal swabs from the dogs were the same in principle as those used for the human nasal swabs. In practice, however, the collection of these samples proved more difficult.

Laboratory procedures

Mannitol salt agar (MSA; Oxoid CM86) was used for the primary isolation of staphylococci from the swabs and Millipore filters. Representative colonies were replated on nutrient agar plates (Oxoid CM178) and morphology was checked by

Table 1. *Basic set of international phages*

Group I	29, 52, 52A, 79, 80, 81
Group II	3A, 3B, 3C, 55, 71
Group III	6, 7, 42E, 47, 47C, 53, 54, 75, 77, 83A
Group IV	42D
Miscellaneous	187, B5, 77Ad, D

Table 2. *Phages used by N. E. Nairn*

Ex Davidson's bovine strains	101, 102, 107
Ex Frost's bovine strains	367, 425, 600, 13
Ex Nairn's bovine strains	36, 95, 140, 149, 164
Ex international set	29, 80, 71, 31B, 42D, 6, 53, 77

microscopic examination of Gram-stained smears. Second passage colonies were emulsified in normal saline and the slide coagulase test (Williams & Harper,1946) was performed at the station. Representative strains were stab-inoculated into nutrient agar, incubated at 37·5° C. for 14 hr and stored at 10° C., later to be transported to the Institute of Medical and Veterinary Science (IMVS), Adelaide.

Here the slide coagulase test was repeated and, if necessary, the tube coagulase test was also performed (Fisk, 1940). All specimens were tested for sensitivity to a standard range of antibiotics, and phage typing (Blair & Williams, 1961), using phages from the International Set, was done on all coagulase-positive specimens. Phages were used at RTD and 1000 RTD and are listed in Table 1.

Canine strains were tested with the human phages, and then sent to Dr N. E. Nairn, Veterinary Pathologist, Department of Agriculture, Government of Western Australia, who retested them with 12 bovine phages at RTD and a selection from the International Set (Table 2).

RESULTS

Human staphylococci

Forearm skin

Staphylococci were isolated from all subjects during the year, but were obtained consistently from only 10 men. The majority of forearm skin strains were mannitol negative.

Table 3. *Monthly carriage rates for skin and nasal staphylococci*

		Number carrying			
		On skin		In nose	
Date	No. of subjects	Staphylo-cocci	*S. aureus*	Staphylo-cocci	*S. aureus*
8. iii. 65	27	6	3	21	10
1. iv. 65	27	10	1	24	10
19. iv. 65	27	15	0	27	13
11. v. 65	27	12	0	27	12
9. vi. 65	27	11	0	27	13
25. vi. 65	27	12	0	27	13
20. vii. 65	27	11	0	27	13
24. viii. 65	23	9	0	23	11
5. x. 65	27	10	0	27	13
17. xi. 65	18	4	0	17	10
20. xii. 65	18	6	1	18	10
26. i. 66	27	7	2	27	12

Swabs from the axillae of six men in February 1966 revealed a scanty population, similar to the forearms, though their groin folds supported a denser population. Two of the subjects yielded a heavy growth of mannitol-positive coagulase-negative staphylococci from the groin swabs similar to the organisms commonly isolated from the bedding and from the air in the sleeping huts.

Only five of the 1965 party carried detectable numbers of coagulase-positive staphylococci at the forearm site during the 14 months of testing (Fig. 1). Five of these eight isolations were obtained between January and April 1965. Then followed a period when repeated testing failed to detect *S. aureus* on the forearm skin. Suddenly, three further isolations were made in December 1965 and January

1966—all from men who were persistent nasal carriers of *S. aureus*. The penicillin sensitivity and phage type of each subject's skin and nose strains correlated well.

Anterior nares

In March 1965 staphylococci were isolated from 75% of the subjects, but by April 1965 100% of them harboured these bacteria and this state remained constant for the rest of the study period (Table 3). Approximately half of these subjects carried mannitol-positive as well as mannitol-negative strains and, in many cases, the former predominated. All the mannitol-positive strains were coagulase-positive, while one man (subject 21) persistently carried a mannitol-negative coagulase-positive strain.

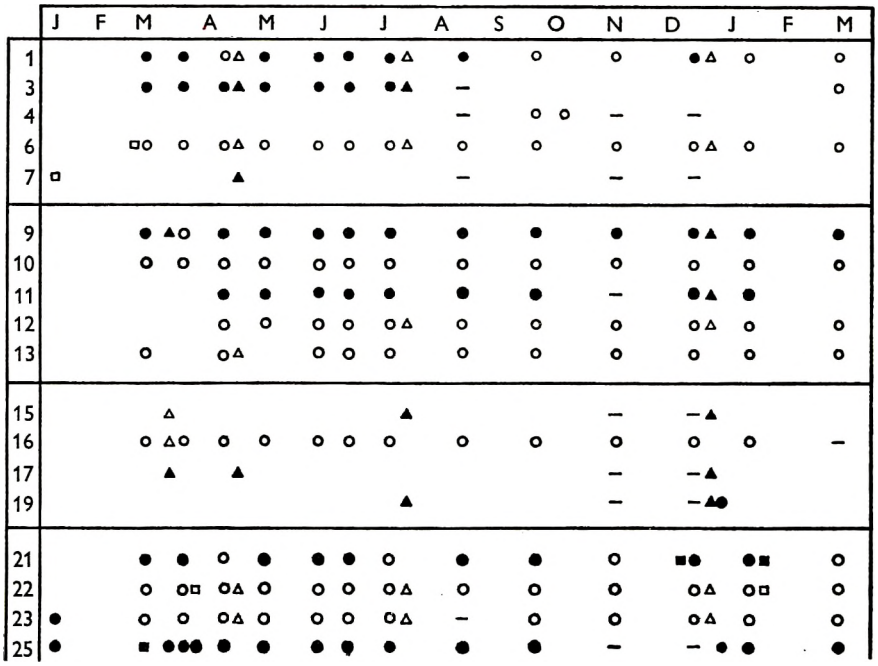

Fig. 1. Schematic representation of the carriage of *S. aureus*. ○, Nasal carrier; □, skin carrier; △, oropharyngeal carrier. The blocked-in symbols represent penicillin-resistant strains.

In all, 15 men carried *S. aureus* in their anterior nares at some time during the study period (Fig. 1). Only two of these were occasional carriers. The first (subject 4) carried a coagulase-positive strain for 2 weeks following his return from a long dog-sledge trip. His strain was closely related to that carried by subject 3, who also went on the trip and who ceased to carry the strain (Group I and III) forthwith. The second occasional carrier was subject 19, who held a penicillin-resistant strain, type 80, for a short period following his return from the spring tractor trip. Subjects 17 and 25, other members of this party, carried similar strains. Subject 3 could be classed as an intermittent carrier, losing his nasal strain for at least 5 months and resuming with a different organism. Though some men (subjects 11,

12 and 16) did not carry their nasal strains for the complete study period, their pattern of constant carriage of organisms of similar phage type for over 9 months allows their inclusion in the category of persistent carriers (Gould & McKillop, 1954). The remaining nasal carriers were persistent carriers of *S. aureus* with similar phage types at each collection, except subject 13, who changed from type 52 to type 6 during June, and who carried the latter strain for the rest of the year. Six of the 13 long term carriers of *S. aureus* had penicillin-resistant strains.

Oropharynx

The isolation of staphylococci from throat swabs, though not performed regularly, allowed a pattern of carriage to be determined (Fig. 1). Fourteen men carried coagulase-positive staphylococci on at least one occasion between March 1965 and January 1966. Ten of these were found to be carriers on more than one occasion. Nine of the 14 throat carriers were also persistent nasal carriers of *S. aureus*, while subject 3 ceased carrying his oropharyngeal strain when he lost his comparable nasal strain. Of the 11 men who carried nose and throat strains concurrently, the phage types were comparable in all but four cases. Seven subjects carried penicillin-sensitive strains and six carried penicillin-resistant strains. Subject 15 changed from a penicillin-sensitive type 80, 6/47/53/83A, 187 B5, to a penicillin-resistant type 53*w* and later to a type 53 strain resistant to penicillin, tetracycline and erythromycin. The latter was the only strain detected with a similar resistance pattern and this man had not been in contact with any of these antibiotics while in Antarctica.

Phage types of staphylococci, Mawson 1965–1966

The individual phage types will not be detailed, but Table 4 summarizes the frequencies of phage groups isolated from the 1965 party.

Table 4. *Phage groups of staphylococci, Mawson*, 1965–1966

	Number of strains from		
Group	Skin	Anterior nares	Oropharynx
I	3	12	4
I and III; and I, III and Miscellaneous	0	5	4
I, II and III	1	0	0
III	0	2	1
Miscellaneous	0	0	1
Non-typable	1	0	0

Staphylococci in the environment

Airborne staphylococci

Air volumes of 10 ft.^{3} in the mess hut and recreation room were collected during and after periods of occupation but relatively insignificant numbers of bacteria were isolated (numbers of colonies ranged from 1 to 23) and all were coagulase negative strains. Air volumes of 20 ft.^{3} were sampled in the sleeping huts. Again

very low counts (1 to 2 colonies) were found during quiet conditions. Counts rose to about 40 when there was a minor disturbance, when, for instance, one man climbed into bed. With a lot of disturbance of bedding and clothing the counts rose to around 500. By collecting air samples for consecutive half-hour periods it was found that significant numbers of bacteria remained circulating in the hut atmosphere for at least 30 min., then dropped rapidly to insignificance. The major cause of the decrease in bacterial load would be settling of bacteria-carrying particles as dust. Some of the loss is also attributable to air exchange, for one volume of hut air was sucked out as draught through the stove flue every 2 hr. under average wind conditions. No coagulase-positive staphylococci were isolated from air samples collected in the buildings.

Canine staphylococci

Twenty-seven sledge dogs, both adults and pups, were investigated. Seventeen were found to carry staphylococci in the anterior nares and thirteen of the strains were coagulase-positive. The cells were larger than the human strains and were sensitive to all antibiotics tested. No lytic reactions were noted when these canine strains were tested with bovine phages at RTD but seven gave a weak lytic reaction with phage 47C.

DISCUSSION

This study demonstrated that living under Antarctic conditions may affect the carriage of coagulase-negative staphylococci on the forearm skin. In fact, the carriage rate tended to increase with the onset of colder weather. A more striking seasonal effect was noted on the coagulase-positive skin strains which were isolated only during the summer months, when the skin carrier rate was similar to that in temperate climates (Gillespie, Devenish & Cowan, 1939; Marples, 1965). Thus a definite ecological advantage was found to exist, favouring the survival of non-pathogenic over pathogenic staphylococci on the skin during the Antarctic winter. The seasonal nature of this change implies that temperature is an important factor in these host–parasite relationships. The bacterial flora of the forearm was exposed to relatively low environmental temperatures, even though the arm was clothed (Winslow & Herrington, 1949). Doctor J. Hudson (Medical Officer, Mawson 1966–1967) did a series of skin temperature readings on men in appropriate dress, exposed to routine work conditions, and found that forearm skin temperatures ranged on the average from 31·7° C. when in the mess, down to 18·2° C. when doing work in the field. Nasal temperatures under these conditions were 33·1° and 14·5° C. respectively. Note that the nasal temperatures actually fell lower than the skin temperatures and that the present study showed that both pathogenic and non-pathogenic staphylococci thrive in the nose. There are, however, two other factors that should be considered:

(1) The dense colonization of the anterior nares, which would permit rapid regeneration of the population if it was depleted during periods of extreme cold exposure.

(2) The flora of the anterior nares had a much more humid environment than the

flora of the forearm skin because rhinorrhoea was common during cold exposure. The results of this survey are consistent with the data recorded by Mayyasi, Birkland & Dodd (1955), who found humidity to be an important factor in the survival of Gram-positive bacteria in the nasal passages of mice, and that of Blank & Dawes (1958), who also established that desiccation decreased the survival rates of skin bacteria, though they failed to show that only altering the relative humidity favoured the survival of non-pathogens over pathogens.

The number of isolations of oropharyngeal coagulase-positive staphylococci tended to increase during the year, for it was perhaps a more favourable situation than the anterior nares, being humid and probably not as cold.

The number of nasal carriers of *S. aureus* in this group is comparable with the carriage rate for normal populations in temperate zones, but is a little higher than Sladen (1965) found in his Hope Bay, Signy Island, Wilkes and Hallett groups. The Mawson results tend to confirm Sladen's proposal that there is a low incidence of occasional carriers in Antarctica, compared with temperate climates, when from 40 % (Gould & McKillop, 1954) to 60 % (Hutchinson, Green & Grimson, 1957) of a normal population will be found to carry staphylococci for short periods if observed over several months. The relative protective effect of an established coagulase-negative population in the anterior nares (Shinefield, Ribble, Boris & Eichenwald, 1963) may have been a factor preventing the appearance of occasional carriers, for it was noted that the coagulase-negative carrier rate in the Mawson party quickly rose to 100 %.

The range of phage types isolated from the 1965 party is peculiar to that population, having a high percentage of group I strains, a few group III and a small proportion of strains difficult to classify satisfactorily. There were no group II isolates, but this is not a polar phenomenon because Sladen records several group II strains and more group III than group I isolations.

McLean (1919) demonstrated that if men stayed in a cold environment for a sufficient length of time they ceased to carry *S. aureus* in their noses and throats. His expedition did not have an effectively heated hut and, within 3 months, no 'golden staphylococci' could be isolated. This was the same stage at which the coagulase-positive skin strains disappeared from the 1965 party. McLean found that *S. albus* could persist where *S. aureus* could not, and this is confirmed by later studies. In contrast, the studies on modern expeditions indicate that nasal and throat carriers continue to hold *S. aureus*, apparently because their cold exposure is neither as great nor as constant as that experienced on Mawson's 1911–14 expedition. It is perhaps significant that subject 3 had been a constant carrier of *S. aureus* in his nose and throat until leaving on a long winter dog-sledge trip. This involved continuous exposure to cold, with long periods of heavy breathing while running with the dogs and sleeping at night in an unheated tent in which temperatures dropped to near ambient (down to -28° C.). Subject 23 was the only other persistent carrier of *S. aureus* on this trip, and he retained his type 52 strain. It was on this trip that one instance of transfer of a coagulase-positive strain to an occasional carrier (subject 4) was noted, but colonization was precarious and the coagulase-negative population remained large. It is important

to note that the transferred strain came from subject 3 and that he and subject 4 lived in adjacent cubicles in the same sleeping hut for the whole of the year, yet no similar transfer was noted to have occurred under station conditions. Another long field trip revealed a second occasional carrier (subject 19). He also lived in the same hut with a possible donor (subject 17) for the year at Mawson. Sladen's subjects O.B. and J.O. perhaps demonstrate this same phenomenon.

Sladen also records that some transfer of pathogenic strains to occasional carriers was noted at Hope Bay and Signy Island. Only two instances of *S. aureus* transfer were noted at Mawson station, and involved subjects 11 and 12, who both continued as persistent carriers from April 1965. This may be analagous to the report by Hicks *et al.* (1964), that some occasional carriers become persistent carriers when living in a cold environment. Subjects 11 and 12 were hut-mates and the latter apparently became colonized with the nasal strain from subject 13, another occupant of the same hut. The source of subject 11's strain is more obscure, for it was difficult to classify satisfactorily, being group I and III, a relatively common pattern at the station.

The studies of the inanimate environment revealed that no significant contamination by *S. aureus* occurs in the sleeping huts or on the sleeping bags, though *S. albus* was found in both situations. Transfer of *S. albus* may well have occurred through the agency of blankets (Rountree & Beard, 1962) and sleeping bags, but the transfer of *S. aureus* may have to be explained by aerial spread from nose and throat.

The sledge dogs were unexpectedly found to have a high rate of carriage of coagulase-positive staphylococci compared with dogs in temperate regions, for Rountree, Freeman & Johnson (1956), Pagano *et al.* (1960) and Smith (1961) found these strains in only 10–15% of animals tested. Smith (1961) noted a high incidence of coagulase-negative strains in his animals, but they were detected in only three animals in this Antarctic series. It is considered that these Antarctic dogs and their nasal flora represent a unique and specialized ecosystem with little pathological significance for their human handlers.

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REFERENCES

Blair, J. E. & Williams, R. E. O. (1961). Phage typing of staphylococci. *Bulletin of the World Health Organization* **24**, 771.

Blank, I. H. & Dawes, R. K. (1958). The water content of the *Stratum corneum*. IV. The importance of water in promoting bacterial multiplication on cornified epithelium. *Journal of Investigative Dermatology* **31**, 141.

BUDD, G. M. (1964). General acclimatization to cold in men studied before during and after a year in Antarctica. *ANARE Reports*, No. 70, Australian National Antarctic Research Expeditions, Melbourne.

CAMERON, A. S. & MOORE, B. W. (1968). The epidemiology of respiratory infection in an isolated Antarctic community. *Journal of Hygiene* **66**, 427.

CHRISTIE, R. W. (1958). Bacterial variations in the nasopharynx and skin of isolated arctic scientists. *New England Journal of Medicine* **258** (ii), 531.

FISK, A. (1940). The technique of the coagulase test for staphylococci. *British Journal of Experimental Pathology* **21**, 311.

GILLESPIE, E. H., DEVENISH, E. A. & COWAN, S. T. (1939). Pathogenic staphylococci—their incidence in the nose and on the skin. *Lancet* ii, 870.

GOULD, J. C. & MCKILLOP, E. J. (1954). The carriage of *Staphylococcus pyogenes* var. *aureus* in the human nose. *Journal of Hygiene* **52**, 304.

HARE, R. & THOMAS, C. G. A. (1956). The transmission of *Staphylococcus aureus*. *British Medical Journal* ii, 840.

HICKS, C. C., POE, R. H. & DOOLEY, E. S. (1964). The nasal and skin carriage of *Staphylococcus aureus* in an artificial cold environment. *Military Medicine* **129** (i), 264.

HUTCHINSON, J. G. P., GREEN, C. A. & GRIMSON, T. A. (1957). Nasal carriage of *Staphylococcus aureus* in nurses. *Journal of Clinical Pathology* **10**, 92.

LIVE, I. & NICHOLS, A. C. (1961). The animal hospital as a source of antibiotic resistant staphylococci. *Journal of Infectious Diseases* **108**, 195.

LIVE, I. & NICHOLS, A. C. (1965). Serologic typing of staphylococci as an aid in epidemiological studies. *Journal of Infectious Diseases* **115**, 197.

MCLEAN, A. L. (1919). Bacteriological and other researches. In *Australasian Antarctic Expedition*, 1911–14, *Scientific Reports*, Series C, VII (4), Sydney.

MARPLES, M. J. (1965). *The Ecology of the Human Skin*, p. 578. Springfield: Thomas.

MAYYASI, S. A., BIRKLAND, J. M. & DODD, M. C. (1955). Effect of temperature and humidity on nasal flora of mice. *Proceedings of the Society for Experimental Biology and Medicine* **90**, 446.

PAGANO, J. S., FARRER, S. M., PLOTKIN, S. A., BRACKMAN, P. S., FEKETY, F. R. & PIDCOE, V. (1960). Isolation from animals of human strains of staphylococci during an epidemic in a veterinary school. *Science* **131**, 927.

ROUNTREE, P. M. & BEARD, M. A. (1962). Observations on the distribution of *Staphylococcus aureus* in the atmosphere of a surgical ward. *Journal of Hygiene* **60**, 387.

ROUNTREE, P. M., FREEMAN, B. M. & JOHNSTON, K. G. (1956). Nasal carriage of *Staphylococcus aureus* by various domestic and laboratory animals. *Journal of Pathology and Bacteriology* **72**, 319.

SHINEFIELD, H. R., RIBBLE, J. C., BORIS, M. & EICHENWALD, H. F. (1963). Bacterial interference: its effect on nursery-acquired infection with *Staphylococcus aureus*. I. Preliminary observations on artificial colonization of newborns. *American Journal of Diseases of Children* **105**, 646.

SLADEN, W. J. L. (1965). Staphylococci in noses and streptococci in throats of isolated and semi-isolated Antarctic communities. *Journal of Hygiene* **63**, 105.

SMITH, J. E. (1961). The aerobic bacteria of the nose and tonsils of healthy dogs. *Journal of Comparative Pathology* **71**, 428.

WILLIAMS, R. E. O. & HARPER, G. J. (1946). Determination of coagulase and alpha-haemolysin production by staphylococci. *British Journal of Experimental Pathology* **27**, 72.

WINSLOW, C. E. A. & HERRINGTON, L. P. (1949). *Temperature and Human Life*, 1st ed. p. 80, Princeton University Press.

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Observations on the carrier state and related antibody titres during an outbreak of foot-and-mouth disease

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SUMMARY

An outbreak of foot-and-mouth disease in a partially immune population of cattle in Botswana is described. The results show that when cattle immunized by vaccination were presented with natural field challenge of FMD, many animals with immunity sufficient to protect them against clinical disease were, however, susceptible to pharyngeal infection and subsequently became virus carriers. The proportion of animals becoming carriers appeared to vary with the degree of severity of the challenge.

Vaccination before exposure to virus appeared to have little effect on the duration of the carrier state. No evidence was obtained of the spread of carrier virus to immune herds following the outbreak.

Antibody titres during the outbreak were higher in the clinically infected animals than in the carrier animals and the uninfected animals. Evidence suggested that natural challenge boosted the titres of immune animals. After the outbreak, however, it was not possible to distinguish by their antibody titres between the carrier animal and the virus-negative animal.

Antigenic studies on the strains of virus isolated are described.

INTRODUCTION

The carrier state in foot-and-mouth disease (FMD) is now well established as a sequel to infection (van Bekkum *et al.* 1959; Sütmoller & Gaggero, 1965; Burrows, 1966; Hedger, 1968), but the infection may not necessarily involve the appearance of lesions at the predilection sites. Thus Sütmoller, McVicar & Cottral (1968) found that the exposure of susceptible cattle to minimal amounts of virus could establish the carrier state with only a subclinical infection with fever and viraemia. They had also shown that immune cattle exposed to contact infection became carriers without developing lesions or viraemia. Hedger (1968) suggested on the basis of his results in Botswana that transmission of virus from carriers in a herd may occur without the appearance of overt disease.

The observations presented here were made during a natural outbreak of FMD caused by a type SAT 1 virus. They show that when animals in a herd partially immune following vaccination are challenged by natural infection in the field, many with immunity sufficient to protect them from clinical disease do nevertheless become carriers of virus. The carrier state was also demonstrated in vaccinated

herds which, although contiguous to naturally infected herds, did not show any clinical evidence of FMD either during or after the outbreak. No evidence was obtained of the spread of carrier virus to immune herds after the outbreak.

Antigenic studies were made on the virus strains isolated.

Sera were assayed for FMD antibodies and the levels demonstrated are correlated with the state of the immunity of the various groups.

Previous history

The outbreak of FMD was confirmed on 1 January 1968 in cattle in the Satau area of the Chobe River district of Botswana close to its northern border with the Caprivi Strip. The virus was typed SAT 1. In this remote area, isolated from the remainder of Botswana by a game reserve to the east and south and by tsetse fly country to the west, FMD has occurred infrequently. The last recorded outbreak was in 1950 and the virus type was SAT 1. In recent years some movement of cattle into the area has taken place, but at the time of the outbreak there were no reports of FMD occurring or having recently occurred in other parts of Botswana or in neighbouring territories.

In September 1967, the Chobe River area was included in the annual prophylactic FMD vaccination campaign which covers the whole of northern and western Botswana. The herds in the area had received their first vaccination with a bivalent types SAT 1 and SAT 3 inactivated vaccine.

Contact of domestic stock with wild life, generally indirect, does occur in the area and there was a reported contact of some of the infected herds with buffalo in November 1967. A type SAT 1 virus strain was recovered from a clinically normal buffalo some months after the outbreak (Hedger, Condy & Falconer, 1969). Although this strain of virus was shown to be antigenically similar to the outbreak strain, it is not possible to state whether the infection passed from the cattle to the buffalo or vice versa.

MATERIALS AND METHODS

Virus

Samples of epithelium from animals with lesions were collected in 50 % glycerol phosphate buffer.

Oesophageal/pharyngeal (O/P) samples for the isolation of carrier virus were taken and transported on dry ice to the laboratory. The collection, transport and the isolation of virus from O/P samples on bovine thyroid cell tissue culture monolayers has already been described in detail (Hedger, 1968).

Whole blood with an anticoagulant (EDTA) was taken from the clinically normal animals to check for the presence of viraemia. These samples were also transported on dry ice and virus isolation attempted on bovine thyroid monolayers.

Virus specificity

The specificity of all the virus strains isolated was checked by complement-fixation (CF) tests using the microtitre technique described by Casey (1965). Subtype variations were checked by screening in CF test all virus strains isolated against a range of subtype-specific antisera.

A virus strain isolated from one of the epithelial samples was adapted to guinea-pigs for the production of a specific antiserum and, using cross-complement-fixation tests (Davie, 1964), antigenic differences between this strain, the vaccine strain, carrier virus strains and previously known subtype strains were studied.

Serum neutralization tests

Sera were assayed by the cell metabolic inhibition test or colour test (Martin & Chapman, 1961), using primary monolayers of pig kidney cells and a type SAT 1 virus strain (Rho 5/66) isolated from an outbreak of FMD in Rhodesia in 1966. Before its use in the test the virus was adapted to grow in pig kidney cell cultures by serial passage.

Neutralization titres are expressed as the reciprocal of the final dilution of serum present in the serum-virus mixture at the 50 % end point estimated according to the method of Kärber (1931).

Vaccine

The vaccine used during the prophylactic vaccination campaign and for control during the outbreak was a bivalent A.E.I. inactivated B.H.K. suspension culture vaccine incorporating a saponin adjuvant. The virus strains used were type SAT 1 (Rho 5/66) and type SAT 3 (Bec. 1/65).

Cattle and area

The cattle (*Bos indicus*) were native-owned indigenous Tswana stock ranched with a low standard of husbandry. Grazing is communal and herds mix freely. Herds were examined in three areas: (1) the infected area (Satau), (2) the contiguous area (Katchekau), (3) the clean area (Kazangula).

The village of Satau is about 16 miles from the village of Katchekau, but the grazing areas of the two villages overlap and some movement of stock takes place between the two areas. Kazangula, although ecologically similar and administratively part of the Chobe District, is approximately 70 miles from Satau and there was little likelihood of recent intermingling of stock between the two areas. There were no sheep and only a few goats in the areas selected for study.

The stocking rate in the district was low and in recent years had been reduced to well below the carrying capacity of the land, probably owing to the combined effects of streptothricosis, tick-borne disease and the tsetse fly.

The examination and sampling of herds in these three areas made it possible to study any evidence of previous undetected infection in the district as a whole and also to study the possible subsequent transmission of carrier virus into cattle in the contiguous area following the withdrawal of the quarantine restrictions after the outbreak.

RESULTS

General survey

Clinical examination of several hundred head of cattle in the clean and contiguous areas failed to show evidence of current or recent disease. O/P samples from 60 head of cattle at Kazangula (clean area) and 30 head at Katchekau (contiguous area) were taken at random from different herds. No foot-and-mouth disease virus (FMDV) was recovered from any of these animals. There was thus no evidence of previous undetected infection in the district and it may be assumed that antibody levels in the cattle in these two areas were due to vaccination and not infection.

The immune and partially immune herds in the infected area (Satau) comprised about 1200 head and fell roughly into three groups: (1) herds with an approximate 70 % morbidity, (2) herds with an approximate 25 % morbidity, (3) herds in contact with diseased herds but in which no clinical disease was recorded.

Carrier virus was recovered from O/P samples from clinically normal animals in all three groups. Table 1 shows the incidence of carrier virus recovery in each of the groups.

Table 1. *The recovery of carrier virus from clinically normal cattle which resisted natural challenge with foot-and-mouth disease virus*

Morbidity in groups	No. of animals sampled	No. virus-positive	% carriers in clinically normal animals
Group 1, 70 %	27	23	85
Group 2, 25 %	18	6	33
Group 3, 0 %	20	6	30

As it was possible that, in the presence of active infection, some of these apparently immune animals were in the prodromal phase of the disease (Burrows, 1968) or were undergoing subclinical infection, whole blood was examined in parallel with the O/P samples for the presence of viraemia. No viraemia was detected.

Available animals in the infected area and the contiguous area were resampled at periods of 6 and 12 months after the outbreak. Table 2 summarizes the results of the follow-up samplings.

All the virus strains isolated were typed SAT 1. Of the 13 virus-positive animals

Table 2. *Percentage of virus-positive animals during and after the outbreak*

	During outbreak			6 months after outbreak			12 months after outbreak		
	No. examined	Virus-positive	%	No. examined	Virus-positive	%	No. examined	Virus-positive	%
Infected area (Satau)	93*	63	68	34*	13	38	37*	2	5·4
Contiguous area (Katchekau)	30	0	0	30	0	0	30	0	0

* Includes both clinically normal and clinically infected animals at time of outbreak.

at 6 months after the outbreak, five had not been clinically infected. One of the two positive animals at 12 months had not been clinically infected.

Serology

Serum was collected from all animals sampled for carrier virus. In addition, in the infected area numbers of animals actually undergoing infection or having recently undergone infection were also bled.

Table 3 presents a summary of the antibody titres to type SAT 1 virus (strain Rho 5/66) at the termination of the outbreak. The results are recorded as the geometric means of the reciprocal antibody titres of each of the groups. The numbers of the animals sampled and tested in each group are given in parentheses.

Table 3. *The geometric mean reciprocal antibody titres of groups of cattle in infected and non-infected herds*

	Clinically infected animals	Carriers (no overt disease)	Virus-negative animals
Infected area (Satau)			
Group I. 70 % morbidity	631 (24)*	247 (23)	1318 (4)
Group II. 25 % morbidity	3090 (4)	513 (6)	513 (12)
Group III. 0 % morbidity	—	794 (6)	166 (13)
Contiguous area (Katchekau)			
Group IV	—	—	112 (29)
Clean area (Kazangula)			
Group V	—	—	112 (22)

* The numbers in parentheses are the numbers of cattle in each group.

Serum samples were taken again at 6 months and 12 months after the outbreak. As expected, antibody titres had dropped considerably. There was no significant difference between the antibody titres in the carrier animals and the virus-negative animals, nor was it possible by their antibody titres to differentiate between those animals which had undergone clinical disease and those which had resisted infection.

Antigenic studies

In an earlier survey of FMDV carrier animals previously infected with a type SAT 3 virus (Hedger, 1968), antigenic variation between different strains of carrier virus from animals in the same herds was observed. It was not possible to state whether there had been a variation in the virus at the time of the outbreak or whether such variation had occurred while the virus was in the carrier state after clinical disease.

Therefore, in this survey, in addition to the various carrier strains of virus isolated during the outbreak, lingual epithelium was collected from a number of affected animals and virus isolations were made. All the virus strains isolated were typed SAT 1.

Virus from one of the epithelial samples was adapted to grow in guinea-pigs for the production of a specific antiserum. Using a range of type SAT 1 subtype-specific antisera and including this homologous antiserum, all the strains of virus

isolated during the outbreak were tested in complement-fixation tests for possible antigenic variation. With each virus strain the serum titres were plotted as histograms and compared with each other and with histograms from other type SAT 1 subtype strains. No antigenic variation was seen among the 40 strains isolated during the outbreak. A number of the carrier virus strains recovered at periods after the outbreak have also been tested. Antigenic variation from the original outbreak strain has not been observed in any of these strains so far examined.

In cross-complement-fixation tests the outbreak strain was shown to be antigenically similar to strain Rho 5/66 with a complement-fixation product of 0·83 (Davie, 1964) equivalent to an *R* value of 91 % (Ubertini *et al.* 1964). Rho 5/66 is a standard World Reference Laboratory subtype strain originating from an outbreak in Rhodesia in 1966 and used since as a vaccine strain.

DISCUSSION

The results show that when cattle immunized by vaccination were presented with a severe natural field challenge with FMDV (Group 1), nearly all those animals (85 %) with sufficient immunity to protect them against clinical disease were, however, susceptible to local pharyngeal infection and became virus carriers.

In Group II immunity was higher and a smaller proportion of animals became carriers.

Most significant was the finding of carrier animals in vaccinated herds which had been in close contact with field virus but in which no clinical disease was observed.

The percentages of demonstrable persisting carrier animals at 6 and 12 months after the outbreak (Table 2) are of a similar order to those found in a previous survey involving a type SAT 3 virus (Hedger, 1968). The results suggest that vaccination before exposure to virus has little effect on the subsequent duration of the carrier state.

Repeated sampling in the vaccinated contiguous herds failed to reveal carrier animals. This indicates that after an outbreak there may be little likelihood of carrier virus spreading in a vaccinated population under natural conditions.

While it is not possible to differentiate by the antibody level of an individual animal between infection and resistance to infection with or without an accompanying carrier status, differences in the mean antibody level of the groups in the areas are worthy of comment.

Experience has shown that, following infection, where high antibody levels are found in a sample of a population, carrier virus will also be recoverable from that population. Conversely, if significant antibody levels cannot be demonstrated in some animals in a population it is highly unlikely that carrier virus will be recovered. Thus the antibody levels in the contiguous area (Katchekau) and the disease-free area (Kazangula) where no carrier virus was isolated may be accepted as due to vaccination.

The antibody levels of Groups I and II in the infected area where animals have been either infected or in direct contact with natural infection are considerably higher. In Group III, however, where there was no apparent clinical disease, but

where there had been sufficient contact with infection for some of the animals to become virus carriers, the mean antibody titre of the negative animals is of the same order as the control groups (IV and V) and is markedly lower than the other virus-negative animals in the herds where frank clinical disease occurred. This supports the observation that clinical disease had not in fact occurred in these herds, although some of the animals had assumed the carrier status.

In Groups I and II where overt disease had occurred, the mean antibody titres in the clinically affected animals were, as expected, higher than in those which had resisted challenge. The mean titres of the negative animals in these groups were, however, also considerably higher than in the control groups in the non-infected areas. This suggests that the presence of virulent infection in an immune or semi-immune population actively boosts the immunity of animals which resist challenge.

The very high antibody titres in the virus-negative animals in Group I are not fully understood, but they may suggest that if animals possess a strong enough induced immunity, they may not only resist clinical disease but also carrier infection.

The close antigenic similarity of all the strains of virus isolated at the time of the outbreak was expected, and is similar to the findings when multiple strains have been examined during other outbreaks. For example, no significant antigenic differences were recorded in the many strains of virus isolated during the British type O_1 epizootic of 1967/68 (J. Davie, personal communication). However, the close similarity of all the carrier virus strains isolated after the type SAT 1 outbreak is at variance with the findings of an earlier survey involving a type SAT 3 virus, when antigenic differences were observed not only between some of the carrier strains and the outbreak virus strain but also between carrier strains isolated from different animals in the same herd (Hedger, 1968). The evidence suggests that the SAT 1 virus strain involved in this outbreak and in the previous outbreak in Rhodesia is more stable than the SAT 3 strain mentioned, in which antigenic variations were recorded in the carrier phase. The possibility of antigenic change occurring in the SAT 1 carrier virus at a later date, however, is not precluded.

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REFERENCES

VAN BEKKUM, J. G., FRENKEL, H. S., FREDERIKS, H. H. J. & FRENKEL, S. (1959). Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschrift voor Diergeneeskunde* **84**, 1159–64.

BURROWS, R. (1966). Studies on the carrier state of cattle exposed to foot-and-mouth disease virus. *Journal of Hygiene* **64**, 81–90.

BURROWS, R. (1968). Excretion of foot-and-mouth disease virus prior to the development of lesions. *Veterinary Record* **82**, 387–8.

CASEY, H. L. (1965). Standardized diagnostic complement fixation method and adaptation to micro test. *Public Health Monographs, Washington* **74**, 1–34.

DAVIE, J. (1964). A complement-fixation technique for the quantitative measurement of antigenic differences between strains of the virus of foot-and-mouth disease. *Journal of Hygiene* **62**, 401–11.

HEDGER, R. S. (1968). The isolation and characterization of foot-and-mouth disease virus from clinically normal herds of cattle in Botswana. *Journal of Hygiene* **66**, 27–36.

HEDGER, R. S., CONDY, J. & FALCONER, J. (1969). The isolation of foot-and-mouth disease virus from African Buffalo (*Syncerus caffer*). *Veterinary Record* **84**, 516–17.

KÄRBER, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Archiv für experimentelle Pathologie und Pharmakologie* **162**, 480–3.

MARTIN, W. B. & CHAPMAN, W. G. (1961). The tissue culture colour test for assaying the virus and neutralising antibody of foot-and-mouth disease and its application to the measurement of immunity in cattle. *Research in Veterinary Science* **2**, 53–61.

SÜTMOLLER, P. & GAGGERO, A. (1965). Foot-and-mouth disease carriers. *Veterinary Record* **77**, 968–9.

SÜTMOLLER, P., MCVICAR, J. W. & COTTRAL, G. E. (1968). The epizootiological importance of foot-and-mouth disease carriers. I. Experimentally produced carriers in susceptible and immune cattle. *Archiv für die gesamte Virusforschung* **23**, 227–35.

UBERTINI, B., NARDELLI, L., DAL PRATO, A., PANINA, G., & SANTERO, G. (1964). Subtype variation of foot-and-mouth disease virus and vaccination. *Wiener tierärtzliche Monatsschrift* **51**, 99–110.

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Growth in chick chorioallantoic membranes of strains of Newcastle disease virus of differing virulence

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SUMMARY

The growth of eight strains of Newcastle disease virus in chick embryo chorioallantoic membranes was studied by comparing, at different times after infection, the amounts of haemagglutinin released into the allantoic fluid (extracellular haemagglutinin) with that associated with the membrane (cell-associated haemagglutinin). The virulence of the strains examined differed in that some killed chick embryos more rapidly than others. All strains released similar amounts of extracellular haemagglutinin and maximum titres were achieved about 12 hr. after infection. With virulent strains cell-associated haemagglutinin titres increased exponentially until the death of the host and maximum titres were much higher than those of extracellular haemagglutinin. With avirulent strains cell-associated haemagglutinin titres increased exponentially for only a limited time and titres were always lower than the titres of extracellular haemagglutinin.

Similar results were obtained when the titres of neuraminidase and viral ribonucleoprotein were measured during the growth of two virulent and two avirulent strains. Virulence appears to be associated with the continued intracellular accumulation of viral antigens.

INTRODUCTION

Newcastle disease virus (NDV) has been suggested as a suitable virus for the study of virulence (Waterson, Pennington & Allan, 1967). Many strains are available and their virulence, defined here as the ability to cause disease or death in a host, ranges from highly virulent strains, which are extremely pathogenic for chickens, kill chick embryos rapidly and destroy infected chick embryo fibroblast cells in culture, to strains isolated from apparently normal chickens which are not cytopathic and kill chick embryos infrequently or not at all. Virulence can be measured accurately and reproducibly in chick embryos and young chickens, and all strains grow readily in the chick embryo chorioallantoic membrane and release high titres of infectious virus into the allantoic fluid. They can, therefore, be cultured in quantity. Virus titres can be accurately and easily measured in terms of infectivity, haemagglutinin and neuraminidase activity. Besides this, NDV is not only an important animal pathogen in its own right, but is biologically akin to some important pathogens of man and other vertebrates, including mumps, the

para-influenzas, measles and distemper viruses: studies with NDV are thus relevant in a wide context.

Studies on the isolated virion show a surprising uniformity among strains: all those so far examined under the electron microscope look alike (Waterson & Cruickshank, 1963) and no major serological differences have been seen using serum neutralization or haemagglutination-inhibition (HAI) tests (W. H. Allan, unpublished observations) or in gel diffusion studies (Pennington, 1967).

Strains which differ in virulence for the chick embryo also differ, in parallel with this, in cytopathogenicity. Schloer & Hanson (1968) have shown, for example, that the most virulent strains for chick embryos produce the largest plaques on chick embryo fibroblast monolayers. This suggests that it is in the intracellular stages of virus development that strain differences might be found.

T. H. Pennington (unpublished observations) has reported differences in the quantity of haemagglutinin found in the chorioallantoic membranes of chick embryos infected with different strains of NDV, although with all strains substantially similar quantities of haemagglutinin (HA) were released into the allantoic fluid. However, these findings were based on samples taken, for all strains, 40 hr. after inoculation of the chick embryos: thus no information was available on the rates at which the haemagglutinin was produced. We have, therefore, measured the production of haemagglutinin in chorioallantoic membranes of chick embryos throughout one cycle of growth of several strains of NDV. We have compared these results with the titres of haemagglutinin released into the allantoic fluid and we have also measured the rates at which neuraminidase (Mucopolysaccharide *N*-acetylneuraminylhydrolase) and viral ribonucleoprotein are produced.

METHODS

Viruses

The following strains of NDV were used:

Herts 33 (Herts), received from W. H. Allan, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey. Isolated at Weybridge in 1933 by Dobson (1939).

Italien, received from Professor R. Rott, Institute of Virology, Giessen, Germany. Isolated in Italy at some date before 1949 (Schäfer, Schramm & Traub, 1949).

Texas GB, (Texas), received from W. H. Allan; isolated in 1948 near Austin, Texas, U.S.A.

Field Pheasant, isolated at Weybridge in 1962 from pheasants; clinically more virulent than Herts 33 (W. H. Allan, personal communication).

Beaudette C, received from Professor R. Rott. This is a heat stable variant isolated from the Beaudette strain by Granoff (1959).

Strain F, received from W. H. Allan, isolated in England by Asplin in 1949 (Asplin, 1952).

Queensland V4 (Queensland), received from W. H. Allan, isolated in 1966 by Rylie, in Brisbane, Australia (W. H. Allan, personal communication).

Ulster, received from J. B. McFerran, Ministry of Agriculture for Ireland, Belfast. Isolated in 1966 in Northern Ireland from the faeces passed by healthy chickens.

The viruses were grown in the allantois of 10-day-old chick embryos incubated at 37° C., and virus-infected allantoic fluids were stored at −70° C.

Growth cycles in chick embryo chorioallantoic membranes

For each experiment about 50 10-day-old chick embryos were inoculated with 0·5 ml. of infected allantoic fluid containing about 10^9 Egg $_{ID_{50}}$, and incubated at 37° C. At intervals thereafter three chick embryos were harvested and the allantoic fluid pooled. The chorioallantoic membranes were pooled after three washes with ice cold saline (0·15 M). All samples were stored at −70° C. until assayed.

Allantoic membranes were thawed and blended in homogenizers with motor-driven Teflon pestles to give 50 % (w/v) suspensions in phosphate buffered saline, pH 7·2 (PBS).

Haemagglutination titrations

Plastic plates were used with 0·2 ml. volumes and 1 % (v/v) chicken erythrocytes; titres are expressed as the reciprocal of the last dilution giving complete agglutination.

Neuraminidase assays

Sample volumes of 0·1 ml. were incubated at 37° C. for 10 min. with 0·1 ml. fetuin and 0·3 ml. 0·2 M-KH_2PO_4-Na_2HPO_4 buffer (pH 6·0). Fetuin was prepared from foetal calf serum by the method of Graham (1961). The *N*-acetyl neuraminic acid (NANA) released was assayed by the method of Aminoff (1961). Results are expressed as μg. NANA liberated per ml. of sample.

Incorporation of ^3H-uridine

The allantoic fluid of each egg at the start of the growth cycle was inoculated with 100 μC. ^{3}H-uridine 5-T (purchased from the Radiochemical Centre, Amersham).

The estimation of ^{3}H-uridine incorporated into ribonuclease-insensitive acid-insoluble material was based on the method of Plagemann (1968). For each sample, 0·1 ml. was diluted with 0·5 ml. 0·01 M sodium deoxycholate, 0·1 ml. ribonuclease (100 μg. per ml. in PBS) and incubated at 37° C. for 30 min.; 2·0 ml. 1 N perchloric acid was added and, after centrifugation at 1000 ***g*** for 10 min., the precipitate was washed three times with ice cold 5 % (w/v) trichloracetic acid. The precipitate was dissolved by adding 0·5 ml. 1 N sodium hydroxide and incubating at 60° C. for 30 min.: 0·2 ml. of this solution was added to 10 ml. of scintillation fluid containing 8·0 g. butyl PBD (Ciba Ltd), 80·0 g. naphthalene, 400 ml. oxitol (ICI Ltd) and 600 ml. toluene. Radioactivity was measured using an ABAC SL 40 Liquid Scintillation Spectrometer (Inter-technique Ltd) with an efficiency of about 35 %.

RESULTS

The growth of seven strains of NDV in chick embryo chorioallantoic membranes was examined by measuring the amount of haemagglutinin released into the allantoic fluid (extracellular haemagglutinin) and that still associated with the allantoic membrane (cell-associated haemagglutinin). The strains examined differed in virulence in that some killed chick embryos more quickly than others; Table 1 shows the mean death time (MDT) of chick embryos inoculated with one minimum lethal dose. Herts was the most virulent strain examined, with an MDT of 49 hr. Neither Queensland nor Ulster consistently killed chick embryos, many of which survived even high inocula of these strains. With all strains an increase in haemagglutinin was first detected in the allantoic fluid about 4 hr. after inoculation, and thereafter titres increased exponentially until 12–14 hr. after inoculation (Fig. 1).

Table 1. *Relative virulence of Newcastle disease virus strains (from Waterson, Pennington & Allan, 1967)*

Strain	Mean death time (hr.)	Intracerebral index
Herts	49	1·88
Italien	50	1·86
Texas	50	1·80
Beaudette C	62	1·48
F	168	0·25
Queensland	∞	0·25
Ulster	∞	0·00

Mean death time is the average time in which eggs inoculated with one minimum lethal dose of virus are killed. The intracerebral index is estimated from the time taken for chicks to die after intracerebral inoculation. The results are based on a scoring system in which the maximum index is 2 (100% mortality in one day) and the minimum is 0 (no recorded symptoms after 8 days).

All strains produced approximately the same levels of extracellular haemagglutinin but marked differences were apparent when haemagglutinin associated with the allantoic membranes was assayed. With strains Herts, Italien and Beaudette C the production of cell-associated haemagglutinin continued exponentially until at least 20 hr. after inoculation and only diminished when at least 50% of the chick embryos were already dead (Table 2). Cell-associated haemagglutinin titres of these virulent strains greatly exceeded those measured in the allantoic fluid at the end of the growth cycle.

Strains Queensland, Ulster and F, on the other hand, produced only low levels of cell-associated haemagglutinin and the rate of production appeared to diminish after approximately 12 hr. The titres of cell-associated haemagglutinin in these avirulent strains never exceeded those measured in the allantoic fluid.

All chick embryos inoculated with the strains Herts, Italien and Beaudette C had died after approximately 30 hr.; of the less virulent strains only F consistently killed the majority of chick embryos (90% dead within 96 hr.) whereas

50 % of chick embryos inoculated with Queensland and Ulster survived for seven days after inoculation (Table 2).

Four strains (Herts, Texas, Queensland and Ulster) were examined in greater detail to see if other structural antigens making up the NDV virions, viz. neuraminidase and ribonucleoprotein, also accumulated in the allantoic membranes infected with virulent virus but not in those infected with avirulent strains. The results obtained supported this possibility: ^{3}H-uridine incorporated into ribonuclease-resistant, acid-insoluble material and neuraminidase titres both increased exponentially in allantoic membranes infected with the virulent strains Herts and

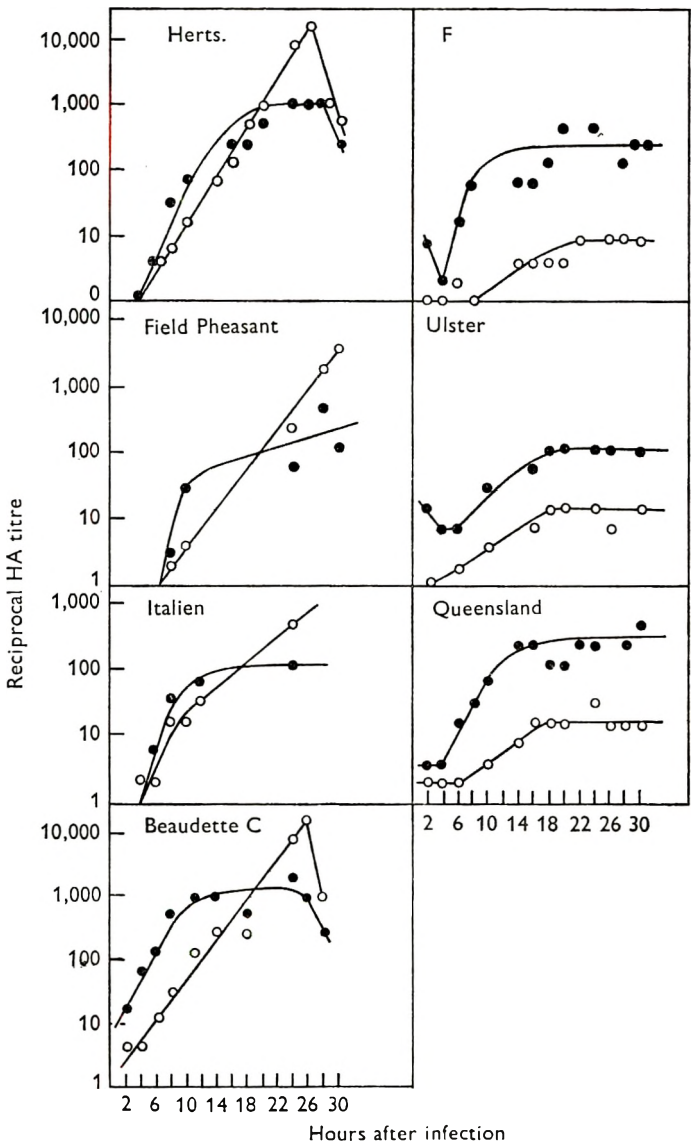

Fig. 1. The comparison of cell-associated (○—○) and extracellular (●—●) haemagglutination titres during the growth of seven different strains of NDV in eggs.

Texas until the embryos died (Fig. 2). We have assumed that the ^{3}H-labelled product is either viral RNA or ribonucleoprotein: both double-stranded RNA (Plagemann, 1968) and ribonucleoprotein (Kingsbury & Darlington, 1968) resist digestion with ribonuclease whereas single-stranded (cellular) RNA would be destroyed. In allantoic membranes infected with the avirulent strain Queensland, titres of neuraminidase and ribonuclease-resistant, acid-insoluble ^{3}H-labelled material increased exponentially only for the first 14–16 hr. after infection and then levelled off; with strain Ulster the exponential phase extended rather longer but levelled off within 24 hr. after infection (Fig. 3).

Table 2. *Percentage death of chick embryos in eggs inoculated with different strains of Newcastle disease virus*

Time after inoculation (hr.)	Strains of NDV					
	Herts	Italien	Beaudette C	F	Queensland	Ulster
22	0	0	0	0	0	0
24	7·5	—	0	0	0	0
26	15	59	9	0	0	0
28	34	78	56	0	0	0
29	—	100	—	0	0	0
30	44	.	100	0	0	0
32	100	.	.	0	0	0
48	.	.	.	0	0	0
69	.	.	.	57	7	20
74	.	.	.	70	7	20
89	.	.	.	97	20	27
96	.	.	.	100	23	30
114	.	.	.	.	36	43
138	.	.	.	.	40	53
142	.	.	.	.	57	53

DISCUSSION

The results obtained show that some strains of Newcastle disease virus had higher titres of viral components associated with the chorioallantoic membrane than with the allantoic fluid, suggesting two discrete groups of strains; (1) Virulent strains which kill chick embryos rapidly and accumulate all the major viral components in the chorioallantoic membrane to titres higher than in the allantoic fluid, and (2) avirulent strains which kill embryos slowly or not at all and produce similar extracellular antigen titres to virulent strains but in which the intracellular accumulation of virus antigen is limited.

Our results show that virulence is associated with the continued exponential production of cell-associated viral antigens without concomitant release of mature virions, a process terminated only by the death of the chick embryo.

Newcastle disease virus, like other myxoviruses, is assembled at the cell surface and the envelope may consist of or contain cell membrane (Waterson, 1968). The modification of the cell membrane presumably has a drastic effect on the cell

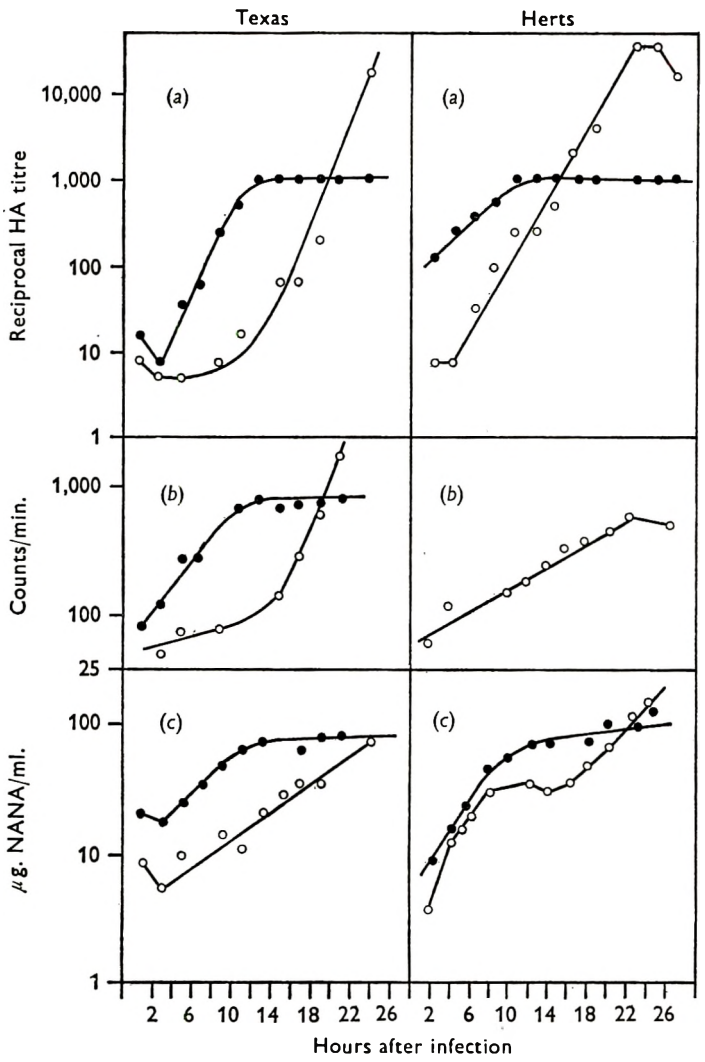

Fig. 2. Comparison of the extracellular (●—●) and cell-associated (○—○) major antigens of two virulent strains of NDV during growth in eggs. (*a*) Haemagglutinin. (*b*) Material incorporating ^{3}H-uridine, in an acid-insoluble RNase-resistant fraction. (*c*) Neuraminidase, as μg. NANA released.

permeability and metabolism. It could therefore be postulated that the strains of virus producing the most intracellular haemagglutinin would be the most virulent. The loss of ability to carry out cellular repair would enhance this effect and Wilson (1968) has shown that infection with the strain Texas GB, which is highly virulent, inhibits host cell protein synthesis in chick embryo fibroblasts.

Compans *et al.* (1966) in a morphological study showed that BHK21-F cells infected with SV5 virus accumulated viral ribonucleoprotein and disintegrated after extensive fusion although little mature virus was released. In contrast, primary rhesus monkey kidney (MK) cells infected with SV5 did not accumulate RNP, released infective virus, but were not destroyed. Virulence in their system

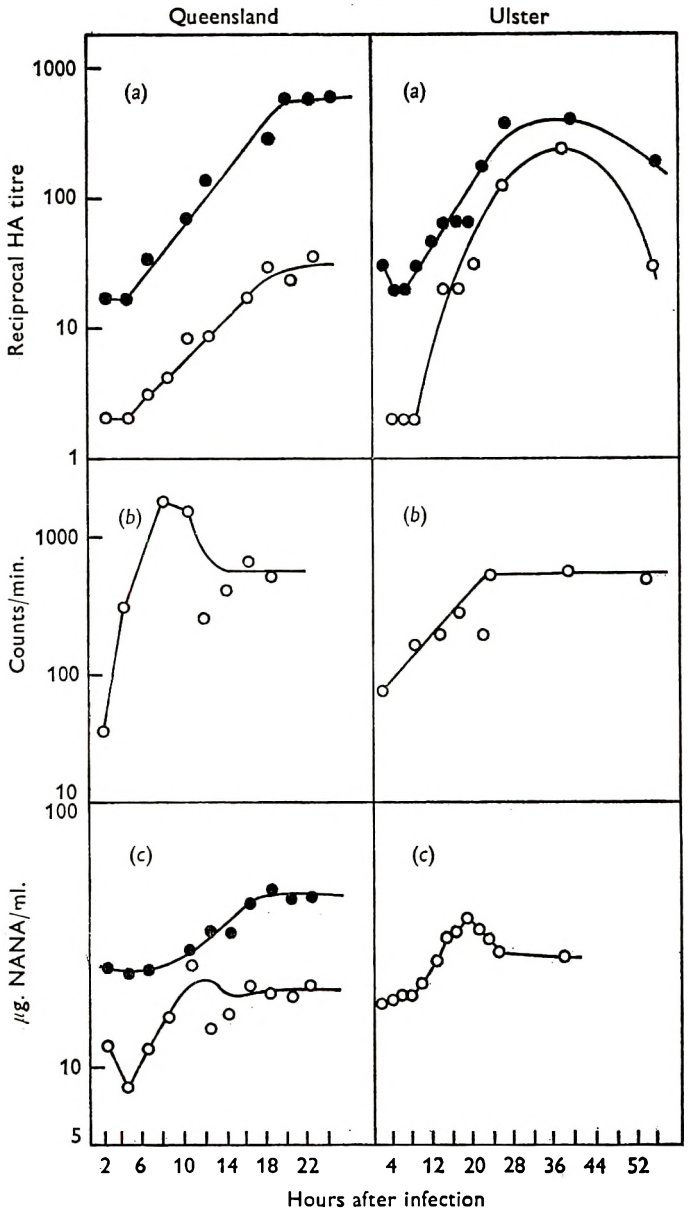

Fig. 3. Comparison of the extracellular (●—●) and cell-associated (○—○) major antigens of two avirulent strains of NDV during growth in eggs. (*a*) Haemagglutinin. (*b*) Material incorporating ^{3}H-uridine in an acid-insoluble RNase-resistant fraction. (*c*) Neuraminidase, μg. NANA released.

was determined by host cell differences but was also mediated by changes in the cellular surface and accumulation of viral antigen. In our system, unlike that studied by Compans *et al.*, virulence is determined by differences in the virus strain, the same host system being used throughout. Although avirulent strains did not appear to accumulate viral antigens in the chorioallantoic membrane we do not

know if all the cells were infected. Fewer cells may have been infected by avirulent strains than by virulent strains, but since extracellular virus titres are similar with all strains this would require cells infected with avirulent viruses to release relatively more virus into the allantoic fluid than cells infected with virulent virus.

If the same number of cells is infected the difference between strains may be that the intracellular production of viral antigen is limited in cells infected with avirulent strains either by some host cell response or some limiting factor in the virus antigen production mechanism.

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REFERENCES

AMINOFF, D. (1961). Methods for quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochemical Journal* **81**, 384.

ASPLIN, F. D. (1952). Immunisation against Newcastle disease with a virus of low virulence (strain F) and observations on sub-clinical infection in partially resistant fowls. *Veterinary Record* **64**, 245.

COMPANS, R. W., HOLMES, KATHRYN V., DALES, S. & CHOPPIN, P. W. (1966). An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV 5. *Virology* **30**, 411.

DOBSON, N. (1939). Newcastle Disease. *Proceedings of the 7th World's Poultry Congress, Cleveland*, p. 250.

GRAHAM, E. R. B. (1961). Some aspects of the structure of the carbohydrate moiety of fetuin. *Australian Journal of Science* **24**, 140.

GRANOFF, A. (1959). Studies on mixed infection with Newcastle disease virus. *Virology* **9**, 636.

KINGSBURY, D. W. & DARLINGTON, R. W. (1968). Isolation and properties of Newcastle disease virus nucleocapsid. *Journal of Virology* **2**, 248.

PENNINGTON, T. H. (1967). Studies with Newcastle disease virus. Ph.D. Thesis, University of London.

PLAGEMANN, P. W. (1968). Mengovirus replication in Novikoff rat hepatoma and mouse L cells: Effects on synthesis of host-cell macromolecules and virus specific synthesis of ribonucleic acid. *Journal of Virology* **2**, 461.

SCHÄFER, W., SCHRAMM, G. & TRAUB, E. (1949). Untersuchungen über das Virus der atypischen Geflügelpest. *Zeitschrift für Naturforschung* **46**, 157.

SCHLOER, G. M. & HANSON, R. P. (1968). Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. *Journal of Virology* **2**, 40.

WATERSON, A. P. (1968). *Introduction to Animal Virology*. 2nd ed., Cambridge University Press.

WATERSON, A. P. & CRUICKSHANK, J. G. (1963). The effect of ether on Newcastle disease virus: a morphological study of eight strains. *Zeitschrift für Naturforschung* **18**B, 114.

WATERSON, A. P., PENNINGTON, T. H. & ALLAN, W. H. (1967). Virulence in Newcastle disease virus. A preliminary study. *British Medical Bulletin* **23** (2), 138.

WILSON, D. E. (1968). Inhibition of host-cell protein and RNA synthesis by Newcastle disease virus. *Journal of Virology* **2**, 1.

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The platelet aggregation test in group B arbovirus infections

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SUMMARY

A new serological method, the platelet aggregation (PA) test, was used to study Group B arbovirus infections, which are often associated with thrombocytopenia. The method is based on the interaction of antigen-antibody complexes with platelets. The PA test was nearly as good as the haemagglutination-inhibition (HI) test for epidemiological studies of tick-borne encephalitis (16 out of 18 HI positive sera) and was superior to the complement-fixation test which was positive in 10 out of 18 HI positive sera. The PA test could be used in the serological diagnosis of Group B arbovirus infection. The titres with the PA test were of the same magnitude as the HI titres, and about ten times higher than the CF titres. Two pairs of dengue haemorrhagic fever sera showed high initial PA titres. The role of immune complexes in the pathogenesis of haemorrhagic fevers is discussed.

INTRODUCTION

Thrombocytopenia is often found during infections casued by the arboviruses of Group B (Clarke & Casals, 1965). In the haemorrhagic forms (yellow fever, haemorrhagic dengues, Omsk haemorrhagic fever and Kyasanur forest disease) it is an almost constant finding. Platelets can be aggregated *in vitro* by complexes of viral antigens and antibodies, and the platelet aggregation (PA) test has been developed to assay this activity (Penttinen & Myllylä, 1968; Myllylä, Vaheri, Vesikari & Penttinen, 1969). In this study the antibodies associated with Group B arbovirus infections were investigated by the PA test and by haemagglutination inhibition (HI) and complement fixation (CF). An attempt is made to use the findings for explaining some features of the pathogenesis of Group B arbovirus infections.

METHODS

Sera

The following human sera were studied: 24 from a region where tick-borne encephalitis (TBE) is endemic; 19 samples from nine virologically verified cases of clinical TBE; one sample taken 3 years after Omsk haemorrhagic fever; and samples from a man vaccinated once with yellow fever 17 D vaccine and a woman vaccinated four times with inactivated TBE cell culture vaccine (Institute of Poliomyelitis and Viral Encephalitis, Moscow). All these sera were stored at $-20\,^{\circ}C$.

Dr T. J. Smith (SEATO) kindly sent two paired lyophilized sera obtained from two cases of dengue haemorrhagic fever in 6-year-old girls.

Antigens

TBE virus antigens were prepared from the local strain 'Kumlinge A 52' (CABV no. 188) in cell cultures of a continuous line of human amnion cells (strain 'Utrecht'), treated with protamine (Salminen, 1962), and inactivated by heat or Tween-ether treatment (Halonen, Stewart & Hall, 1967). Yellow fever antigen was prepared from 17 D vaccine strain ('Arilvax') in the same cells and inactivated with Tween-ether treatment.

Dr J. Casals (Yale Arbovirus Research Unit) kindly sent lyophilized sucrose-acetone mouse brain antigens of dengue virus types 1–4.

Haemagglutination inhibition

Standard methods (Clarke & Casals, 1958) modified for microtechnique were used. Sera, not inactivated, were treated with kaolin and absorbed with goose cells. The borate buffer contained 0·4 % bovine albumin. The reagents were diluted in tubes and then pipetted onto plates in volumes of 0·025 ml. TBE antigen was used in concentrations of 4–16 units/0·025 ml. For this, heat-inactivated antigen was diluted five times, and the Tween-ether treated antigen 50 times. Antigen-serum mixtures were incubated overnight at 4° C. or at room temperature, and 0·05 ml. of 0·3 % male goose cells at pH 6·2–6·4 were added. The control TBE immune serum gave 4–8 times higher titres with the Tween-ether treated antigen.

Complement fixation

The standard micromethod (Lennette, 1964) was used with minor alterations. Sera were heated at 56° C. for 30 min., diluted in tubes and pipetted onto plates. Two units of guinea-pig complement and two units of antigen (Tween-ether antigen undiluted) were used. Antigen-serum mixtures were incubated overnight at 4° C. before adding the haemolytic system. Haemolysis of 25 % or less was regarded as positive.

Platelet aggregation

The method has been described in detail earlier (Penttinen & Myllylä, 1968; Myllylä *et al.* 1969). Washed human platelets (200,000/mm^3.) were suspended in buffered (pH 7·4–7·8) saline solution. Serum and antigen dilutions (0·05 ml. of each) were mixed and 0·05 ml. of platelet suspension was added. The sera were heated at 56° C. for 30 min. before use. Microplates were kept overnight at 5° to 8° C., and aggregation was read the following morning. In TBE titrations Tween-ether inactivated antigen was used in dilutions of 3 and 10 (reciprocal values are given). A negative prozone was frequently found in PA titrations, and it was more marked with smaller amounts of antigen. With dengue antigens and sera 'checkerboard' titrations were used. These indicated that the antigen titre was 80, and that an antigen dilution of 1/20 gave the highest antibody titres.

RESULTS

Sera from TBE-endemic region

One serum aggregated platelets directly without added antigen. The PA, HI and CF titres of the other 23 sera with TBE virus antigen are shown in Table 1. Eighteen of the sera were positive by HI, 16 of these were also positive by PA and 10 by CF. There was a good correlation between titres in the PA and HI tests, but

Table 1. *Antibody titres of sera from TBE-endemic region against TBE antigen using three different methods*

Serum no.	PA	HI	CF
Kn 7724	0	20	0
Kn 7804	0	0	0
Kn 7806	0	20	0
Kn 7809	0	0	0
Kn 7831	0	0	0
Kn 7852	0	0	0
Kn 7921	0	0	0
Kn 7746	40	20	0
Kn 7770	40	20	0
Kn 7792	40	40	0
Kn 7812	40	40	0
Kn 7813	80	80	20
Kn 7723	160	80	0
Kn 7749	160	80	0
Kn 7752	160	80	20
Kn 7801	160	80	10
Kn 7815	160	320	10
Kn 7820	160	80	20
Kn 7753	320	320	40
Kn 7757	320	160	40
Kn 7777	320	160	40
Kn 7791	320	320	40
Kn 7782	640	320	80

PA = Platelet aggregation; HI = haemagglutination inhibition (heat-inactivated antigen); CF = Complement fixation.

the PA test tended to give slightly higher titres than the HI test with the less sensitive heat-inactivated TBE virus antigen. Two sera were weakly positive by HI but negative by PA (the specificity of the HI reactions was verified with the tissue culture neutralization test). CF was apparently less sensitive than the other two tests.

Sera from vaccinated persons

The serum of the man vaccinated against yellow fever did not react with TBE virus antigens in any of the tests (homologous HI titre against yellow fever antigen was 160). Similarly, no reaction with TBE virus antigens could be detected by any test in the serum of the woman injected with TBE vaccine (low grade neutralizing activity was seen in the tissue culture neutralization test).

Sera from patients

Two samples of serum from TBE patients reacted directly with platelets without added antigen. The titres of the other 17 sera in the PA, HI and CF tests with TBE virus antigen are shown in Table 2. In two cases samples taken during the early

Table 2. *Antibody titres in sera from TBE patients against TBE antigen using three different methods*

	Time	PA	HI	CF
A. A.	−0	0	0	0
	122	320	640	40
R. S.	6	0	0	0
	260	320	640	40
G. S.	15	0	320	0
	23	320	640	10
Å. R.	15	0	320	10
	28	640	1280	20
K. V.	42	320	1280	80
	52	160	640	40
	156	160	320	20
L. E.	18	320	320	20
	31	160	640	80
	52	160	2560	80
K. P.	84	1280	1280	10
U. L.	44	320	160	80
E. R.	15	320	1280	40

PA = Platelet aggregation; HI = haemagglutination-inhibition (tween-ether-treated antigen); CF = Complement fixation. Time in days after onset of disease.

stages of the disease were positive only in HI, or in HI and CF. In other samples there was a good correlation between the PA and HI titres, but with the more sensitive Tween-ether antigen HI gave higher titres. Two or more samples were obtained from six patients. Diagnostic changes in titre (at least fourfold) could be detected by PA and CF in four and by HI in five cases. Serum from the man who had Omsk haemorrhagic fever 3 years earlier had a PA titre of 320 and an HI (heat-inactivated TBE virus antigen) titre of 160. The serum was anticomplementary.

The later serum of one patient (sample no. 36945) with dengue haemorrhagic fever aggregated platelets directly to a titre of 200. Dengue type 4 antigen, which was treated with protamine as well as sucrose-acetone, reacted directly with platelets without added serum. The results with other dengue antigens and sera from patients are shown in Table 3. Even the early serum sample reacted strongly in the PA test. The PA titres were generally higher than the HI titres (Dr Smith) in early samples but lower in late samples. Cross-reactions were as wide in both tests. Dengue serum no. 33431 was fractionated in a sucrose gradient, and the antibody that was active in PA was 7S.

Table 3. *Antibody titres in sera from two patients with haemorrhagic dengue, tested against* 3 *serotypes of dengue virus*

Serum no.	Time	Dengue 1		Dengue 2		Dengue 3	
		PA	HI	PA	HI	PA	HI
36,522	2	640	320	1,280	320	320	320
36,945	30	3,200	5,120	6,400	⩾ 20,480	3,200	10,240
33,122	3	800	640	800	320	400	640
33,431	30	6,400	10,240	1,600	10,240	1,600	10,240

Time in days after onset of disease. HI titres according to Dr T. Smith.

DISCUSSION AND GENERAL CONCLUSIONS

The results show that the PA technique can be used to measure group B arbovirus antigens and antibodies. The PA titres were higher than the CF titres, and frequently equal to the HI titres. The PA test detected antibody in nearly as many as the HI test. Similar results were found in the rubella system (Myllylä *et al.* 1969).

The results, though limited, suggest that the serological cross-reactions between members of the TBE and dengue complexes might be as extensive with PA as with HI.

The PA test can be used in the serological diagnosis of group B arbovirus infections. In TBE infections the results with a few samples suggest that PA antibodies may appear later than HI antibodies. So far only 7S antibodies seem to be active in PA. These appear later than 19S antibodies which are active in HI also (see also Myllylä *et al.* 1969; Penttinen *et al.* 1969).

In dengue the very limited results suggest that the PA technique is nearly as sensitive as the HI technique. It is interesting that the PA titres could be higher than the HI titres in early sera, but with late sera the reverse was true. The high initial PA titres might be connected with the development of thrombocytopenia in haemorrhagic dengue. However, the high PA titres do not necessarily entail platelet damage *in vivo*. In the PA test platelets are not affected by antibodies but by antigen-antibody complexes. The immune complexes may damage platelets also *in vivo*. Soluble viral antigens are probably present in the circulation during active infection, though there is no direct evidence for this. The development of thrombocytopenia in haemorrhagic forms of dengue seems to require the presence of anti-dengue antibodies at the beginning of the infection (Halstead, 1968). They may be cross-reacting antibodies acquired by preceding infection with other types of dengue (or related viruses) or by transplacental passage from the mother. Thus circulating antibodies should be present while viral antigens are being produced. In this situation soluble antigen-antibody complexes may damage platelets *in vivo*, and thus cause thrombocytopenia. The observation of direct high-titre PA activity in one dengue serum is of interest in this connexion.

The platelet aggregation technique depends on the interaction of immune complexes with target cells, and thus is a useful method for investigating the action and significance of immune complexes.

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REFERENCES

CATALOGUE OF ARTHROPOD-BORNE VIRUSES OF THE WORLD (1967). (Compiled by Richard M. Taylor.) *Public Health Service Publication*, no. 1760. U.S. Government Printing Office, Washington, D.C.

CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropodborne viruses. *American Journal of Tropical Medicine and Hygiene* **7**, 561.

CLARKE, D. H. & CASALS, J. (1965). Arboviruses; Group B. In *Viral and Rickettsial Infections of Man*, 4th ed. pp. 606–58. Ed. F. L. Horsfall and I. Tamm. London: Pitman Medical.

HALONEN, P. E., STEWART, J. A. & HALL, A. D. (1967). Rubella hemagglutinin prepared in serum free suspension culture of BHK-21 cells. *Annales medicinae experimentalis et biologiae Fenniae* **45**, 182.

HALSTEAD, S. B. (1968). Consideration of possible immunologic mechanisms in dengue hemorrhagic fever (Dengue hypersensitive disease) (DHD). Abstracts of the Eighth International Congresses on Tropical Medicine and Malaria, Teheran, 1064.

LENNETTE, E. H. (1964). In *Diagnostic Procedures for Viral and Rickettsial Infections*, 3rd ed. p. 57, Ed. E. H. Lennette and Nathalie J. Smith, New York: American Public Health Association.

MYLLYLÄ, G., VAHERI, A., VESIKARI, T. & PENTTINEN, K. (1969). Interaction between human blood platelets, viruses and antibodies. IV; Post-rubella thrombo-cytopenic purpura and platelet aggregation by rubella antigen–antibody interaction. *Clinical and Experimental Immunology* **4**, 323.

PENTTINEN, K. & MYLLYLÄ, G. (1968). Interaction of human blood platelets, viruses and antibodies. I. Platelet aggregation test with microequipment. *Annales medicinae experimentalis et biologiae Fenniae* **46**, 188.

PENTTINEN, K., MYLLYLÄ, G., MÄKELÄ, O. & VAHERI, A. (1969). Soluble antigen–antibody complexes and platelet aggregation. *Acta pathologica et microbiologica scandinavica* **77**, 309.

SALMINEN, A. (1962). A method for production of arthropod-borne viral hemagglutinins in tissue culture. *Annales medicinae experimentalis et biologiae Fenniae* **40**, 174.

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Degradation of influenza virus by non-ionic detergent

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SUMMARY

Preparations of influenza virus A0 PR8/34 and A2 Malaysia/68 have been studied in the electron microscope. They were similar in appearance to preparations made by others. Each preparation was degraded by Triton N 101. The process of degradation appeared to be different from that observed using ether and, by inference, a number of other agents.

INTRODUCTION

Corbel & Rondle (1970) described the biological properties of soluble materials obtained by treatment of influenza virus with Triton N 101 (Nonylphenoxypolyethoxyethanol). The physical process of virus disruption was followed using an electron microscope. As far as is known to the authors the probable sequence of events was unique and is therefore reported in this paper.

MATERIALS AND METHODS

Virus strains and virus purification

The work was done with A0 PR8/34 and A2 Malaysia/68. The history of the strains and full details of purification are described by Corbel & Rondle (1970). Most work was done with virus purified by centrifugation in sugar density gradients. Experiments using ether as degrading agent were done with virus purified only by differential centrifugation.

Virus degradation

Purified virus was removed from suspension by centrifugation at 100,000 *g* for 1 hr. The supernatant fluid was discarded. In most experiments virus pellets were resuspended in phosphate-buffered saline containing 1% Triton N 101 and stored 1 hr. at 4° C.; in one experiment a pellet of A0 PR8/34 was resuspended in phosphate-buffered saline and an equal volume of ether was added and the mixture shaken for 1 hr. at 4° C. After treatment the preparations were centrifuged at 100,000 *g* for 1 hr. and the supernatant fluids discarded.

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Electron microscopy

Drained pellets of purified virus and drained pellets of degraded material were kept at 4° C. and processed within 1 hr. of preparation. Pellets were resuspended in distilled water (0·1 ml.). A drop of each suspension was mixed with one drop of 3% (w/v) neutralized phosphotungstic acid on a clean microscope slide and the suspension applied immediately to carbon grids. Excess fluid was removed after 15 sec. and the grids allowed to dry. They were examined in a GEC/AEI EM 6 electron microscope and photographs were taken on Ilford N 50 film.

RESULTS

A representative specimen of A 0 PR 8/34 is shown in Pl. 1, fig. 1. Virus particles reasonably regular in size and each possessing external projections occupy most of the field. There is little debris to be seen.

A probable early effect of treatment with Triton N 101 is shown in Pl. 1, fig. 2. The external projections are stripping off the virus particle but remaining attached to each other. Plate 2, fig. 1 shows a probable later stage in this process. The field is littered with strings of projections and contains a few uncoated particles of the size of influenza virus. At a later stage, as shown in Pl. 2, fig. 2, the strings of projections cannot be found and only 'ghost' particles, some penetrated by stain, can be seen. Finally, as stated by Corbel & Rondle (1970), more drastic treatment with Triton N 101 leads to complete disruption of the virus with production of soluble material not sedimented by centrifugation at 100,000 *g* for 1 hr.

This probable series of events differs from that occurring after treatment with ether. As shown in Pl. 3, fig. 1, ether treatment of A 0 PR 8/34 caused 'rounding-up' of the external projections followed by complete disruption of virus particles and release of internal components (Pl. 3, fig. 2). The observed effects of ether are in agreement with the results of Hoyle, Horne & Waterson (1961).

The effect of treating influenza virus with Triton N 101 was studied also using A 2 Malaysia/68. A typical purified virus preparation is shown in Pl. 4, fig. 1. It was not possible to obtain preparations of this virus as uniform or as free from debris as the preparations of A 0 PR 8/34. This was possibly due to its relatively recent isolation and poor degree of adaptation to the chick chorio-allantois. However, as shown in Pl. 4, fig. 2, individual particles appeared as typical influenza virus and when treated with Triton N 101 (Pl. 5, fig. 1) the external projections were removed and ghost particles finally produced (Pl. 5, fig. 2).

DISCUSSION

Examination of purified influenza virus preparations in the electron microscope gave pictures similar to those published previously (Hoyle *et al.* 1961). The virus strain A 2 Malaysia/68 was more pleomorphic than A 0 PR 8/34 which is well adapted to the chick chorio-allantois. Both viruses, however, were degraded in a similar way by Triton N 101. The external projections were removed probably

at first as a continuous string, and virus ghosts were formed. The strings of external projections were apparently degraded in turn to smaller subunits morphologically similar to the monovalent haemagglutinin preparations obtained by Choppin & Stoeckenius (1964). However, the effects of Triton N 101 and ether differ considerably in the early and intermediate stages of treatment.

Other methods have been used to disrupt influenza virus. Thus Valentine & Isaacs (1957) used hydrochloric acid and trypsin; Laver (1963) used sodium deoxycholate and sodium dodecyl sulphate; Waterson, Hurrell & Jensen (1963) used formaldehyde and chloroform; and Choppin & Stoeckenius (1964) used ultrasonic vibrations. The effect of these reagents ranged from destruction of external projections to complete fragmentation of virus. Blough (1963*a*, *b*) studied the effect of ionic and non-ionic surface-active agents on influenza virus but did not report effects similar to those seen with Triton N 101.

Again, the effect of enzymes on influenza virus has been studied. Thus Simpson & Hauser (1965, 1966) used phospholipase C. This enzyme caused progressive disruption of virus without release of discrete surface structure. Finally, the effect of a number of proteolytic enzymes on influenza virus has been studied; Reginster (1965) used pronase, and Biddle (1968) used a bacterial protease. Under certain conditions these enzymes destroyed the external projections.

The results suggest that Triton N 101 differs from ether and other agents in its effect on influenza virus. Mild treatment with Triton N 101 leads initially to removal only of the outer coat of the virus. This mild degradation leaves a particle still bounded by a membrane. The finding supports the view of Kates, Allison, Tyrrell & James (1962) who suggested that influenza virus has two outer membranes. They stated that one membrane might be derived from the host nuclear membrane and the second manufactured in host-cell cytoplasm or derived from host-cell membrane. It is possible that isolation and further investigation of the early products of Triton N 101 degradation might lead to a better understanding of the structure of influenza virus.

REFERENCES

Biddle, F. (1968). The action of protease on influenza A 2 virus. *Journal of General Virology* **2**, 19–28.

Blough, H. A. (1963*a*). The role of the surface state in the morphogenesis of influenza virus filaments. *Virology* **19**, 112–14.

Blough, H. A. (1963*b*). The effect of vitamin A alcohol on the morphology of myxoviruses. I. The production and comparison of artificially produced filamentous virus. *Virology* **19**, 349–58.

Choppin, P. W. & Stoeckenius, W. (1964). Interactions of ether-disrupted influenza A 2 virus with erythrocytes, inhibitors and antibodies. *Virology* **22**, 482–92.

Corbel, M. J. & Rondle, C. J. M. (1970). Soluble antigens obtained from influenza virus by degradation with non-ionic detergent. *Journal of Hygiene* **68**, 81–96.

Hoyle, L., Horne, R. W. & Waterson, A. P. (1961). The structure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether. *Virology* **13**, 448–59.

Kates, M., Allison, A. C., Tyrrell, D. A. J. & James, A. T. (1962). Lipids of influenza virus and their relation to those of the host cell. *Biochimica et Biophysica Acta* **52**, 455–66.

LAVER, W. G. (1963). The structure of influenza viruses. 3. Disruption of the virus particle and separation of neuraminidase activity. *Virology* **20**, 251–62.

REGINSTER, M. (1965). Inactivation of influenza virus by caseinase C from *Streptomyces albus* G culture filtrate. *Journal of General Microbiology* **40**, 157–69.

SIMPSON, R. W. & HAUSER, R. E. (1965). Structures associated with influenza virus suspensions treated with phospholipase C. *Virology* **27**, 642–6.

SIMPSON, R. W. & HAUSER, R. E. (1966). Influence of lipids on the viral phenotype. I. Interaction of myxoviruses and their lipid constituents with phospholipases. *Virology* **30**, 684–97.

VALENTINE, R. C. & ISAACS, A. (1957). The structure of viruses of the Newcastle disease–mumps–influenza (Myxovirus) group. *Journal of General Microbiology* **16**, 680–85.

WATERSON, A. P., HURRELL, J. M. W. & JENSEN, K. E. (1963). The fine structure of influenza A, B and C viruses. *Archiv für die Gesamte Virusforschung* **12**, 487–95.

EXPLANATION OF PLATES

Magnification *ca.* 200,000 except for Pl. 4, fig. 2 which was *ca.* 400,000. All preparations were negatively stained with phosphotungstic acid.

PLATE 1

Fig. 1. Influenza virus A0 PR 8/34 purified by centrifugation in a sugar density gradient.
Fig. 2. Preparation shown in Fig. 1 treated with Triton N 101.

PLATE 2

Fig. 1. Preparation shown in Pl. 1, fig. 1 treated with Triton N 101. A probable further stage in degradation.

Fig. 2. Preparation shown in Pl. 1, fig. 1 treated with Triton N 101. Probably a late stage in degradation.

PLATE 3

Fig. 1. Influenza virus A0 PR 8/34 treated with ether.
Fig. 2. Probably a further stage of degradation from Fig. 1.

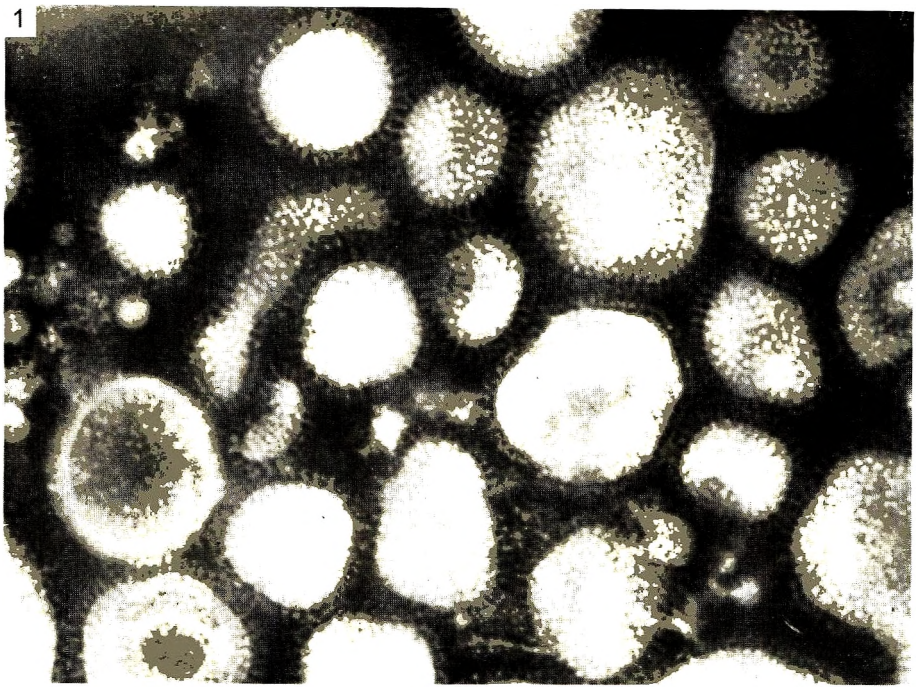
PLATE 4

Fig. 1. Influenza virus A2 Malaysia/68 purified by centrifugation in a sugar density gradient.
Fig. 2. Single particle showing myxovirus morphology.

PLATE 5

Fig. 1. Particle from preparation shown in Pl. 4, fig. 1 after treatment with Triton N 101. External projections mostly removed.

Fig. 2. Preparation shown in Pl. 4, fig. 2 after treatment with Triton N 101. Only 'ghost' particles remain.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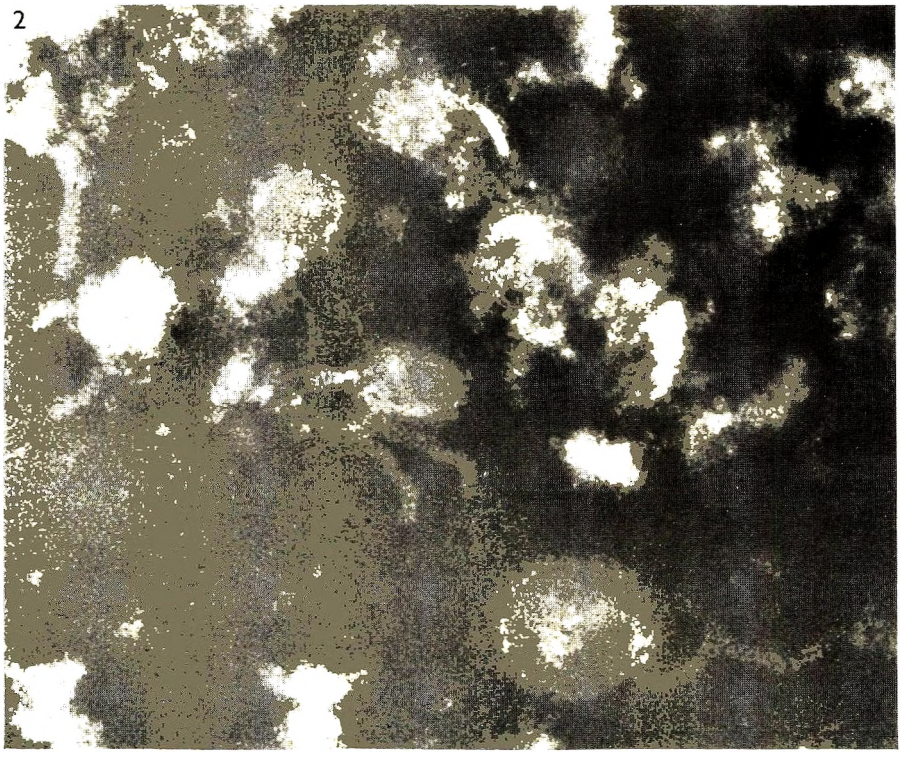
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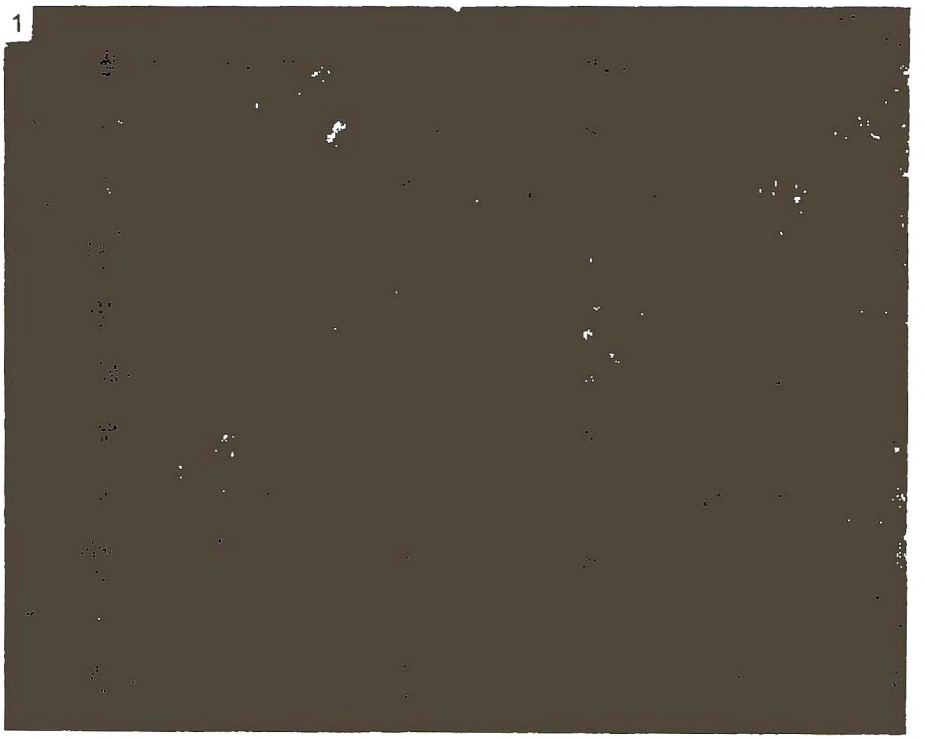
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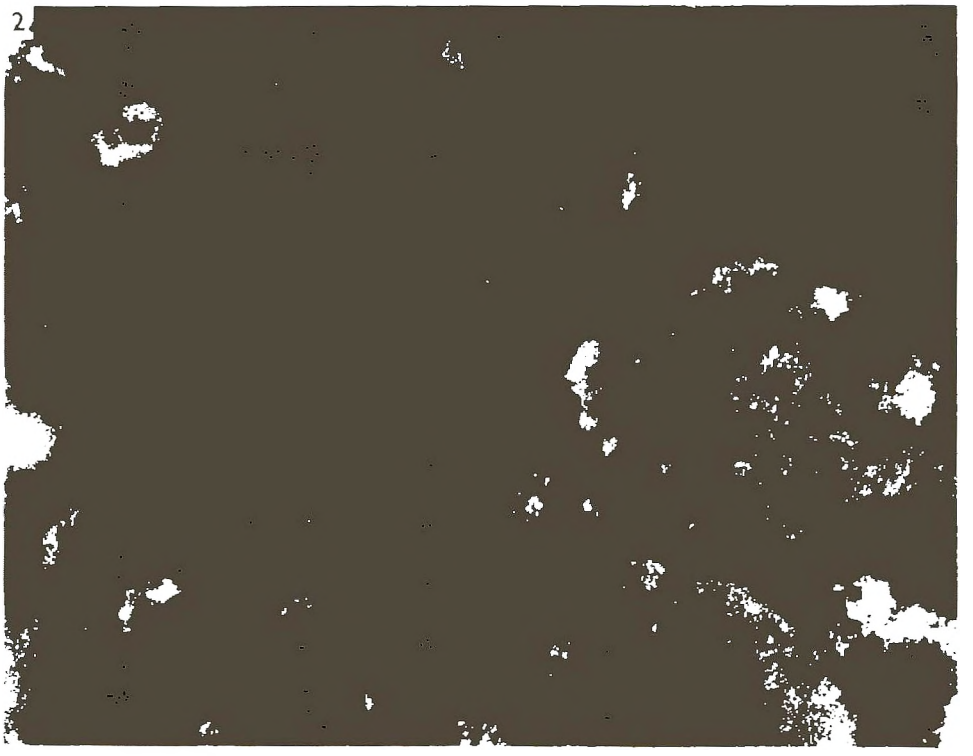
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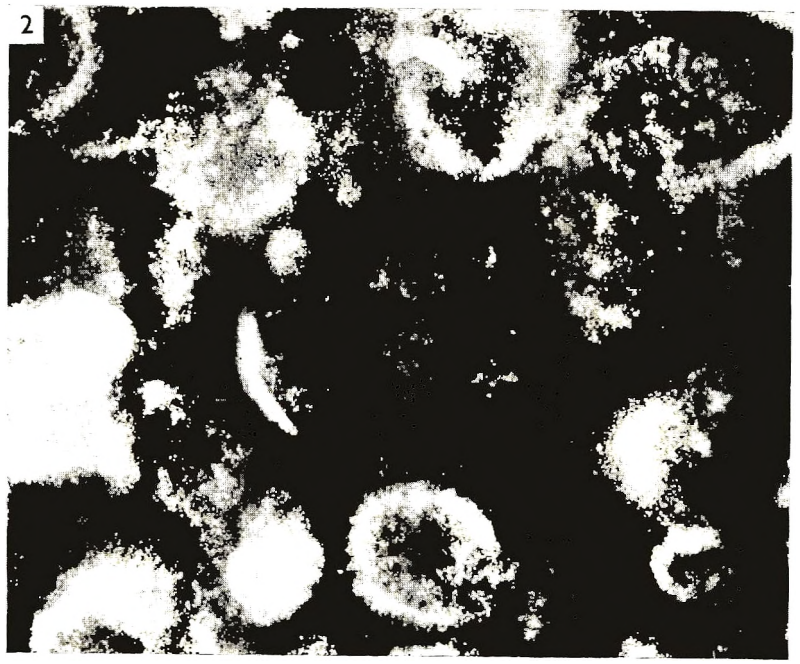
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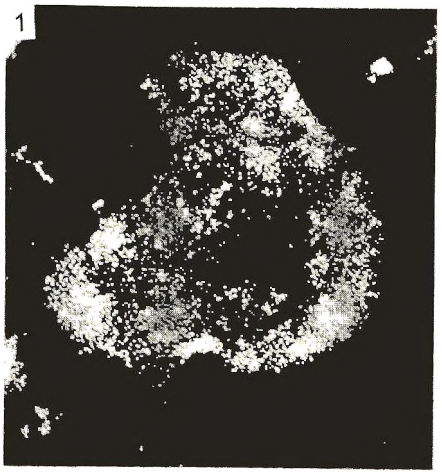
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Soluble antigens obtained from influenza virus by treatment with non-ionic detergent

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SUMMARY

Highly purified influenza virus was degraded using anionic and non-ionic detergents. Best results were obtained using the non-ionic detergent Triton N101. Tests showed that virus extracts contained neuraminidase and a substance that reacted specifically with rabbit antibody to virus haemagglutinin (specific serum blocking substance). Haemagglutination-inhibiting antibody was produced when virus extracts were inoculated into guinea-pigs. Immunodiffusion tests showed that extracts were complex. Host-specific material was regularly found. Under appropriate conditions S-antigen was detected as a single line pattern component. Two or more virus-specific materials were also present. One of these was probably neuraminidase and the other the specific serum blocking substance.

INTRODUCTION

It has been shown previously that influenza virus contains a group-specific 'S' or 'g' antigen (Lennette & Horsfall, 1941; Hoyle, 1945; Lief, Fabiyi & Henle, 1958), a strain-specific, haemagglutinating 'V' antigen (Fabiyi, Lief & Henle, 1958) and an antigenic neuraminidase. The neuraminidase also shows strain specificity although the serological cross-reactions of strains related by their neuraminidases do not exactly parallel the cross-reactions given by their haemagglutinins (Paniker, 1968).

In addition to these three antigens, which are generally regarded as being virus-specific, the virus also contains antigens derived from the host tissue in which it is grown (Knight, 1946; Hoyle, 1948, 1950, 1952; Smith, 1952; Smith, Belyavin & Sheffield, 1955; Howe, Lee, Harboe & Haukenes, 1957).

When the host tissue is that of the chick chorioallantoic membrane (CAM) then the host-derived antigens associated with the virus may include a heterophile antigen of the Forssman type (Kosyakov & Rovnova, 1965) and an acidic mucopolysaccharide possibly associated with the haemagglutinin (Harboe, Borthne & Berg, 1961; Haukenes, Harboe & Mortensson-Egnund, 1965). Finally Klamerth (1961) and Neurath & Sokol (1963) demonstrated the presence of host-derived

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adenosine diphosphatase and adenosine triphosphatase in purified preparations of influenza viruses.

Hoyle (1950) first showed that ether could be used to disrupt the virus and separate the haemagglutinating component from the complement-fixing internal antigen. Subsequently this method was modified and extended for use in the study of other myxoviruses. Norrby (1962) included Tween 80 in the virus-ether mixture and showed that this modification permitted more uniform results to be obtained in the disruption of measles virus. This procedure was adopted by Davenport *et al.* (1964) for the production of an effective influenza vaccine containing haemagglutinating sub-units of the virus.

Ether treatment, however, even in the presence of Tween 80, does not yield a uniform product (Choppin & Stoeckenius, 1964) and this has led to a search for more efficient agents. Laver (1963) adopted the anionic detergents, sodium deoxycholate (SDC) and sodium dodecyl sulphate (SDS) for this purpose and showed that complete virus disruption could be obtained although biological activity was not always retained. Schild & Pereira (1969) used immunodiffusion tests to show that SDS extracts of virus contained precipitable S-antigen and neuraminidase. They claimed similar results using the non-ionic detergent Non-idet P40.

Hobson (1966) combined the use of SDC with ether extraction and was able to obtain a product from the WS strain of influenza A 0 which was non-haemagglutinating yet capable of blocking the haemagglutination-inhibiting (HI) activity of specific antiserum. The material was strain-specific and gave rise to HI antibodies in guinea-pigs. This work suggested that haemagglutinating activity (HA) was not essential for an influenza vaccine to be capable of evoking HI and possibly neutralizing antibody, a finding of considerable importance, as present methods of influenza vaccine standardization are based on haemagglutinin titration.

We found that the results obtained by Hobson (1966) could be reproduced with virus extracts made using non-ionic detergent. We studied our extracts in immunodiffusion tests and compared them with those made using SDC and SDS. The results of the investigation are given in this paper.

MATERIALS AND METHODS

The virus used

Strains used were: A Swine/31 (SW); A 0 WS/33 (WS); A 0 PR 8/34 (PR 8); A 1 FM 1/47 (FM 1); A 2 Jap/305/EFME/57 (EFME); A 2 Rus/IKSHA/57 (IK); A 2 Eng/1/66 (AE); A 2 Malaysia/68 (MAL); B Eng/939/59 (B); B Eng/5/66 (B 2).

With the exception of MAL, AE and B 2, virus strains were supplied by Dr D. Hobson, Bacteriology Department, Liverpool University.

MAL was generously provided by Dr Lim Teong Wah, Institute for Medical Research, Kuala Lumpur, Malaysia. This strain was isolated from a patient infected with the A 2 Hong Kong virus. The virus strains AE and B 2 were derived from infected calf kidney tissue cultures and were a gift from Dr A. D. Kanarek of Burroughs Wellcome Research Laboratories.

On receipt all virus strains were grown in the chorioallantois of 10-day-old fertile

hen eggs. Infected allantoic fluids were harvested after 48 hr. incubation at 35° C. Appropriate pools were made and the fluids then stored in 0·1 ml. amounts in sealed glass ampoules at −60° C. A fresh ampoule was used as seed virus in each subsequent experiment.

Virus growth and purification

Seed virus was diluted to a mean 10^4 egg infective doses per ml. and propagated in chick-chorioallantois. Dilutions were made in 0·01 M phosphate buffer, pH 7·4 (PBS), containing 50 μg./ml. chloramphenicol (PBSC). Infected allantoic fluids were harvested and pooled. Pooled fluids were clarified by centrifugation at 1200 *g* for 30 min. and partially purified virus obtained by the barium sulphate absorption–elution method of Mizutani (1963). Eluates in neutralized 0·25 M sodium citrate were combined. Failure to neutralize the sodium citrate resulted in virus preparations of reduced infectivity. Virus was sedimented by centrifugation at 50,000 *g* for 60 min. and resuspended in PBSC using an MSE-Mullard ultrasonic disintegrator. The virus suspension was then clarified at 1500 *g* for 15 min. Where possible manipulations were done at 4° C. Virus purified in this way was used for inoculation into rabbits.

Final purification was by centrifugation in discontinuous sucrose density gradients. Gradients were prepared by the successive layering of 0·8 ml. volumes of 60, 50, 40, 30 and 20 % sucrose in PBSC into 5 ml. cellulose nitrate tubes and allowing diffusion to take place for several hours. Partially purified virus concentrate (1·0 ml.) was then layered on top of each gradient and centrifuged at 100,000 *g* for 15 hr. using a Spinco SW 39 L rotor.

Fractions were collected dropwise after piercing the bottom of each tube. Fractions containing virus (50–60 % sucrose) were pooled, diluted tenfold in PBSC and the virus recovered by centrifugation at 100,000 *g* for 30 min. Virus pellets were resuspended in PBSC as described previously.

Virus extracts

Extracts were made by disrupting virus with non-ionic detergents of the Triton series (Rohm & Haas Co., Philadelphia). Experiments with different Tritons showed that for this purpose Triton N 101 (Nonylphenoxypolyethoxyethanol) was to be preferred. Usually a 10 % aqueous solution of detergent was added to virus concentrates, containing not less than 10^5 HAU/ml., to a final concentration of 1 to 2 %.

After 12 hr. at 4° C. debris was removed by centrifugation at 1500 *g* for 30 min. and samples of the clarified extract diluted to a maximum Triton N 101 concentration of 0·1 %.

In the text the suffix 'T', as in SW/T, is used to denote extracts made in this way.

Non-haemagglutinating extracts were prepared also using SDC as described by Hobson (1966) and using SDS.

For electrophoresis and some immunodiffusion tests a virus pellet was dissolved in the minimum quantity of 10 % Triton N 101. Detergent was not removed, as it

was found that over a wide range of concentrations Triton N101 did not interfere in electrophoretic separations and unlike SDC and SDS did not give non-specific precipitation with antisera or other antigens in immunodiffusion tests.

Normal tissue extracts

Normal allantoic fluid (NAF) was collected from uninfected 12-day-old chick embryos. Material was clarified by centrifugation at 1200 *g* for 30 min., dialysed against distilled water at 4° C. for 48 hr. and dried from the frozen state. For use as antigen in immunization and serological tests 100 mg. of the dried material was dissolved in 1 ml. of sterile saline.

Normal CAM extracts (NCAM) were prepared from uninfected CAM harvested from 12-day-old chick embryos. Membranes were washed, minced at 4° C. and the minced tissue centrifuged at 1500 *g* for 30 min. The clear supernatant was dialysed overnight against distilled water at 4° C. and dried from the frozen state. For use as antigen in immunization and serological tests 100 mg. of dried NCAM was dissolved in 1 ml. of sterile saline.

Antisera used

Antisera were prepared in adult New Zealand white rabbits. Before immunization trial bleedings were taken from all animals. All pre-immunization sera failed to react with virus antigens, NAF and NCAM when tested by ring or gel precipitation. Moreover, no HI antibodies to any of the virus strains used were detectable.

Antiviral sera were prepared using the virus purified as described above. Virus concentrates were diluted to 10,000 HA units (HAU) per ml. and homogenized with an equal volume of Freund's complete adjuvant containing 2 mg./ml. heat-killed *Mycobacterium tuberculosis* var. *hominis* (FCA). Each rabbit received a primary injection of homogenate (1 ml.) divided over a number of intramuscular and subcutaneous sites. One month later two intravenous injections of purified virus (5000 HAU) were given 1 week apart. Serum was collected 1 week after the final injection.

Antisera to NAF and NCAM were prepared by giving primary intramuscular and subcutaneous injections of 1 ml. of antigen homogenized in FCA followed after one month by a series of weekly intravenous injections of 1 ml. of antigen without adjuvant.

The prefix 'a' as in aSW is used throughout to denote an antiserum raised against a particular virus or substance.

A sample of serum from a patient convalescent from infection with MAL was kindly donated by Dr Lim Teong Wah of the Medical Research Institute, Kuala Lumpur, Malaysia.

Serum inactivation

Non-specific serum inhibitors of virus haemagglutinin were inactivated by periodate as described by Davenport *et al.* (1964). All inactivated sera were tested for fowl haemagglutinins and positive sera absorbed with packed fowl erythrocytes.

Serum absorption

Antisera to several virus strains were absorbed exhaustively with NCAM to remove antibody to normal chick tissue antigens. Excess dried NCAM extract was added to antiserum and kept at 4° C. overnight. Particulate matter was removed by centrifugation at 25,000 *g* for 60 min. Lipid which collected on the antiserum surface during centrifugation was drawn off and discarded. Absorbed antisera gave no ring or gel precipitin reaction with NCAM but reacted with aNCAM, indicating the presence of excess absorbing material.

Absorbed antisera were stored at 4° C. after addition of sodium azide to a final concentration of 0·1 %.

Biological tests

Haemagglutination and haemagglutination-inhibition tests

These tests were done using the standard World Health Organization (1953) 'Perspex' plate method.

Strain-specific serum blocking tests

Tests for strain-specific serum blocking activity (SSB) were done essentially as described by Hobson (1966).

Neuraminidase activity

Enzymic activity was estimated from the ability of the material under test to destroy the HI property of Collocalia mucoid.

Collocalia mucoid was prepared by the method of Howe, Lee & Rose (1961) from crude swallows' nest cementing substance ('Chinese birds' nest') purchased from the Wing Lee Co., Liverpool. The available sialic acid content of the final material was estimated by the thiobarbituric acid method of Aminoff (1961) after hydrolysis of samples with 0·1 N-H_2SO_4 at 80° C. for 1 hr.

For test, mucoid was dissolved at 100 μg./ml. available sialic acid (usually 10 mg. mucoid/ml.), in 0·01 M phosphate buffer (pH 6·0). Mucoid solution (0·25 ml.) was incubated with an equal volume of 'enzyme' sample at 37° C. for 1 hr. Some of the mixture (0·25 ml.) was then diluted serially in doubling dilutions using saline buffered at pH 6·0. To each dilution 0·25 ml. indicator virus was added. The indicator virus used was influenza B heated at 56° C. for 30 min. and diluted to 32 HAU/ml. Fowl erythrocytes (0·25 ml. of a 0·5 % suspension) were then added and tests were read after 60 min. at 4° C. Scoring of results was as for HI tests. For each test a 'blank' titration was performed using mucoid incubated with an equal volume of buffer as 'enzyme'. Neuraminidase activities of samples were expressed as a percentage survival of the HI activity of the mucoid used. This figure is related to the amount of available sialic acid released on enzymic digestion of mucoid.

The method described was consistently more reproducible than simple estimation by the thiobarbituric acid reaction of sialic acid release by enzymic digestion of mucoid.

Antineuraminidase activity

The antineuraminidase titres of a number of antisera were determined. A virus suspension was prepared that contained sufficient neuraminidase in 0·25 ml. to destroy 50 % of the HI activity of a standard Collocalia mucoid solution after 1 hr. incubation at 37° C. and pH 6·0. To 0·25 ml. volumes of this preparation were added equal volumes of serially doubling dilutions of periodate-inactivated antisera. Serum-enzyme mixtures were incubated at room temperature for 30 min. after which 0·25 ml. volumes were removed and tested for neuraminidase activity as described above. Antineuraminidase titres were expressed as the reciprocal of that dilution of serum giving 50 % inhibition of the virus neuraminidase.

Immunodiffusion tests

The double diffusion technique of Ouchterlony (1948) was used. The diffusion medium was filtered 1 % Oxoid No. 1 agar in 0·85 % saline containing 0·1 % sodium azide as preservative. Reagent wells 5 mm. in diameter with centres 10 mm. apart were cut in isometric patterns in diffusion medium of a depth of 3 to 4 mm. contained in 90 mm. diameter polystyrene Petri dishes. Tests were read after 3 days development at room temperature in a saturated atmosphere. Results were recorded by photography under dark-ground illumination after gels had been washed in saline containing 0·1 % sodium azide.

Analytical disk electrophoresis

This was done in 7 % acrylamide gel as described by Davis (1964). Gels were stained with 1 % Amido-Black 10B in 7 % acetic acid and differentiated by prolonged washing in 7 % acetic acid or by electrophoresis.

Chemical analysis

Protein was estimated by the Folin–Biuret method of Sutherland, Cori, Haynes & Olsen (1949) using crystalline bovine serum albumin (Sigma) as standard.

A modified diphenylamine reaction (Burton, 1956) was used to determine 2-deoxyribose.

Absorption spectra

These were determined using an 'Optica' double-beam recording spectrophotometer, Model CF4NI.

RESULTS

Purity of virus preparations

The highly purified virus used for immunodiffusion and electrophoresis contained 50 to 80 HAU/μg. protein. No 2-deoxyribose was detected in this material and it was considered to be devoid of DNA.

Disk electrophoresis revealed a single component staining with Amido-Black 10B, which was near the junction of the large and small pore gels.

Some preparations were stained with phosphotungstic acid and examined in the

electron microscope. As shown by Corbel, Rondle & Bird (1970), little debris or non-virus-like material was seen.

Virus concentrates gave positive ring precipitin reactions when tested against antisera to the virus or to NCAM. They failed to react, however, when tested against these reagents in immunodiffusion experiments unless tests were incubated at room temperature for long periods.

These results suggested that the virus used was chemically and physically pure and was not associated with diffusible material.

Physical properties of extracts

Clarified virus extracts prepared with Triton N101, SDC and SDS contained approximately 5 mg./ml. material of virus origin. This material did not pass through 'Visking' dialysis tubing, indicating molecular weights in excess of 10,000 Daltons.

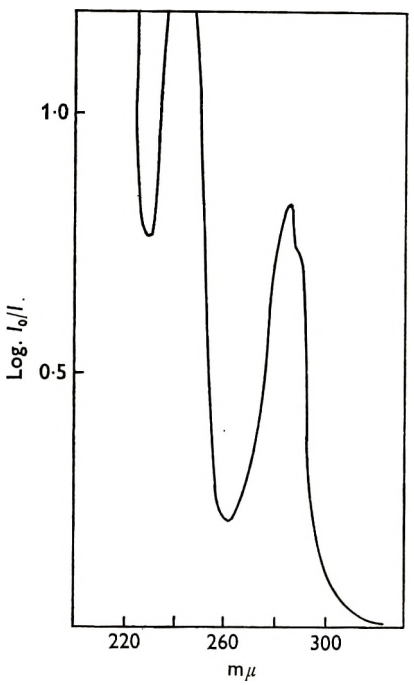

Fig. 1. Ultraviolet absorption spectrum of Triton N 101 (1/10,000 w/v).

Ultraviolet absorption spectra indicated the presence of nucleic acid and protein. Triton N101 interfered with protein detection since in addition to an absorption maximum at 240 mμ it had two absorption maxima between 280 mμ and 290 mμ (Fig. 1). The absorption maximum at 287 mμ which appears as a 'shoulder' in Fig. 1 was useful as it facilitated the detection of Triton N101 in virus extracts.

Polyacrylamide gel electrophoresis showed that virus extracts contained up to six substances staining with Amido-Black 10B. It was essential to remove SDC and SDS, although not Triton N101 before test, because extracts contaminated with anionic detergents gave a single broad fast-moving band on electrophoresis.

Extracts prepared with SDC formed thixotropic gels at 4° C. This property was not lost even after prolonged dialysis against water containing Bio-deminerolite resin (Permutit Co. Ltd.). The salicylaldehyde reaction of Szalkowski & Maeder (1952) showed that the procedure used reduced the SDC content of extracts to less than 0·01 % measured as deoxycholic acid.

Biological properties of extracts

All extracts were non-infective within the limits of the tests employed. Extracts prepared with Triton N 101 were diluted 1/100 before test because concentrations of detergent greater than 0·01 % were toxic to chick embryos.

Diluted Triton N 101 extracts were also used for HA tests. This was necessary because concentrations of detergent greater than 0·01 % lysed 0·5 % suspensions of fowl erythrocytes. The diluted extracts did not contain detectable HA.

The lytic effect on fowl cells might explain the toxicity of Triton N 101 for chick embryos.

TABLE 1. *Specific serum blocking tests with Triton N* 101 *extracts of virus*

	Reciprocal of HI titre of serum to homologous strain after treatment with:								
		A	A0		A1,	A2			B,
Serum	Saline	SW/T	WS/T	PR8/T	FM1/T	EFME/T	IK/T	MAL/T	B2/T
aSW	5,120	**240**	5,120	5,120	5,120	5,120	5,120	N/T	5,120
aWS	10,240	10,240	**240**	8,960	10,240	10,240	10,240	N/T	10,240
aPR8	10,240	10,240	10,240	**240**	10,240	10,240	10,240	N/T	10,240
aFM1	7,680	7,680	7,680	7,680	**240**	7,680	7,680	N/T	7,680
aEFME	2,560	2,560	2,560	2,560	2,560	**240**	640	1,280	2,560
aIK	5,120	5,120	5,120	5,120	5,120	1,280	**240**	2,560	5,120
aMAL	5,120	5,120	5,120	5,120	5,120	5,120	5,120	**640***	5,120
aB2	5,120	5,120	5,120	5,120	5,120	5,120	5,120	N/T	**340***

* MAL/T and B2/T were prepared from virus concentrates of approx. 20,000 HAU/ml. only.

In a few experiments Triton N 101 treated materials were centrifuged at 50,000 *g* for 1 hr. The sediment, which was small, contained some haemagglutinating material which represented, however, only a very small proportion of the original virus haemagglutinin.

Results of SSB tests with Triton N 101 extracts are shown in Table 1. They demonstrate that the serum-blocking activity of extracts was strain-specific except where two very closely related strains (EFME and IK) were studied. Similar results were obtained with virus extracts made with SDC. Other tests showed that extracts did react with heterologous antisera, but in most cases they blocked only HI antibody cross-reacting with their own strain and not HI antibody directed towards the immunizing strain. For example, aPR 8 'blocked' with WS/T retained its high titre of HI to PR 8 virus. It lost, however, its relatively lower HI titre to WS virus. The range of titres of homologous and heterologous HI antibody in the

sera used is shown in Table 2. For convenience antineuraminidase titres are included in the Table (see Discussion).

The results of SSB tests with both Triton N101 and SDC virus extracts are in agreement with those obtained by Hobson (1966) using SDC and ether.

TABLE 2. *Haemagglutination inhibition (HI) and neuraminidase inhibition (NI) titres of the antisera used*

		Reciprocal of titre with antigens:								
		A	A0		A1	A2			B	
Serum		SW	WSE	PR8	FM1	EFME	IKSHA	MAL	B	B2
aSW	HI	**5,120**	320	240	80	0	0	0	0	0
	NI	160	0	0	0	0	0	—	0	0
aWS	HI	320	**10,240**	2,560	240	0	0	0	0	0
	NI	0	80	0	0	0	0	—	0	0
aPR8	HI	240	1,920	**10,240**	160	0	0	0	0	0
	NI	0	0	160	60	0	0	—	0	0
aFM1	HI	240	320	320	**7,680**	40	0	0	0	0
	NI	0	0	80	640	0	0	—	0	0
aEFME	HI	0	0	0	0	**2,560**	1,920	80	0	0
	NI	0	0	0	0	640	320	—	0	0
aIK	HI	0	0	0	0	3,840	**5,120**	80	0	0
	NI	0	0	0	0	640	1,280	—	0	0
aMAL*	HI	40	40	40	40	160	160	**5,120**	0	0
	NI	—	—	—	—	—	—	—	—	—
aB	HI	0	0	0	0	0	0	0	**2,560**	1,920
	NI	0	0	0	0	0	0	0	480	320

0 Indicates HI or NI titres of less than 1/20.
* Human convalescent serum.
—Not tested.

TABLE 3. *Neuraminidase activity of virus preparations and Triton N101 extracts*

	A	A0		A1	A2		B	
	SW	WS	PR8	FM1	EFME	IK	B	B2
*Untreated	12·5	3·3	12·5	25	87·5	50	25	3·3
*Triton N101 extract	12·5	1·7	12·5	37·5	57·5	25	12·5	5

* Virus used contained 10^3 HAU/ml.

As with SDC extracts, Triton N101 extracts elicited HI antibodies when injected into guinea-pigs. For these tests a single intramuscular injection was given and serum samples taken 2–3 weeks later.

Neuraminidase activity was demonstrated consistently in virus extracts with the exception of those derived from WS. Extracts from this source were variable in their behaviour and on occasion no neuraminidase was found. Some results for Triton N101 extracts are shown in Table 3.

All extracts gave lines of precipitation when tested against homologous and heterologous antisera in immunodiffusion tests. Results obtained with Triton N101, SDC and SDS were similar and showed strain-specific line pattern components (lpc). However, SDS and SDC extracts frequently caused non-specific precipitation of antisera and control antigens. Moreover, patterns obtained with SDS extracts were invariably the least complex and the gelling properties of SDC extracts introduced technical difficulties. For these reasons most work was done with Triton N101 extracts which did not suffer from such disadvantages.

TABLE 4. *Number of lpc given by Triton N101 extracts of virus and antisera absorbed with NCAM*

	Antigens								
	A	A0		A1	A2			B	
Serum	SW/T	WS/T	PR8/T	FM1/T	EFME/T	IK/T	MAL/T	B1/T	B2/T
aSW	2	1	1	0	0	0	0	0	0
aWS	1	2	1	0	0	0	0	0	0
aPR8	1	1	3	1	0	0	0	0	0
aFM1	0	1	1	2	0	0	0	0	0
aEFME	0	0	0	0	2	2	2	0	0
aIK	0	0	0	0	2	2	2	0	0
aMAL*	1	1	1	1	3	3	3–4	0	0
aB	0	0	0	0	0	0	0	2	2

* Human convalescent serum.

The NAF–aNAF and CAM–aCAM control systems also gave several lpc when examined in immunodiffusion tests. It was essential, however, to use a CAM–aCAM control in tests for host materials. Some lpc present in tests on virus extracts were given by this control but were absent from NAF–aNAF or CAM–aCAM reactions. This last observation is shown in Pl. 1, fig. 6.

A typical test using virus extract and host controls is shown in Pl. 1, fig. 1. The lpc in the reaction between IK/T and aIK are labelled arbitrarily 1 to 5. Inspection shows that lpc 1 occurs in the reaction between IK/T and aNCAM and lpc 4 and 5 occur in the reaction between NCAM and aIK. This suggests that lpc 1, 4 and 5 are due to host components present in purified virus preparations. The lpc 2 and 3, however, appear only in the IK/T–aIK reaction and would seem to be virus-specific. The other reactions seen are due to host material: lpc E1 is unique to the NCAM–aNCAM reaction and lpc E2 probably represents host component in the purified virus. The lpc E2 appears to show a reaction of partial identity with lpc 3 but the significance of this is not known. The experimental design does not allow comparison of lpc E1 with lpc 1 (or E2) but was chosen to show the virus-specific nature of lpc 2 and 3.

The strain-specific nature of the virus-specific lpc is illustrated in Pl. 1, fig. 2. Here IK/T is compared with WS/T. The lpc 1, 4 and 5 of Pl. 1, fig. 1 are common to both virus extracts but lpc 2 and 3 are unique to the IK/T–aIK experiment and lpc 6 and 7 are unique to the WS/T–aWS experiment.

Further proof of the strain-specificity of IK/T components is given in Pl. 1, fig. 4 where FM 1/T is included in the experiment. The lpc 2 and 3 of the IK/T–aIK experiment are not given by WS/T or FM 1/T.

Similar results were obtained with extracts prepared from other strains. Results with PR 8/T are shown in Pl. 2, fig. 1. Here the lpc labelled 8 and 9 are probably strain-specific. Results with FM 1/T are shown in Pl. 2, fig. 2. Here lpc 10 is unique to the FM 1/T–aFM 1 system.

An attempt was made to study strain-specific antigens using antisera absorbed with NCAM to remove anti-host antibody. Results are summarized in Table 4. Absorbed antisera gave up to 3 lpc when tested against Triton N 101 extracts of homologous virus. In some cases one of the lpc was common to viruses of the same sub-group but was not found in tests involving viruses of different sub-groups, i.e. it was sub-group specific. The other lpc were strain-specific except that some cross-reactions were found between closely related viruses. For example, the strain-specific antigens in WS/T were distinct from those in FM 1/T. The virus-specific materials in PR 8/T, however, showed some cross-reaction with WS/T and FM 1/T.

Examples of tests using absorbed antisera are shown in Plate 2. Here fig. 3 shows results obtained with aPR 8 and fig. 4 results obtained with aSW. In fig. 3 the PR 8/T-absorbed aPR 8 reaction shows 3 lpc. None of these lpc are given by FM 1/T. One of the lpc (labelled 12) is given by SW/T but another (labelled 11) is unique to PR 8/T. The occurrence or absence of the other lpc in SW/T cannot be determined in this experiment. In fig. 4 the SW/T-absorbed aSW reaction shows two lpc which are not given by NCAM. These reactions are virus-specific. The SW/T–aSW reaction shows these lpc together with other lpc due to host components. Host components and other egg lpc are shown in the reaction between aSW and absorbed aSW. Clearly absorption of antisera with host material facilitated the demonstration of virus-specific materials.

Recently group- and strain-specific antigens have been demonstrated using a human convalescent serum which was free from host antibody. One result is shown in Pl. 1, fig. 3. Triton N 101 extracts of CAM-grown MAL gave at least 3 lpc when tested against the human convalescent serum VR. This serum did not react with NCAM or B/T. In other experiments one of the lpc was found to correspond to S-antigen, another was sub-group specific for influenza A 2 strains and the others were unique to MAL.

The immunological data available are in agreement with previous observations and suggest that influenza B strains possess their own group- and strain-specific antigens and are related to influenza A strains only by the presence of common host antigens. This is illustrated in Pl. 1, fig. 5, where the B/T–aB reaction gives one lpc (labelled B) not given by IK/T or NCAM.

DISCUSSION

It is thought that the virus preparations used in this work were as pure as those used by others. A possible exception was MAL. This virus had been passed only a

few times in CAM, did not grow to high titre and hence was associated with relatively more host material before purification began.

The highly purified virus preparations were agglutinated by aNCAM. This observation supports the view that host material is present in the virus surface; it differs from the results of Ananthanarayan (1954), Kroeger (1962) and Duc-Nguyen, Rose & Morgan (1966).

Purified virus did not react in immunodiffusion tests unless experiments were incubated at room temperature for prolonged periods. It is thought that in this time virus was degraded. Such an explanation would account for the results obtained by Jensen & Francis (1953), Hennisch (1960) and Nikolova & Kavaklova (1967).

Following degradation of virus a number of different host components were detected by immunodiffusion tests. For example, Pl. 2, fig. 2 shows up to five lpc common to the PR8/T–aFM1 and the NCAM–aFM1 reactions. This indicates that either more than one host material is present on the virus surface or that some host material is present in the virus core or that on degradation a host component is split into a number of materials having different serologically reactive sites.

Disrupted virus extracts also contained virus-specific material. Neuraminidase and SSB activity were detected by specific tests, and immunodiffusion experiments showed several virus-specific precipitating substances. Using rabbit antisera, at least two virus-specific materials were detected in virus extracts. Detection was facilitated by the use of sera absorbed with NCAM. It was essential to use NCAM and not NAF for this purpose as the latter material lacked some of the host components found in virus extracts. It seems possible that the virus-specific precipitating substances detected by our rabbit antisera were related to SSB and neuraminidase. They were not related to S-antigen, as these sera did not contain anti-S antibody.

S-antigen was present in our virus extracts. It was detected by use of human convalescent serum. In immunodiffusion tests a single lpc appeared common to all extracts of influenza A virus tested. The finding is not illustrated in this paper but one of the lpc shown in the MAL/T–VR reaction in Pl. 1, fig. 3 is due to S-antigen.

The detection of a single S-antigen agrees with the work of Schild & Pereira (1969) but differs from that of Styk & Hána (1966), Hána & Hoyle (1966) and Styk, Hána & Sedíleková (1968), who used the Wadsworth (1957) micro-immunodiffusion technique to investigate virus disrupted with ether and SDC and tested against human convalescent serum. They described multiple S components in virus extracts together possibly with some strain-specific material.

Using virus disrupted with SDS, Schild & Pereira (1969) were unable to detect virus-specific material which did not correspond to S-antigen or neuraminidase. A substance which might have been related to HA was detected in virus disrupted by the non-ionic detergent Non-idet P40. We suspect that, given suitable antisera, virus extracts made with non-ionic detergents give lpc in immunodiffusion tests which correspond to neuraminidase, SSB substance and S-antigen. Support for this view is given by consideration of data presented in Table 2 and Table 4.

With respect to neuraminidase, some of the virus-specific lpc correspond to the occurrence of these enzymes as determined by anti-neuraminidase tests. Strain-specific precipitating substances and strain-specific neuraminidase occur in SW and WS. Strain-specific precipitating substances and neuraminidase of restricted specificity occur in PR 8 and FM 1. In A 2 strains the neuraminidase was sub-group specific and a common precipitating substance was found.

The other virus-specific lpc corresponded to the range of HI antibodies present in the sera used. Thus in immunodiffusion tests cross-reactions were obtained with SW, WS and PR 8 and with WS, PR 8 and FM 1; there was no virus-specific lpc cross-reaction between SW and FM 1 even though there was some cross-reaction between the HI antibodies to these viruses (see Table 2). In A 2 influenza virus strains differences were found between MAL and the closely related EFME and IK. The extracts of influenza B strains examined had virus-specific materials which did not cross-react with any of the extracts of the A strains studied. The significance of other virus-specific substances detected in PR 8/T and MAL/T (see Table 4) is not known.

The work of Tyrrell & Horsfall (1954), Reginster (1965, 1966) and Hobson (1966) suggests that degradation of influenza virus HA leads to the appearance of SSB activity. It is probable therefore that the virus-specific precipitating materials apparently detected by HI antibody are those possessing SSB activity which have arisen by degradation of HA. The wider range of reactivity shown in precipitation tests as compared with the SSB tests could be explained if the materials responsible for both activities possessed strain and sub-group specific sites. The latter might well be masked in intact virus particles and might not take part in SSB tests done on virus extracts.

Ivaničova (1968) has also used immunodiffusion tests to study strain-specific materials in HA preparations of virus but it is not possible to compare our results with hers.

Most of the work reported was done with virus degraded with Triton N 101. This material did not suffer from the several disadvantages encountered with SDS and SDC. It did not give non-specific precipitates with antisera and virus extracts and apparently did not form ionic complexes or coacervations with virus antigens. Unfortunately it could not be removed from virus extracts by dialysis but on the other hand it was readily separated from virus-specific material by electrophoresis in borate buffer (unpublished observation).

A particular advantage of Triton N 101 was that it gave consistent results with all strains of influenza virus examined irrespective of strain and host tissue. Apart from the results reported for virus grown in CAM and in calf kidney tissue culture similar results have been obtained with virus grown in BHK 21 cells and in mouse lung. This suggests that Triton N 101 might be of value in the production of split virus vaccines. As shown by Corbel *et al.* (1970), this particular non-ionic detergent has a specific effect on virus degradation.

Finally these results, taken in conjunction with those of others, suggest that vaccines prepared from degraded virus do not necessarily require HA activity to be effective in the production of HI antibody. Virus-free preparations containing

SSB activity and strain-specific precipitating substances regularly induced HI antibody in guinea-pigs. Preliminary observations suggest that immunodiffusion tests might be used to assess the immunizing potency of such virus extracts.

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REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetyl neuraminic acid and their application to hydrolysates of sialo-mucoids. *Biochemical Journal* **81**, 384.

ANANTHANARAYAN, R. (1954). The fabric of virus elementary bodies. *British Journal of Experimental Pathology* **35**, 381.

BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal* **62**, 315.

CHOPPIN, P. W. & STOECKENIUS, W. (1964). Interactions of ether-disrupted influenza A2 virus with erythrocytes, inhibitors and antibodies. *Virology* **22**, 482.

CORBEL, M. J., RONDLE, C. J. M. & BIRD, R. (1970). Effect of non-ionic detergent on influenza virus. *Journal of Hygiene* **68**, 77.

DAVENPORT, F. M., HENNESSY, A. V., BRANDON, F. M., WEBSTER, R. G., BARRATT, C. D., Jr, & LEASE, G. O. (1964). Comparison of serological and febrile responses in humans to vaccination with influenza A viruses or their haemagglutinins. *Journal of Laboratory and Clinical Medicine* **63**, 5.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404.

DUC-NGUYEN, H., ROSE, H. M. & MORGAN, C. (1966). An electron microscopic study of changes at the surface of influenza-infected cells as studied by ferritin-conjugated antibodies. *Virology* **28**, 404.

FABIYI, A., LIEF, F. & HENLE, W. (1958). Antigenic analysis of influenza virus by complement fixation. II. The production of antisera to strain-specific V antigens in guinea-pigs. *Journal of Immunology* **81**, 467.

HÁNA, L. & HOYLE, L. (1966). The disintegration of the internal ribonucleoprotein of influenza virus A with the production of serologically distinct components. *Acta Virologica* **10**, 506.

HARBOE, A., BORTHNE, B. & BERG, K. (1961). Antibody against normal egg material resulting from influenza vaccination. *Acta Pathologica et Microbiologica Scandinavica* **53**, 95.

HAUKENES, G., HARBOE, A. & MORTENSSON-EGNUND, K. (1965). A uronic and sialic acid free chick allantoic mucopolysaccharide sulphate which combines with influenza virus HI-antibody to host material. I. Purification of the substance. *Acta Pathologica et Microbiologica Scandinavica* **64**, 534.

HENNISCH, M. P. (1960). Virus antigen antibody reactions by gel diffusion. *International Archives of Allergy and Applied Immunology* **16**, 153.

HOBSON, D. (1966). The strain-specific serological activity of a non-haemagglutinating fraction of influenza virus. *British Journal of Experimental Pathology* **47**, 257.

HOWE, C., LEE, L. T., HARBOE, A. & HAUKENES, G. (1967). Immunochemical study of influenza virus and associated host tissue components. *Journal of Immunology* **98**, 543.

HOWE, C., LEE, L. T. & ROSE, H. M. (1961). Collocalia mucoid: a substrate for Myxovirus neuraminidase. *Archives of Biochemistry and Biophysics* **95**, 512.

HOYLE, L. (1945). An analysis of the complement fixation reaction in influenza. *Journal of Hygiene* **44**, 170.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *British Journal of Experimental Pathology* **29**, 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *Journal of Hygiene* **48**, 277.

HOYLE, L. (1952). Structure of the influenza virus. The relation between biological activities and the chemical structure of virus fractions. *Journal of Hygiene* **50**, 229.

IVANIČOVA, S. (1968). Immunodiffusion reaction with influenza virus haemagglutinin. *Acta Virologica* **12**, 171.

JENSEN, K. E. & FRANCIS, T. (1953). Antigen-antibody precipitates in solid medium with influenza virus. *Journal of Immunology* **70**, 321.

KLAMERTH, O. (1961). Virus und enzymatische Eigenschaffen. Über ein Adenosindiphosphat spaltendes Enzym und seine Assoziation mit dem Influenza-Virus. *Zeitschrift für Naturforschung* **16**b, 781.

KNIGHT, C. A. (1946). Precipitin reaction of highly purified influenza viruses and related materials. *Journal of Experimental Medicine* **83**, 281.

KOSYAKOV, P. N. & ROVNOVA, Z. I. (1965). Antigenic host components in the virus structure. *Voprossy Virusologii* **10**, 17.

KROEGER, A. V. (1962). Evidence for the absence of anaphylactogenic host protein in highly purified PR8 influenza virus. *Journal of Immunology* **89**, 136.

LAVER, W. G. (1963). The structure of influenza viruses. 3. Disruption of the virus particles and separation of neuraminidase activity. *Virology* **20**, 251.

LENNETTE, E. & HORSFALL, F. L. (1941). Studies on influenza virus. The complement-fixing antigen of influenza A and swine influenza viruses. *Journal of Experimental Medicine* **73**, 581.

LIEF, F., FABIYI, A. & HENLE, W. (1958). Antigenic analysis of influenza viruses by complement fixation. *Journal of Immunology* **80**, 53.

MIZUTANI, H. (1963). A simple method for purification of influenza virus. *Nature* **198**, 109.

NEURATH, A. R. & SOKOL, F. (1963). Association of myxoviruses with an adenosine diphosphatase/adenosine triphosphatase as revealed by chromatography on DEAE-cellulose and by density gradient centrifugation. *Zeitschrift für Naturforschung* **18**b, 1050.

NIKOLOVA, Z. & KAVAKLOVA, L. (1967). Reaktsia immunodiffuzii v agare u virusof grippa. *Trudy Nauchno-Issledovatel'skogo Instituta Epidemiologii i Mikrobiologii Sofia* **12**, 211.

NORRBY, E. (1962). Haemagglutination by measles virus. 4. A simple procedure for production of high potency antigen for haemagglutination inhibition (HI) tests. *Proceedings of the Society for Experimental Biology and Medicine* **111**, 814.

OUCHTERLONY, O. (1948). Antigen-antibody reactions in gels. *Acta Pathologica et Microbiologica Scandinavica* **32**, 231.

PANIKER, C. K. J. (1968). Serological relationships between the neuraminidases of influenza viruses. *Journal of General Virology* **2**, 385.

REGINSTER, M. (1965). Inactivation of influenza virus by caseinase C from *Streptomyces albus* G culture-filtrates. *Journal of General Microbiology* **40**, 157.

REGINSTER, M. (1966). Release of influenza virus neuraminidase by caseinase C of *Streptomyces albus* H. *Journal of General Microbiology* **42**, 323.

SCHILD, G. C. & PEREIRA, H. G. (1969). Characterisation of the ribonucleoprotein and neuraminidase of influenza A viruses by immunodiffusion. *Journal of General Virology* **4**, 355.

SMITH, W. (1952). The structural and functional plasticity of influenza virus. *Lancet* i, 885.

SMITH, W., BELYAVIN, G. & SHEFFIELD, F. W. (1955). The host tissue component of influenza viruses. *Proceedings of the Royal Society*, Series B **143**, 504.

STYK, B. & HÁNA, L. (1966). Immuno-diffusion studies on the reaction of influenza virus with specific antibody and non-specific β-inhibitor. *Acta Virologica* **10**, 281.

STYK, B., HÁNA, L. & SEDÍLEKOVÁ, M. (1968). Antibody response to natural influenza A2 infection of man as studied by gel double diffusion. *Acta Virologica* **12**, 208.

SUTHERLAND, W. E., CORI, C. F., HAYNES, R. & OLSEN, A. S. (1949). Purification of the hyperglycaemic-glycogenolytic factor from insulin and from gastric mucosa. *Journal of Biological Chemistry* **180**, 825.

SZALKOWSKI, C. R. & MAEDER, W. J. (1952). Colorimetric determination of desoxycholic acid in ox bile. *Analytical Chemistry* **24**, 1602.

TYRRELL, D. A. J. & HORSFALL, F. L. (1954). Disruption of influenza virus. Properties of degradation products of the virus particle. *Journal of Experimental Medicine* **99**, 321.

WADSWORTH, C. (1957). A slide microtechnique for the analysis of immune precipitates in gel. *International Archives of Allergy and Applied Immunology* **10**, 355.

WORLD HEALTH ORGANIZATION EXPERT COMMITTEE ON INFLUENZA (1953). *World Health Organization Technical Report Series* No. 64.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. The IK/T–aIK reaction shows up to 5 lpc. Lpc 1 is given in the IK/T–aNCAM reaction and lpc 4 and 5 in the NCAM–aIK reaction. Lpc 2 and 3 are probably virus-specific. Other reactions, e.g. E1, E2, are due to CAM materials.

Fig. 2. Lpc 2 and 3 are specific for the IK/T–aIK reaction. Lpc 6 and possibly 7 occur only in the WS/T–aWS reaction. The lpc are probably strain-specific. Other lpc occur in both homologous and heterologous tests.

Fig. 3. The virus extract MAL/T reacts with the aMAL human convalescent serum VR to give at least 3 lpc. The lpc are not given by NCAM or B/T and are thus probably specific for influenza A virus.

Fig. 4. The IK/T–aIK reaction shows the lpc 2 and 3 of figs. 1 and 2. These lpc are not present in the WS/T and FM/T–aIK reactions.

Fig. 5. The B/T–aB reaction shows the lpc B, which is not given by the NCAM or IK/T–aB reactions.

Fig. 6. The NCAM–aNCAM reaction is much more complex than the NAF–aNCAM reaction. This latter test also lacks lpc present in the IK/T–aNCAM reaction.

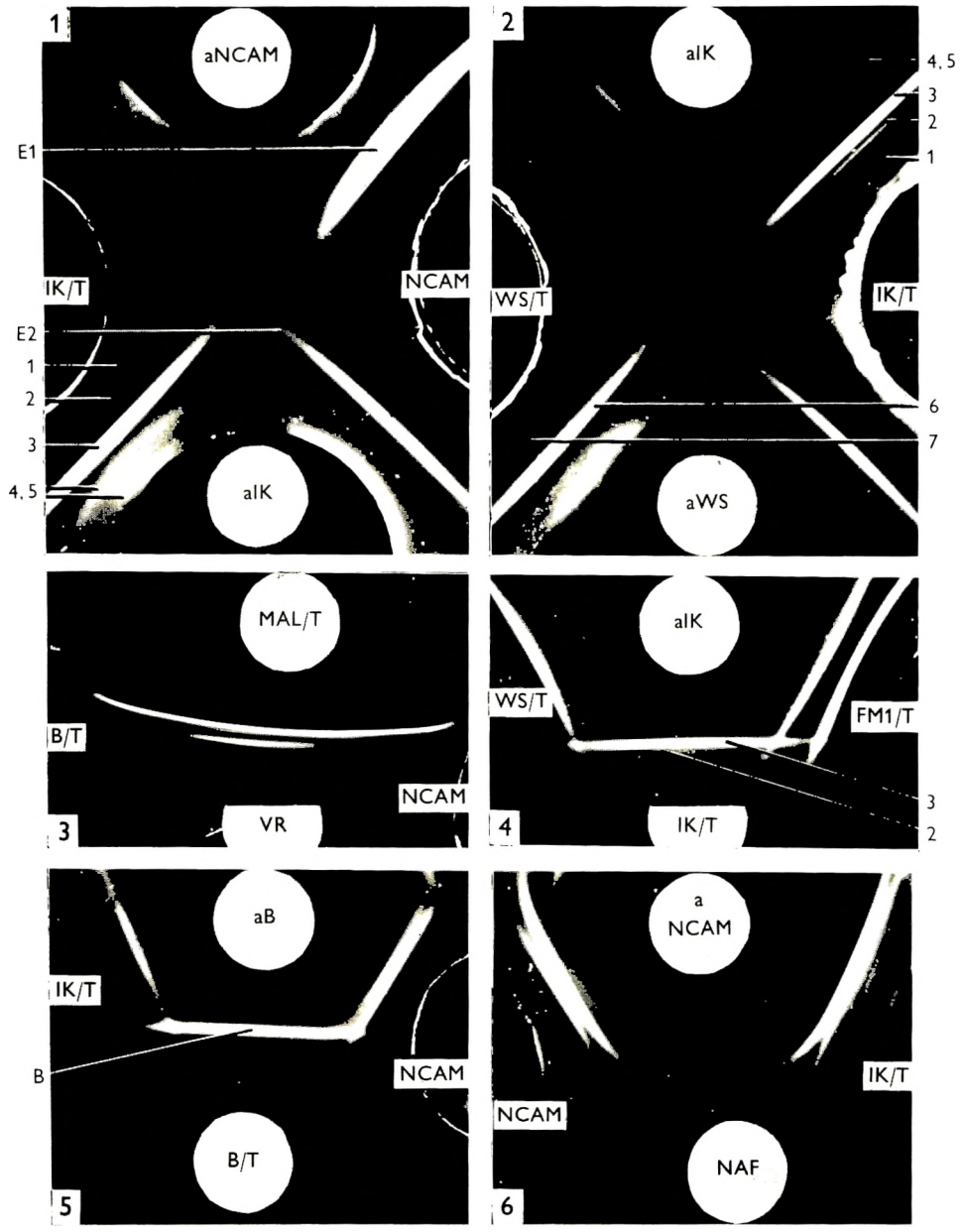
PLATE 2

Fig. 1. The PR8/T–aPR8 reaction shows 2 lpc (labelled 8 and 9) not present in the NCAM–aPR8 reaction. Lpc 9 is present in the FM1/T–aPR8 reaction but lpc 8 is unique to the PR8/T–aPR8 reaction.

Fig. 2. The FM1/T–aFM1 reaction shows an lpc (labelled 10) which is not present in reactions involving PR8/T or NCAM. The distribution of other lpc is marked by the complexity of the reactions.

Fig. 3. The PR8/T–aPR8 (absorbed with NCAM) reaction shows 3 lpc. One lpc (labelled 11) is unique to this test; another (labelled 12) is given also in the SW8/T–aPR8 reaction. The virus extract FM1/T did not react with NCAM-absorbed aPR8.

Fig. 4. The SW/T–aSW (absorbed with NCAM) reaction shows 2 lpc not given by the reaction involving NCAM. Both lpc are given by the SW/T–aSW reaction and probably represent virus-specific reactions. The aSW–aSW (absorbed with NCAM) reaction shows that absorption was complete. It also indicates the host reactions involved in the SW/T–aSW test.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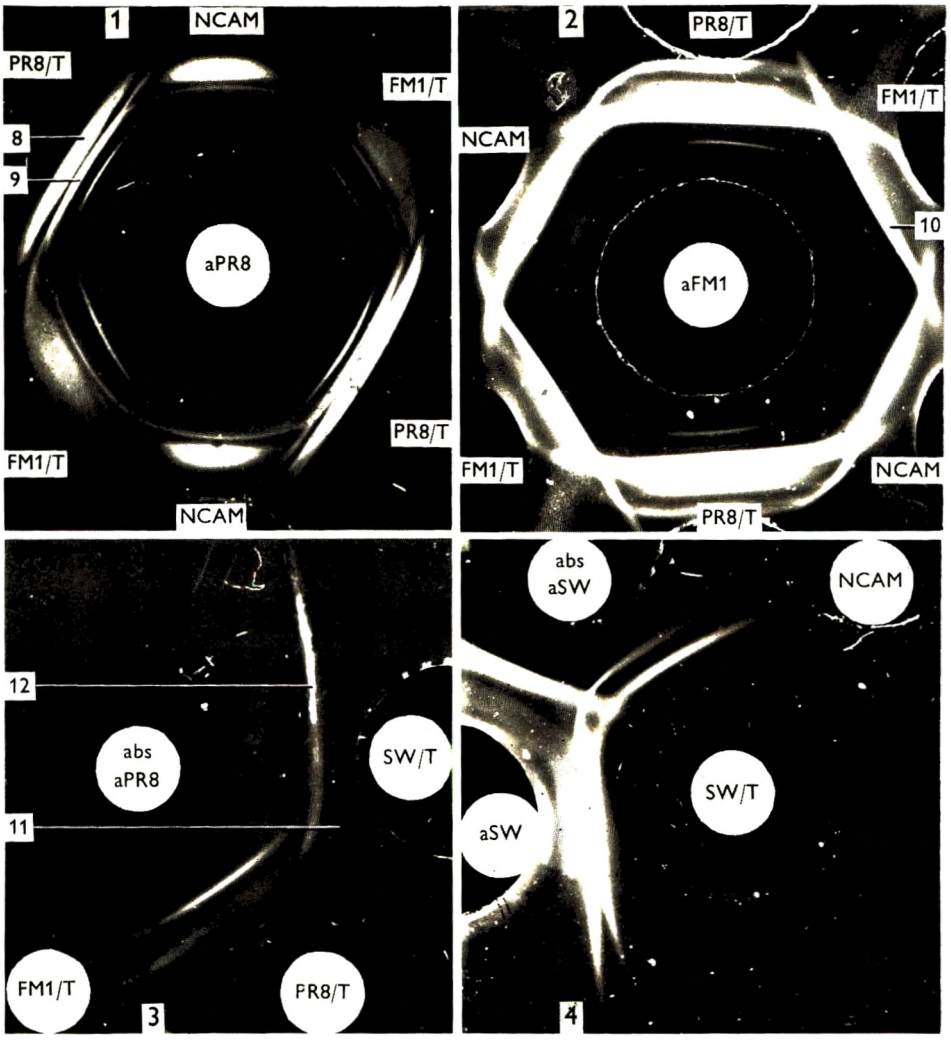
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Isolation of *Mycobacterium xenopei* from water taps

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SUMMARY

An increase in the number of isolations of *Mycobacterium xenopei* from sputum suggested environmental contamination. The organism was recovered from water taps in 61 of 111 pairs of hot and cold water taps in one hospital, 20 of the 74 pairs in another hospital, but from only 3 of 61 pairs of taps in a third hospital and two of 34 pairs in private houses. Scotochromogens were recovered from taps in the first two hospitals only, and *M. kansasii* was isolated twice from the same tap in the first hospital. No mycobacteria were isolated from pigeon droppings or viscera, nor from patients' tooth brushes, tooth powder or razor debris. The source of contamination is not known.

INTRODUCTION

During 1967 there was an increase in the number of isolations of *Mycobacterium xenopei* from specimens of sputum examined at the Epsom Laboratory. Of 435 sputa examined, 16, all from different patients, yielded scanty but pure growths of *M. xenopei*, whereas in the previous year this organism had been isolated once only. Thirteen of these patients were known cases of pulmonary tuberculosis and further sputa from these yielded neither *M. xenopei* nor *M. tuberculosis*. The remaining three patients had left the hospital and could not be traced.

Ten of these patients were in one ward at Hospital A. Contamination from an environmental source was suspected and it was decided to investigate likely sites in this ward and in the laboratory. Subsequently, after the isolation of *M. xenopei* from water taps in both ward and laboratory, the survey of taps was extended to other wards at Hospital A, to Hospitals B and C and to 10 private houses, all supplied by the same water company. Hospitals A and B had water towers housing storage tanks which were not completely protected from dust and bird droppings. Hospital C had water tanks in the roof space; this was not normally accessible to birds, although they had entered it in the past.

METHODS

Collection of material

Swabs were taken from hot and cold water taps and cultures were made from the tooth powder, tooth brushes and electric razors used by the patients. No water

carafes were used in these wards. Droppings from pigeons and from seagulls which congregated during the winter months on the grass surrounding the ward were collected.

The laboratory reagents and apparatus used in the culture of tubercle bacilli were tested.

Bacteriological methods

Sterile cotton wool swabs were used to sample the water taps and centrifuge buckets; each swab was agitated in 1 ml. of quarter-strength Ringer's solution. The fluid was treated by a modified Petroff's method (Cruickshank, 1965) with both incubation at 37° C. and centrifugation at 4° C. for 15 min.; two Lowenstein–Jenson slopes were inoculated and incubated for 8 weeks. At first one swab was used for each pair of hot and cold taps, but later in the survey one swab was used for each tap.

Tooth powder, tooth brushes and razor debris were mixed with a small quantity of quarter-strength Ringer's solution. Pigeon and seagull excreta were suspended in quarter-strength Ringer's solution and allowed to stand for 30 min. The supernatant was concentrated by centrifugation and inoculated on Lowenstein–Jenson slopes after treatment by the modified Petroff's method.

A control consisting of 1 ml. of quarter-strength Ringer's solution in a universal container was included with each batch of tests. This was treated in the same way as the test specimens.

Identification of mycobacteria

Cultures showing acid fast organisms were examined by the methods described by Collins (1967) except that sensitivity tests were restricted to isoniazid and ethionamide.

RESULTS

The isolation rates of *M. xenopei* in pairs of hot and cold taps in Hospitals A and B (55 % and 27 % respectively) were very much higher than at Hospital C (5 %) (Table 1). *M. xenopei* was isolated from the taps of only one of the ten houses swabbed.

Table 1. *Isolation of mycobacteria from water taps*

Building studied	Total no. of pairs of taps sampled (hot and cold)	Mycobacteria isolated		
		M. xenopei	Scoto-chromogens	*M. kansasii*
Hospital A	111	61 (55)	11 (10)	1 (1)
Hospital B	74	20 (27)	37 (50)	—
Hospital C	61	3 (5)	— —	—
Ten private houses	34	2*(6)	— —	—

* These isolations were from one house. Numbers in parentheses indicate percentages.

M. kansasii was isolated from two of five swabs from one tap in hospital A over a period of 9 months. The identity of this strain was confirmed by the Tuberculosis Reference Laboratory, Cardiff.

Scotochromogens (47 strains) were isolated from taps in Hospitals A and B only.

Once *M. xenopei* had been isolated from water taps, hot and cold taps were sampled separately. *M. xenopei* was isolated from 36 hot taps but from only 11 corresponding cold taps.

Hospitals A and B both had water towers which had been contaminated with pigeon droppings. Twenty-seven samples of pigeon droppings from the vicinity of the water tanks, and livers and spleens from eight dead pigeons, were examined over a period of 7 months with negative results. Eleven samples of seagull droppings were also negative.

M. xenopei was not isolated from tooth powder, tooth brushes or electric razors used by the patients in a ward at Hospital A.

M. xenopei was present in most of the water taps in the laboratory and was occasionally isolated from the centrifuge buckets. However, it was not isolated from any of the reagents used, nor from the controls which were included with each of the 28 batches of specimens. During the period of study 187 samples of sputum were cultivated for mycobacteria and all were negative for *M. xenopei*. It seemed highly unlikely, therefore, that laboratory contamination could account for the isolation of *M. xenopei* from taps; (28 % of all taps tested were positive).

DISCUSSION

Mycobacterium xenopei was described by Schwabacher (1959), who isolated it from a skin lesion in a toad (*Xenopus laevis*) in a pregnancy diagnosis laboratory. The role of this organism as an opportunist pathogen is discussed by Marks & Schwabacher (1965) and by Marks (1968). *M. xenopei* is frequently isolated from pathological material but in a high proportion of cases it appears to have no significance. Marks & Schwabacher reported 24 non-significant isolations (including six from urine) amongst 50 cases studied. In the three years, June 1966 to June 1969, 68 of 103 cultures identified as *M. xenopei* at The Regional Centre for Tuberculosis Bacteriology in London were single isolations and probably not significant. Eleven of these were from urine, three from gastric contents, two from endometrium and the remainder from sputum.

Most isolations reported in the United Kingdom are from London and South-Eastern England (Marks & Schwabacher, 1965). The normal habitat of *M. xenopei* is not known, but in view of the high incidence in coastal areas in England and Europe (Marks, 1964) and the high optimal growth temperatures (42°–44° C.), it is possible that sea-birds might be infected. Pigeons are also very common in the Epsom area but our attempts to isolate *M. xenopei* from seagull and pigeon droppings and from dead pigeons were not successful.

M. xenopei was isolated from 300 patients in a single hospital district in Le Havre by Lelieur (1968). The organism was obtained once only from 268 of the 300 patients and environmental contamination was suspected. This mycobacterium

was not, however, recovered from dust, air, pigeon droppings or water, but the water taps were not examined.

At Epsom it seems likely that the organisms entered the water tanks at some time, survived heating in the calorifiers (the temperature of the tap water in the three hospitals varies between 54° and 64° C.) and infected the slime in the water taps. *M. xenopei* is 'thermophilic' (42°–44° C.) and may survive higher temperatures for short periods when the taps are in use. It does not grow appreciably at 25° C. in 3 weeks and this temperature preference may account for the higher rate of isolation from hot taps.

The results of this investigation suggest that strains of *M. xenopei* isolated on one occasion only should not be regarded as significant and that if the organism is encountered several times in the same laboratory over a short period contamination should be suspected.

The isolation of *M. kansasii* is of interest. The natural habitat of this organism is not known and attempts to recover it from the environment in areas where there is a high incidence of *M. kansasii* infection have been unsuccessful (J. Marks, personal communication; Spencer Jones, 1969).

The distribution of scotochromogens was unexpected; only Hospitals A and B yielded these organisms. No scotochromogens were cultured from sputum specimens during the period of study, but in general sputum receives more prolonged treatment with sodium hydroxide than that given to the swabs from water taps, and species of mycobacteria are known to differ in susceptibility to this reagent.

REFERENCES

COLLINS, C. H. (1967). *Microbiological Methods.* 2nd ed. London: Butterworth.

CRUICKSHANK, R. (1965). *Medical Microbiology.* 11th ed. Edinburgh and London: E. & S. Livingstone.

LELIEUR, G. M. (1968). Etude d'une invasion hospitalière de *Mycobacterium xenopei* à propos de 300 observations cliniques. M.D. Thesis, Paris.

MARKS, J. (1964). Aspects of the epidemiology of infection by 'anonymous' mycobacteria. *Proceedings of the Royal Society of Medicine* **57**, 479.

MARKS, J. (1968). Opportunist mycobacteria. In *Recent Advances in Respiratory Tuberculosis*, 6th ed. Ed. F. Heath and N. Lloyd Rusby. London: J. & A. Churchill.

MARKS, J. & SCHWABACHER, H. (1965). Infection due to *Mycobacterium xenopei*. *British Medical Journal* i, 32.

SCHWABACHER, H. (1959). A strain of mycobacterium isolated from skin lesions of a cold-blooded animal, *Xenopus laevis*, and its relation to atypical acid-fast bacilli occurring in man. *Journal of Hygiene* **57**, 57.

SPENCER JONES, J. (1969). *Mycobacterium kansasii* in East Kent. A report of seven pulmonary infections with an environmental study. *British Journal for Diseases of the Chest* **63**, 83.

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A comparative study of two live measles vaccines in Iran

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SUMMARY

The results of immunization of 523 children from 9 months to over 5 years of age with two attenuated measles vaccines, 'Denken' and 'Biken', are described. The clinical reactions following vaccination with both vaccines were mild, but Denken vaccine produced more rashes than the Biken vaccine. The serological conversion was satisfactory for both vaccines but a higher titre of neutralizing and haemagglutination-inhibiting antibodies was found in children immunized with Denken vaccine. The incidence of severe reactions or complications was negligible in this trial. The immunity to diphtheria or tetanus was not altered by vaccination with live measles vaccine.

INTRODUCTION

Since the introduction of the first live attenuated measles vaccine, Edmonston B. Strain (Enders, Katz, Milovanovic & Holloway, 1960) several variants of this vaccine, based on difference in number of passages of the same strain of virus in susceptible host cells, have been developed (Schwarz, Moraten, Beckenham, further attenuated Beckenham 31 and Milovanovic's strains). In Russia (Fadeeva, Dadashyan, Lebedev & Zhdanov, 1960; Smorodintsev *et al.* 1960) vaccines were prepared by adapting two different strains of measles virus isolated by Russian workers to human and chick-embryo cell cultures. In Japan also 'Denken' vaccine was developed by Matumoto *et al.* (1961; 1962) by attenuation of the Sugiyama strain, originally isolated in monkey kidney cell culture. It later underwent six passages in the same cell, six in human conjunctival-cell culture and 45 passages in primary bovine kidney cell. Another Japanese vaccine 'Biken' was first introduced by Okuno *et al.* (1960) from supernatant of chick-embryo amniotic-membrane emulsion. This strain was derived by 61 passages of the Toyoshima strain in chick-embryo amniotic membrane.

Since the Schwarz and Beckenham vaccines have been widely used in rural areas of Iran with a high percentage of protection (Nafyci *et al.* 1967) it was desirable to study the effectiveness of and the reactions produced by the other existing vaccines. This paper presents the results of clinical and serological follow-up of children immunized with Denken and Biken vaccines.

MATERIALS AND METHODS

Study population

The five villages called Hessarak, Mehrdasht, Soufiabad, Heydarabad and Kamalabad located on a dry plateau area at an altitude of about 3000 feet, 40 miles west of Tehran, and around the Razi Institute were chosen for this study. The study population was made up of 523 healthy children, aged 9 months to 7 years, evenly distributed between the two sexes and without a known history of measles infection.

The vaccine trial was conducted at the end of January, when there was some snow most days. The children and their mothers were brought by cars to the dispensary of the Razi Institute, where vaccination was practised, and the children of each village were divided into three groups. One group received Denken vaccine, another group Biken vaccine and the last was injected with physiological saline.

Surveillance

The vaccinated children were observed by three physicians and 20 Public Health technicians who did not know which vaccine had been administered. Medical personnel were well trained to perform the follow-up examination. All information was recorded on a card containing spaces for identifying information, code numbers of vaccines, date of vaccination, daily temperature and significant reactions. The initial survey revealed the occurrence of a respiratory disease in the area. There was, however, no evidence of measles, diphtheria or any other infectious diseases. Rectal temperatures were measured at 9 a.m. and 4 p.m. each day from the 5th to the 19th day after immunization. All children with severe reactions were examined by physicians.

Vaccines

The following two live measles vaccines were used: (1) Denken lyophilized vaccine lot No. 2A, generously granted by Dr Hashizuma, Chief of the Measles Unit, Chiba Serum Institute, Ichikawa Chiba, Japan. (2) Biken lyophilized vaccine lot No. 6803 kindly supplied by Professor Okuno, Director, Research Institute for Microbial Diseases, Osaka University, Suita City, Osaka, Japan.

Physiological saline was used as placebo for inoculation of the children in the control group. Both vaccines were shipped by air-freight in dry ice and were then kept at $-20°$ C. until used. Children were immunized subcutaneously with 500 TCID 50 of vaccine in a volume of 0·25 ml.

Serological techniques

Paired bloods of 449 children (85% of the total children under study) were collected immediately before inoculation of vaccine and 30 days later. The blood was collected from finger pricks using paper disks as described previously (Mirchamsy, Nazari, Stellman & Esterabady, 1968). All specimens were kept in envelopes in a dry and cool place before use. Each pair of sera was tested by neutralization and haemagglutination-inhibition tests.

Neutralization test

Each disk of filter paper was soaked overnight at 4° C. in 0·8 ml. of sterile distilled water and serum was removed by centrifugation. This dilution was accepted as 1/4 serum dilution. Before each test this serum dilution was heated at 56° C. for 30 min. Neutralization was performed in Vero cell cultures as follows. Replicate monolayers containing 5×10^5 Vero cells grown in tubes, as described previously (Mirchamsy & Rapp 1969), were infected with 0·2 ml. of a mixture of equal amounts of 50 TCID 50 of Edmonston strain of measles virus and serum dilution incubated for 1 hr. at 37° C. Serum dilutions of 1/16 to 1/1024 were used. After an adsorption period of 1 hr. at 37° C. 1·3 ml. of maintenance medium was added to each tube. Observation of cytopathic changes was made for 7 days. The neutralization titre (NT) was expressed as negative log, to base 2, of the serum dilution.

Haemagglutination-inhibition (HAI) test

Haemagglutinin (HA) was partly purchased from commercial laboratories and partly prepared according to Rosen's Technique (Rosen, 1961) but using Vero cells instead of KB cells. Three lots of virus prepared by this technique had haemagglutinin titres of 1/64, 1/32 and 1/64.

For the haemagglutination test we used a 0·5 % (v/v) suspension of vervet monkey erythrocytes in phosphate-buffered saline (PBS). The HA titre was determined by incubating at 37° C. a mixture of 0·2 ml. of each of twofold serial dilutions of HA and 0·2 ml. of 0·5 % red cell suspension. The test was performed in special Plexiglass plates (Flow laboratories) made for HA titration and was read after 2 hr. The highest dilution showing complete agglutination of cells was considered to contain one HA unit.

Equal volumes of 0·2 ml. of the 1/4 dilution of serum to be tested and a 25 % (w/v) suspension of acid-washed kaolin in PBS (pH 7·2) were mixed, shaken for 20 min. at room temperature, and centrifuged at 2000 rev./min. for 15 min. The clear supernatant was used as treated serum. For the HAI test, 0·1 ml. of twofold serial dilutions of the treated serum was mixed with 0·1 ml. of haemagglutinin containing four HA units. The tubes were incubated at 37° C. for 1 hr., 0·2 ml. of the monkey erythrocyte suspension was added, and the tubes were incubated at 37° C. for 2 hr. and at 4° C. overnight. The HAI titre was expressed as the negative $\log_2$ of the highest serum dilution showing complete HA inhibition. In each test, HA titration, standard serum titration and red cell controls were included. This test was performed with dilutions of each serum from 1/8 to 1/1024.

Standard serum

The pooled sera of five adults with high neutralization and HAI titres was distributed in ampoules of 0·5 ml. and was kept at − 20° C. In each test a sample of this serum was used as standard serum.

Diphtheria and tetanus antitoxin titration

In order to follow possible variations in immunity to diphtheria and tetanus due to measles immunization, 56 pairs of sera of children under measles study, having a know history of immunization against diphtheria and tetanus, were selected for antitoxin titration according to the procedure described earlier (Mirchamsy *et al.* 1968).

RESULTS

Study population

The assessment of the study group according to vaccines used is as follows: of 523 children initially immunized, 272 received Denken vaccine, 161 were immunized with Biken vaccine and 90 received physiological saline as placebo. Of 272

Table 1. *Age and sex distribution of inoculated children*

Type of vaccine	Inoculated	Sex		Age			
		Female	Male	9–12 months	> 1–2 years	> 2–5 years	> 5 years
Denken	272	124	148	71	65	68	68
Biken	161	78	83	41	43	41	36
Placebo	90	37	53	27	24	18	20

Table 2. *Number of children developing pyrexia*

Type of vaccine	No. of children clinically assessed	Pyrexia					
		37·4–38° C.		38–39° C.		> 39° C.	
		No.	%	No.	%	No.	%
Denken	249	177	71	34	14	9	3·6
Biken	138	64	46	24	17	8	5·8
Placebo	72	44	61	4	5·6	4	5·6

Table 3. *Distribution of children by incubation periods of pyrexia*

Type of vaccine	Onset mean days	Mean duration of pyrexia (days)	Mean duration of maximum temperature (days)
Denken	9·11	3·83	2·7
Biken	9.4	2·66	2·4
Placebo	—	1·17	1

children immunized with Denken vaccine, 249 were clinically observed, paired blood samples were obtained from 256 children, 13 of them had measles antibodies before immunization and were excluded from the analysis. Of 161 subjects vaccinated with Biken vaccine, 138 were clinically examined, 159 paired blood samples were collected before and 30 days after immunization, 15 children had

natural measles antibodies and were not included in this study. Of 90 placebo group, 72 were in the clinical follow-up, 76 paired samples of blood were collected but because five children of this group had natural measles antibodies, 71 pairs of blood samples were included in our study. The age and sex distributions of children are shown in Table 1.

Clinical response

The clinical findings are summarized in Tables 2–6. The onset of fever was 9·11 and 9·4 days on the average after immunization with Denken and Biken vaccines respectively (Table 3). The duration of fever was 3 to 4 days for both vaccines in

Table 4. *Pyrexia variations according to age group*

Type of vaccine	9–12 months		> 1–2 years	
	Mean duration (days)	Mean max. fever (° C.)	Mean duration (days)	Mean max. fever (° C.)
Denken	4	38·1	3	37·4
Biken	3	38·5	4	38·3
Placebo	1	38	3	37·9
	> 2–5 years		> 5 years	
Denken	4	38·3	2	38
Biken	3	38·1	1	37·5
Placebo	2	38	1	37·7

Table 5. *Symptoms reported 7–18 days after immunization*

Type of vaccine	No. of children clinically assessed	Rash		Koplik		Coryza	
		No.	%	No.	%	No.	%
Denken	249	66	26·5	5	2	40	16
Biken	138	12	8·7	3	2·1	17	12·3
Placebo	72	1	1·4	—	—	6	8·3

Table 6. *Reactions and complications of live Denken and Biken measles vaccines in children under 2 years of age*

Type of vaccine	No. of children	Rash		Koplik		Coryza	
		No.	%	No.	%	No.	%
Denken	120	29	24·1	2	1·7	27	22·5
Biken	42	9	21·4	2	4·7	12	28·6
Placebo	42	1	2·4	—	—	5	11·9

children under 5 years, 2 days for Denken and only 1 day for Biken in children over 5 years (Table 4). The pyrexia was mild in immunized children; as a matter of fact the majority of febrile reactions were mild, not exceeding 37·4–38° C. during the period of observation (Table 2). There was, on the other hand, the same range of temperature for the same percentage of controls. The pyrexia of the control

Table 7. *Serological results of the comparative vaccine study 1 month after immunization (neutralization test)*

Treatment group	No. of sera tested	Number of sera showing: No serological response	Neutralization titres (log_2) 4·0	4·5	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5	10·0	Mean titre	Seroconversion (%)
Denken vaccine	243	14	5	4	3	12	19	24	45	33	31	23	16	4	10	7·3	94·2
Biken vaccine	135	25	19	10	20	10	15	6	9	5	3	7	3	0	3	5·87	81·5
Placebo	76	76	0	0	0	0	0	0	0	0	0	0	0	0	0	—	—

Table 8. *Serological results of the comparative vaccine study 1 month after immunization (haemagglutination-inhibition test)*

Treatment group	No. of sera tested	Number of sera showing: No serological response	Haemagglutination-inhibition titres (log_2) 3·0	3·5	4·0	4·5	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5	10·0	Mean titre	Seroconversion (%)
Denken vaccine	243	20	1	5	2	5	9	12	20	20	17	25	40	26	18	11	12	7·38	91·7
Biken vaccine	135	29	8	6	4	15	11	10	14	5	6	7	4	7	5	2	2	5·89	78·5
Placebo	76	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	—

group, as well as coryza, cough and diarrhoea, was due to respiratory infection that was endemic in the area at the time of the study. The temperature of 38–39° C. was observed in 14, 17 and 5·6 % of subjects immunized with Denken vaccine, Biken vaccine and in the placebo group respectively. Despite the fever, malaise or lack of appetite was not observed. An exanthem was recorded in 66 out of 249 patients (26·5 %) immunized with Denken vaccine; this reaction was much lower following Biken vaccination, since only 12 of 138 children (8·7 %) showed the exanthem (Table 5). The duration of rash ranged between 1 and 3 days. It appeared first on the face as a mild maculopapular eruption. In no instance did the rash produce any disability. The Koplik spots were noticed among 2 % of children immunized with either vaccine (Table 5). No evidence of central nervous involvement, bacterial infections, otitis or any other sequelae were observed in the vaccinated children.

Serological findings

In a preliminary study a comparison was carried out between neutralization, haemagglutination-inhibition and complement-fixation (CF) tests in some sera of children and adults. It was found that when Vero cells were used for the neutralization test as described above, a close correlation existed between neutralization titre and HAI titre if only 10–50 TCID 50 of Edmonston measles virus was used as test dose. In this case the test was performed in 5 days without need of changing medium. The tubes were read each day for cytopathic effect and the neutralization end point was determined when only 1/10 virus test dose showed 50 % cytopathic effect in virus titration. With regard to the CF test, lower titres were obtained when four units of CF antigen were used. The results of CF tests were not included in this study. Comparison was made between NT and HAI tests, and Tables 7 and 8 show the range of seroconversion following administration of Denken and Biken vaccines. The seroconversion was 94·2 % (NT) and 91·7 % (HAI) following vaccination with Denken; 81·5 % (NT) and 78·5 % (HAI) after immunization with Biken. None of the children in the placebo group showed seroconversion. The existing titres against diphtheria and tetanus were not altered following immunization with live measles vaccine.

DISCUSSION

The high mortality due to natural measles complications, exceeding 10,000 cases a year in Iran (Nafyci *et al.* 1967) justified a regular mass campaign against the disease in remote rural areas of the country. Since 1965, attenuated and further attenuated measles vaccines have been successfully tested and widely used in various regions of Iran. In a comparative study Nafyci and his associates (1967) have noticed more severe reactions with attenuated measles vaccine than with further attenuated vaccine, and a high conversion rate for Edmonston B, Beckenham 31 and Schwarz vaccines in children of rural communities around Tehran. The present trial was designed to evaluate the two Japanese live measles vaccines 'Denken' and 'Biken', to compare their clinical and serological reactions with the other vaccines already studied and to examine the possibility of large-scale use of these vaccines. The study was carried out at a time when the incidence of a

respiratory disease was high in the area but measles outbreaks had not been observed for 2 years. Both vaccines have been widely used in Japan. The incidence of clinical reactions in our study for Denken vaccine was lower than that observed in Japan (Matumoto *et al.* 1961; 1962). By inoculation of 0·1 to 10 TCID 50 of the Sugyama strain adapted to primary bovine renal cells, Matumoto and his associates (1962) have observed 70 % incidence of pyrexia and 65 % of rash among susceptible children of various age groups. These results are comparable with those noticed for the Edmonston B strain by Enders and for the Leningrad 4 strain used in Russia. The difference in incidence of fever and rash between our results and those of Matumoto and his associates can be attributed to the difference in passage numbers of the Sugyama strain in bovine renal cells. While these authors used the virus at its 28th passage in bovine kidney cells, the vaccine supplied by the Chiba Institute for this study has been prepared with the same strain at its 72nd passage in primary bovine renal cells. The average time of onset of rash recorded by Matumoto *et al.* was 13·4 and 18·5 days when 10 and 1 TCID 50 of virus were inoculated respectively. In our trial this average was reduced to 9·11 days, most probably because each dose of vaccine administered contained 500 TCID 50 of Sugyama virus. The persistence of rash, on average 3 days, is similar to the finding of the Japanese workers. The attenuated measles vaccine 'Biken', first developed by Okuno *et al.* (1960), was derived from the Toyoshima strain of measles virus. This virus was propagated in the amniotic membrane of the developing chick embryo. The Biken vaccine has been largely used in Japan and in Thailand (Ueda, Hosai, Minekawa & Okuno, 1966) but to our knowledge in most field trials this vaccine has been used either by inhalation or after administration of a killed measles vaccine. In both cases the reactions were mild or inapparent. The seroconversion, on the other hand, was satisfactory especially when live Biken vaccine was used as a challenge vaccine, 3–4 weeks after inoculation of the killed vaccine. In our study the clinical reactions due to Biken vaccination were mild and similar to those observed for Denken vaccine. However, rash appeared in only 8·7 % for Biken vaccine compared with 26·5 % after Denken vaccine.

The serological responses are shown in Tables 7 and 8, from which it is evident that both vaccines had a strong immunogenic effect. There was a satisfactory correlation between NT and HAI antibodies; however, the neutralization test, which is more accurate, showed a somewhat higher percentage of seroconversion than the HAI test. This difference may be attributed to the presence of some non-specific inhibitors which were not totally removed by kaolin. The close correlation between NT and HAI tests confirms the similar finding of Kunita, Kitavaki, Funahashi & Toyoshima (1963).

It is also evident that a better serological response was obtained after immunization with Denken vaccine. We believe however that the stability of immunity will not be altered by the rather low antibody titres recorded for children immunized with Biken vaccine. Since the correlation between HAI activity and the immune response is controversial (Norrby, 1964; Parisius & Macmorine, 1969) it was more accurate to evaluate the immune response by neutralization test. From the data presented we can assume that the effectiveness of Denken and Biken vaccines is

similar to that of Schwarz and further attenuated Beckenham 31 vaccines previously studied in the same region around Tehran.

It is a matter of interest to compare data presented here (Table 6) with similar results observed by Nafyci and his associates (1967) with regard to the reactions and complications of attenuated and further attenuated measles vaccines in children under 2 years of age. Both trials have been made in the same plateau area of 3000 feet elevation. In our study 24·1 % and 21·4 % rash, 1·7 % and 4·7% Koplik spots were found after immunization with Denken and Biken vaccines respectively while 50 % and 45 % rash, 7 % and 5 % Koplik spots and 8 % and 7 % otitis were recorded by Nafyci *et al.* following vaccination with Edmonston B and Beckenham 31 vaccines respectively.

The immunization with live measles vaccine did not depress the immunity to diphtheria or tetanus.

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REFERENCES

ENDERS, J. F., KATZ, S. L., MILOVANOVIC, M. V. & HOLLOWAY, A. (1960). A study on an attenuated measles vaccine. I. Development and preparation of the vaccine; technics for assay of effects of vaccination. *New England Journal of Medicine* **263**, 153.

FADEEVA, L. L., DADASHYAN, M. A., LEBEDEV, D. D. & ZHDANOV, V. M. (1960). Attenuated measles virus strain in the U.S.S.R. *American Journal of Diseases of Children* **103**, 379.

KUNITA, N., KITAVAKI, T., FUNAHASHI, S. & TOYOSHIMA, K. (1963). Comparison of the haemagglutination inhibition test for measles with serological tests and its application to a field trial. *Biken Journal* **6**, 45.

MATUMOTO, M., MUTAI, M., OGIWARA, H. & NAKAMURA, M. (1961). Prolifération du virus rougeoleux en culture de cellules rénales bovines. *Comptes Rendus des Scéances de la Société de Biologie* **155**, 1192.

MATUMOTO, M., MUTAI, M., SABURI, Y., FUJI, R., MINAMITANI, M. & NAKAMURA, K. (1962). Live measles-virus vaccine: clinical trial of vaccine prepared from a variant of the Sugyama strain adapted to bovine kidney cells. *Japanese Journal of Experimental Medicine* **32**, 433.

MIRCHAMSY, H., NAZARI, F., STELLMAN, C. & ESTERABADY, H. (1968). The use of dried whole blood absorbed on filter-paper for the evaluation of diphtheria and tetanus antitoxins in mass surveys. *Bulletin of the World Health Organization* **38**, 665.

MIRCHAMSY, H. & RAPP, F. (1969). Role of interferon in replication of virulent and attenuated strains of measles virus. *Journal of General Virology* **4**, 513.

NAFYCI, K., SAIDI, S., NATEGH, R., MOSTATAB, A. & AKBARSHAHY, R. (1967). Comparative study of live attenuated and further attenuated measles vaccines in rural areas of Iran. *Archiv für die gesamte Virusforschung* **22**, 11.

NORRBY, E. (1964). A sensitive measles HAI test. *Proceedings of the International Symposium of Measles Vaccine Standardization. Measles and Rubella Serology*, Lyon, France, page 186.

OKUNO, Y., SUGAI, T., TOYOSHIMA, K., TAKAHASHI, M., YAMAMURA, T., HATA, S., NIKI, T., NAKAMURA, K., UEDA, S. & KUNITA, N. (1960). Studies on the prophylaxis of measles with attenuated living virus. IV. Inoculation tests in children with chick embryo passage measles virus in 1960. *Biken Journal* **3**, 293.

PARISIUS, W. & MACMORINE, H. G. (1969). Effects of tween 80 and Freon 113 on measles virus. *Applied Microbiology* **17**, 379.

ROSEN, L. (1961). Haemagglutination and haemagglutination-inhibition with measles virus. *Virology* **13**, 139.

SMORODINTSEV, A. A., BAICHUK, L. M., SHIKINA, E. S., BATANOVA, T. B., BYSTRYAKOVA, L. V. & PERADZE, T. V. (1960). Clinical and immunological response to live tissue culture vaccine against measles. *Acta Virologica* (English edition), **4**, 201.

UEDA, S., HOSAI, H., MINEKAWA, Y. & OKUNO, Y. (1966). Studies on the combined use of killed and live measles vaccine. III. Conditions for the 'take' of live vaccine. *Biken Journal* **9**, 97.

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Distribution of mercury resistance among *Staphylococcus aureus* isolated from a hospital community

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SUMMARY

Results from clinical isolations confirmed that mercury resistance is common among antibiotic-resistant strains of *Staphylococcus aureus* present in a large general hospital although the correlation is not as high as that found by Moore (1960).

The distribution of mercury-resistant strains among infections and carriers in surgical, medical, obstetric and geriatric patients and staff was studied. Attention was directed to the distribution among carriers since there are fewer extraneous factors operating among them, and a statistical analysis was made on the total number of mercury-resistant strains and the number of non-endemic strains; this latter figure was obtained by subtracting the dominant type 80/81, which is nearly always mercury-resistant and antibiotic-resistant, from the total. Analysis showed the geriatric patients to have a significantly higher proportion of mercury-resistant strains in both cases, and obstetric patients to have a significantly lower proportion when the total number of mercury-resistant strains was considered. Among the surgical, medical and staff categories, no significant difference in proportions could be found although a trend, in that order, of decreasing proportions of mercury-resistant strains present was noted.

In those cases infected on admission with tetracycline-resistant strains, although mercury-resistant strains still predominate, mercury-sensitive strains make a sizeable contribution. This is a reflexion of their dominance in the non-hospital environment.

INTRODUCTION

It was observed (Moore, 1960) that *Staphylococcus aureus* were either resistant or sensitive to a discriminatory concentration of mercury salts. Furthermore, staphylococci of phage-types associated with hospital epidemics were more often mercury-resistant than non-epidemic strains. Moore found a close correlation between resistance to mercury salts and to antibiotics in general but to no antibiotic in particular. Within the different phage-types there were strains of the same phage-type and same mercury reaction that had different antibiotic sensitivity spectra, indicating that mercury resistance and specific antibiotic resistance are probably independent properties. Surveys carried out in West Africa (Akinlade,

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1962), England (Turner & Willis, 1962) and Denmark (Jessen *et al.* 1963) show a variation in this association.

The aim of this paper is to investigate the distribution of mercury resistance among strains of hospital staphylococci isolated from different departments of a hospital community. It is important to delineate the epidemiological pattern of infection with mercury-resistant staphylococci in order to assess the possible significance of mercury resistance as an epidemiological marker. A major effort to control antibiotic-resistant staphylococcal infection in the hospital was being planned and as this entailed a major sampling programme involving the taking of many swabs over a period of time from the general hospital population, it was decided to take advantage of this in order to obtain the necessary epidemiological information regarding mercury-resistant strains (Stokes, Hall, Richards & Riley, 1965).

Description of hospital

This has been described in full elsewhere (Hall, 1966). Briefly, the main hospital houses medical and surgical wards while adjacent buildings with communicating tunnels house the Obstetric Hospital, Ear, Nose and Throat Hospital and Private Patients' Wing. The geriatric wards are part of a completely separate hospital block one mile distant. In all about 1000 beds are served and there is an active outpatient department.

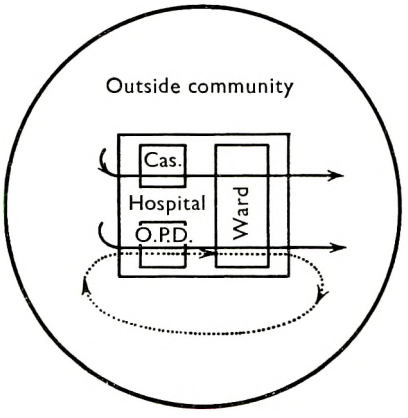

Fig. 1. Pathway of patients to and from the hospital community.

The hospital may be regarded as a community set aside from the outside world into which patients are admitted and from which they are discharged. The total number of inpatients is fairly constant, this being a function of the total number of beds. Patients may attend Casualty or an outpatient department. If the former they are unlikely to have had previous hospital contact and any staphylococci isolated from their lesions are likely to be representative of those present in the community at large. Patients attending outpatient departments will in many cases have been inpatients previously and may be carrying antibiotic-resistant strains. From both these departments they may be transferred to the wards and the

duration of hospital stay will be one factor in determining the chances of acquiring hospital staphylococci. If readmission is necessary, the time interval after previous discharge is important; if relatively short the patients may still carry hospital staphylococci, but if a longer period has elapsed the strain may have exchanged with one commonly present in the patient's normal home environment. The probable general pattern of the epidemiological situation may be summarized diagrammatically (Fig. 1). In addition there is the turnover among the staff although this does not approach that of the patients. Changes in their nasal flora are also less likely as they are not receiving therapeutic doses of antibiotics.

MATERIALS AND METHODS

Plan of investigation

To obtain a complete picture of the distribution of staphylococci in the hospital and outside population, ideally nasal swabs and wound swabs should be taken of the population surrounding the hospital, all patients attending Casualty and outpatient departments, all staff and all patients in wards; also nose swabs of the latter should be taken immediately before discharge. This is clearly impracticable and for the practical purposes of the control programme a more feasible plan of campaign was devised.

A sampling procedure for the detection of antibiotic-resistant strains was initiated by the nasal swabbing of the following categories: (*a*) patients already in hospital; (*b*) hospital staff in contact with inpatients; (*c*) new patients admitted and those transferred within hospital; (*d*) new staff in contact with inpatients; (*e*) nursing staff about to work in theatres; (*f*) nurses and students about to work in the obstetric hospital. In addition, all infected lesions were also swabbed (Stokes *et al.* 1965). Reference to Fig. 1 will show that these categories are related to the principal pathways and sources of infection.

For 2 years all tetracycline-resistant strains and some sensitive strains from infected patients and staff and from carriers were phage-typed and all tetracycline-resistant strains were tested for sensitivity to mercury salts. Tetracycline-sensitive strains were also tested for a period of 12 months (Stokes *et al.* 1965).

Mercury sensitivity test

The agar plates containing mercuric chloride as described by Moore were found too laborious for the large number of strains to be tested. Paper strips impregnated with mercuric chloride solution were used and provided that mercury-resistant and -sensitive control organisms were included, thin plates were poured and the concentration of mercuric chloride (usually 1/5000 w/v) was adjusted to the nutrient agar employed, this modification was satisfactory. Large batches of strips were made at a time and dried in the incubator; they were stable when stored at 4° C. for several months. Plates were streaked from colonies or coagulase-broth cultures, in a parallel manner, six test strains and two controls per plate.

The resistant control (a type 80 strain resistant to penicillin and tetracycline) gave no zone in contrast to the sensitive Oxford staphylococcus, which gave a

small sharply defined zone of about 2 mm. Size of inoculum made a slight difference to zone size but the test was adjusted to give optimal results with heavily seeded streaks.

RESULTS

Analysis of all *Staph. aureus* isolated from noses and wounds of different categories of persons in five different hospital departments during 12 months is shown in Fig. 2; it was only for this period that the majority of tetracycline-sensitive strains were tested for their mercury reaction. The *Staph. aureus* are divided into tetracycline-sensitive and tetracycline-resistant strains and further subdivided into those which are mercury-sensitive and mercury-resistant.

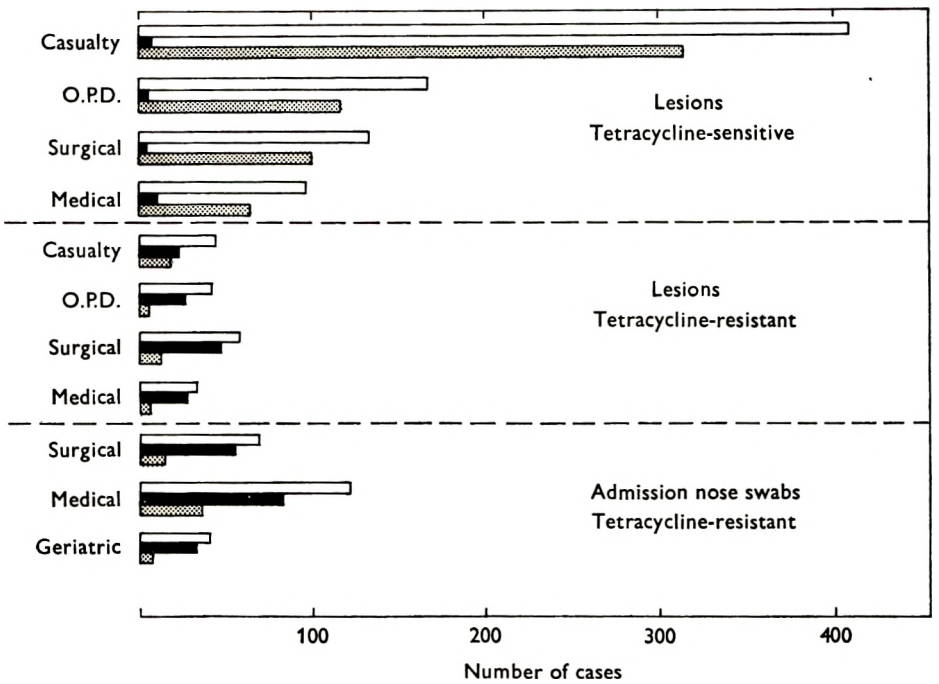

Fig. 2. Analysis of all strains isolated from different departments during a 12-month period. □, Total; ■, mercury-resistant; ▨, mercury-sensitive.

Mercury resistance is seen to follow a closely similar distribution pattern to tetracycline resistance. Less than 5% of tetracycline-sensitive strains were also mercury-resistant except in medical wards, where the figure is 13% (no tetracycline-sensitive strains from the geriatric wards were mercury tested). Amongst tetracycline-resistant strains, the mercury-resistant strains predominate in all departments, the proportion being least in Casualty and highest in medical and surgical wards. Analysis of the admission nose swabs shows that the proportion of mercury-resistant strains is highest in geriatric wards.

The percentage of mercury-resistant strains among the total *Staph. aureus* isolated over the same period is: Casualty 9%, outpatient departments 20%,

Table 1. *Mercury sensitivity of antibiotic-resistant strains isolated from different departments* 1961–64

	Infections (hospital and admitted)					Carriers				
			80/81 and allied types	Other than 80/81				80/81 and allied types	Other than 80/81	
Department	Total tested	Total Mercury-resistant		Mercury-resistant	Mercury-sensitive	Total tested	Total mercury-resistant		Mercury-resistant	Mercury-sensitive
Surgical	137	111	77	34	26	151	119	83	37	31
Medical	86	61	50	15	21	229	162	113	51	65
Geriatric	47	36	24	12	11	87	77	38	39	10
Obstetric	12	5	3	2	7	14	7	4	3	7
Staff	23	12	13	3	7	112	77	51	25	36

surgical 31 % and medical 33 %. Thus as one goes from Casualty to outpatient departments to hospital wards the incidence rises.

Analysis of the phage-types showed that type 80/81 and allied types (including types 52/52A/80, 52/52A/80/81) were predominant among strains isolated in this hospital. This corroborates the findings of Stokes & Milne (1962) (Stokes *et al.* 1965).

The proportion of mercury-resistant strains isolated from infections and carriers in surgical, medical, obstetric and geriatric departments and the staff is shown in Table 1. Since the predominant strain is type 80/81, which is nearly always mercury-resistant, the contribution of this to the total is given. Mercury resistance may appear to be important simply because it is a property of the dominant strain. If, however, mercury resistance is itself an advantage for survival in hospital it should still be dominant even when the number of prevalent strain isolations is subtracted from the total figures. In fact mercury-sensitive strains of other types predominate in infections and in carriers among medical and obstetric patients and staff but in the surgical and geriatric patients the proportions are reversed.

The figures from the Obstetric Hospital, although small, show that the proportion of strains of the 80/81 complex is low both in infections and admitted carriers despite the connexion via the nursing staff with the main hospital.

Among the geriatric patients the proportion of mercury-resistant strains in both infections (77 %) and carriers (89 %) is high. Considering infections first, when the dominant type 80/81 is subtracted from the total, the number of mercury-resistant and mercury-sensitive strains other than type 80/81 are not very different from the other categories of patient. When these figures for nasal carriers are compared however, there were 39 mercury-resistant as compared with 10 mercury-sensitive strains. More emphasis should perhaps be placed on the distribution of mercury resistance among carriers than infections since in this location there are not so many extraneous factors operating. When χ^2 test is applied to nasal carriers of types other than 80/81, it is revealed that: (1) it is not reasonable to assume that all departments have the same proportion of resistant strains ($\chi^2 = 22{\cdot}86$, $P < 0{\cdot}001$ on 4 degrees of freedom); (2) geriatric patients have a significantly higher proportion of resistant strains than the remainder; (3) there are no significant differences between the proportions of resistant strains in the surgical, medical and obstetric carriers and staff ($\chi^2 = 3{\cdot}76$, $P > 0{\cdot}1$ on 3 degrees of freedom); (4) the differences between the proportions are too small to be detected on the amount of data available. When χ^2 test is applied to the total carriers of mercury-resistant strains it again follows that (1) it is not reasonable to assume that all departments have the same proportions of resistant strains ($\chi^2 = 18{\cdot}85$, $P < 0{\cdot}001$ on 4 degrees of freedom); (2) the geriatric patients have a significantly higher proportion of resistant strains than the remainder; (3) there is no significant difference between the proportions of resistant strains in the surgical, medical and staff categories ($\chi^2 = 4{\cdot}16$, $P > 0{\cdot}1$ on 2 degrees of freedom). However the proportion of mercury-resistant strains in the obstetric patients is significantly lower than in the other 3 categories ($\chi^2 = 7{\cdot}62$, $P = 0{\cdot}05$ on 3 degrees of freedom).

Mercury sensitivity of tetracycline-resistant strains

The mercury reaction of tetracycline-resistant strains isolated during 1960–64 from clinical infections is shown in Table 2. The infections are divided into those acquired in hospital and those admitted infected, and are contrasted with the nasal carriers. The proportions are seen to be significantly different in each category. If, as before, the incidence of the dominant epidemic type is subtracted from the total, the figures show more clearly the distribution of mercury resistance among the remaining strains, (Table 3). Among medical patients the proportions of mercury-resistant strains isolated from hospital-acquired infections differ significantly from those infected on admission ($\chi^2 = 7{\cdot}15$, $P < 0{\cdot}01$ on 1 degree of freedom). The distribution pattern for the surgical patients is similar ($\chi^2 = 4{\cdot}45$, $P < 0{\cdot}05$).

Table 2. *Mercury sensitivity of tetracycline-resistant strains*, 1960–64

	Mercury-resistant strains	Mercury-sensitive strains	Proportion of mercury-resistant strains	95 % confidence limits
Hospital infections	231	34	0·872	0·831–0·913
Admitted infections	62	40	0·608	0·511–0·704
Nasal carriers	446	142	0·758	0·723–0·793

Table 3. *Mercury sensitivity of tetracycline-resistant strains other than those of the* 80/81 *complex* (1961–63)

	Infections			
	Hospital		Admitted	
	Mercury-resistant	Mercury-sensitive	Mercury-resistant	Mercury-sensitive
Medical	11	6	4	15
Surgical	28	15	6	11

Overall pattern of nasal carriage

The results are given in detail elsewhere (Stokes *et al.* 1965; Hall, 1966).

Patients

Annual swabbing. The carrier rate of tetracycline-resistant staphylococci in all inpatients swabbed in October of each year varied between 4·3 and 8·2 % during 5 years of testing.

Admission nose swabs. Between 11,509 and 13,395 patients admitted or transferred were swabbed annually. The carrier rate of tetracycline-resistant strains remained more or less constant at 1·4–1·6 %. The mercury-resistant strains were between 2·4 and 3·4 times more numerous than the sensitive in the 4 years of

testing. There was a high preponderance of 80/81 and allied types among these strains.

When the admitted carriers among the medical, surgical and geriatric patients are compared it is seen that the carrier rate for tetracycline-resistant staphylococci is highest in the geriatric wards (10 %) and higher in the medical (3·6 %) than surgical wards (1·8 %) (Stokes *et al.* 1965). In terms of absolute numbers, there are many more carriers admitted to medical wards than to surgical or geriatric wards. In addition, the percentage for obstetric patients was consistently less than 0·4 % and for ear, nose and throat patients less than 0·9 %.

Staff

The carrier rate of tetracycline-resistant staphylococci among the nursing staff when swabbed in October of three consecutive years was 4·9 % of 749, 3·9 % of 793 and 3·0 % of 703. The mercury-resistant strains outnumber the mercury-sensitive by a factor of 5 in the first, 2 in the second and 2·5 in the third year.

DISCUSSION

These data have shown that:

(*a*) Mercury resistance is common in carriers who have been exposed to the hospital environment for long periods and is rare in carriers newly admitted or who stay in hospital for short periods.

(*b*) Although in strains isolated from both nasal carriers and infections mercury resistance and antibiotic resistance are commonly found together, the proportion of mercury-resistant strains is higher than would be expected in geriatric patients if this were only a chance association.

The number of mercury-resistant strains in the admitted infections category is probably artificially high since many of these patients may have had previous hospital experience and the actual difference between the two groups may be more pronounced than the figures in Table 2 would suggest.

Statistical analysis has shown that the higher proportion of mercury-resistant strains among surgical (79 %) than medical patients (71 %) is not significant both for the non-endemic strains ($\chi^2 = 1{\cdot}84$, $P > 0{\cdot}01$ on 1 degree of freedom) and the total mercury-resistant strains ($\chi^2 = 2{\cdot}35$, $P > 0{\cdot}1$ on 1 degree of freedom). Indeed, distinguishing between these two categories is difficult because of their previous histories. Many medical patients who are admitted carrying tetracycline-resistant mercury-sensitive strains have been receiving maintenance doses of tetracycline at home and their strains may have developed resistance to tetracycline as a result, or they may have acquired a tetracycline-resistant strain outside hospital where mercury-sensitive strains are common. Inpatients receiving tetracycline are likely to acquire mercury-resistant staphylococci which are more prevalent in hospital. The situation is further complicated because many surgical patients may previously have received treatment in medical wards. The proportions in the staff are not significantly different although there is a trend for the proportion to be less here than in the surgical and medical departments.

The incidence of strains of the 80/81 complex amongst infections and admitted carriers in the Obstetric Hospital is lower than in the other categories. This is in keeping with the expected low carrier rates; these patients have had little previous hospital history. The figures from the geriatric department suggest that mercury resistance itself is a factor in survival of staphylococci in this environment.

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REFERENCES

AKINLADE, N. D. (1962). A trial of mercuric chloride for the rapid identification of epidemic strains of Staphylococcus pyogenes. *West African Medical Journal* **11**, 35.

HALL, B. M. (1966). A study of antibiotic-resistant *Staphylococcus pyogenes* in a hospital community with particular reference to Moore's mercury sensitivity test as a guide to strains of epidemic importance. Ph.D. Thesis, University of London.

JESSEN, O., ROSENDAL, K., FABER, V., HOVE, K. & ERIKSEN, K. R. (1963). Some properties of Staphylococcus aureus possibly related to pathogenicity. *Acta Pathologica et Microbiologica Scandinavica* **58**, 85.

MOORE, B. (1960). A new screen test and selective medium for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet* ii, 453.

STOKES, E. J., HALL, B. M., RICHARDS, J. D. M. & RILEY, D. J. (1965). Control of hospital staphylococci. *Lancet* ii, 197.

STOKES, E. J. & MILNE, S. E. (1962). Effect of Naseptin cream prophylaxis on staphylococcal infection in adult surgical wards and infant nurseries. *Journal of Hygiene* **60**, 209.

TURNER, G. C. & WILLIS, A. T. (1962). Staphylococcal invasion of a new surgical ward. *Journal of Pathology and Bacteriology* **84**, 349.

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Mercury resistance of *Staphylococcus aureus*

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SUMMARY

Reasons for the accumulation of mercury-resistant strains of *Staphylococcus aureus* in hospital have been studied. A collection of paired strains, that is staphylococci similar in every respect except sensitivity to mercury salts, was made. Tests were made in an attempt to demonstrate a link between mercury resistance and some other factor which might aid survival, viz. resistance to drying and heat, production of bound coagulase, growth in the presence of sublethal amounts of tetracycline, survival in human blood at 37° C. and uptake by polymorphs at 30° C. and 37° C., development of resistance to antibiotics and competition in mixed cultures. It was not possible to demonstrate any consistent link between mercury resistance and any of these properties. Paper strips impregnated with the mercurial diuretic, Mersalyl, were shown to differentiate between mercury-resistant and -sensitive strains *in vitro*. Furthermore, development of resistance to mercury by passage in mercuric chloride-broth was demonstrated.

It is proposed that mercury resistance has developed as a result of exposure to the mercury ion. Mercurial diuretics have been frequently used in medical and geriatric patients and it is among these that the higher carrier rates of mercury-resistant strains are found even when the local endemic strain is disregarded. In obstetric patients, where mercurials are seldom used, mercury-resistant strains are rare.

Nasal carriage of factory workers exposed to mercury products showed that this group is likely to carry resistant or partially resistant strains.

INTRODUCTION

The origins of mercury resistance and the reasons for the accumulation of resistant strains of *Staphylococcus aureus* in the hospital environment might be due to one of the following causes:

(i) Mercury resistance may be a marker of epidemic propensity and may be linked genetically with multiple-antibiotic resistance and therefore automatically selected in hospital.

(ii) Resistant strains may be selected by some other factor, such as penicillinase,

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or some biological property which enables them to survive in the hospital environment.

(iii) Resistant strains may have developed as a result of exposure to mercury-containing products.

This paper sets out to assess these hypotheses.

The first suggested cause is partly answered by reason of the fact that epidemics of antibiotic-resistant mercury-sensitive strains have occurred in maternity hospitals (Moore, 1960). Therefore sensitive strains cannot be regarded as harmless from an epidemiological point of view. Furthermore although mercury-resistant strains are commonly also resistant to penicillin, tetracycline and often to other antibiotics, they are not invariably antibiotic-resistant, neither are they invariably of phage-types associated with epidemics (Moore, 1960; Akinlade, 1962; Turner & Willis, 1962; Jessen *et al.* 1963; Meyer, 1966). With reference to the second proposition, they are commonly high penicillinase producers but there is no constant link so that mercury resistance cannot be satisfactorily explained in these terms. Theoretically there is no reason why antibiotic-resistant high penicillinase producing mercury-sensitive strains should not predominate in hospitals, since such strains exist. If mercury resistance *per se* is no advantage in hospitals it is difficult to comprehend why these strains have not proliferated since they appear to possess all the other required properties.

A variety of tests was made in an attempt to demonstrate a link between mercury resistance and some other biological property which might aid survival and thus account for the predominance of mercury-resistant staphylococci in the hospital environment.

MATERIALS AND METHODS

Staphylococcal strains. A collection of 'paired strains' was made from clinical material and nose swabs. Each member of a pair was of the same phage-type, had the same antibiotic resistance pattern, but one was sensitive to mercury salts, the other resistant. They were tested in parallel.

Mercury impregnated paper strips. The method of preparation is described elsewhere (Hall, 1970).

Resistance to physical agents

Drying. Staphylococcal suspensions were filtered through membrane filters so that the cocci were retained on the membranes and these were cultured at intervals (days) to obtain a quantitative survival figure.

Exposure to heat (56° *C.*). Broth cultures were incubated at 56° C. and explanted to blood agar at 15 min. intervals to assess possible differences in survival.

Effect of incubation temperature on growth rate

Growth rate curves of broth cultures were plotted and compared during incubation at 30 and 37° C.

Capacity to develop resistance to inhibitory compounds

Antibiotic-sensitive strains were passaged on solid and in liquid media and the number of passages required to develop tolerance to tetracycline and erythromycin compared. Likewise mercury-sensitive strains were passaged in mercuric chloride-broth and tested for the development of resistance.

Growth of staphylococci

The effect of tetracycline on growth rate. Growth was compared in broth and tetracycline-broth to see if a sub-inhibitory dose of tetracycline might selectively stimulate the mercury-resistant strains to account for their predominance among those that are tetracycline-resistant.

Competition in mixed cultures. This was investigated to determine whether mercury-resistant strains might outgrow mercury-sensitive strains in the presence of small quantities of antibiotics. Small counted numbers of a mercury-resistant and mercury-sensitive strain (both resistant to tetracycline) were inoculated into broth and broth containing tetracycline and viable counts were made after 24 and 48 hr. incubation. As a refinement serum-broth was used in later experiments to resemble more closely conditions in the patient.

Coagulase

Bound coagulase was estimated by a direct assay method using human plasma. Washed suspensions of staphylococci were added to serial dilutions of plasma and shaken and the highest dilution of plasma in which visible clumping occurred was recorded as the titre. It was also estimated using the antibody inhibition test (Duthie, 1955).

Fate of staphylococci in freshly drawn human blood

Phagocytosis. Counted numbers of washed staphylococci were added to a leucocyte-rich preparation, prepared by a modification of the method described by Li, Mudd & Kapral (1963), in siliconed tubes. These tubes were placed on a roller drum and rotated at 30 and 37° C. After 10 and 30 min. intervals smears were made and the number of cocci in each cell of a counted number of cells per slide was recorded.

Survival in defibrinated blood. Counted numbers of staphylococci were added to freshly drawn defibrinated blood and explants were made to blood agar after hourly intervals of incubation at 37° C.

In vitro *and* in vivo *action of Mersalyl (an organic mercurial diuretic)*

Blotting paper strips were dipped in dilutions of Mersalyl ranging from undiluted (40 mg./ml) to 1/1000 (4×10^{-2} mg./ml). An agar-diffusion method was used to test the action of this compound against mercury-resistant and -sensitive strains.

The serum of patients receiving Mersalyl 30 min. after intra-muscular injection was tested in a similar way with blotting paper disks.

Investigation of factory workers handling mercury compounds

Since mercury resistance and antibiotic resistance are commonly found together and both are used in treatment, both are likely to act as selective agents but it is difficult to distinguish between these two factors in a hospital community. It is already well established that penicillin-resistant strains are prevalent in nasal carriers engaged in preparing penicillin (Gould, 1958). If mercury salts can also act as selectors in the same way it should be possible to demonstrate an increased prevalence of mercury-resistant strains in nasal carriers exposed to mercury compounds.

One large factory handling mercury compounds for battery manufacture provided 101 nose swabs. This factory was divided into four sections and the concentrations of mercury were as follows: amalgam room and cell assembly room 70 μg./m.^{3} air; depolarizing room 40–60 μg./m.^{3} air and packaging room 20 μg./m.^{3} air. Another factory in the same district was used as a control and yielded 108 nose swabs.

A chemical factory manufacturing mercury salts provided 11 swabs from workers handling these substances. In this factory, mercury concentrations were tested every 2 weeks and were 45 μg./m.^{3} air on average but had been as high as 100 μg./m.^{3} air on occasions. Full protective clothing including face masks was employed and showers were taken after leaving the exposed area. In addition urine levels were tested every 3 months. Other sections of this factory provided 37 controls.

A factory which handled mercury for instrument manufacture including thermometers and barometers provided 45 nose swabs. Here the concentration was said to be 'within safe limits' but no figure was given. Other departments of this factory provided 32 controls. A fifth factory manufacturing soap and glue provided 40 controls.

Although the nature of the investigation was explained to the employees, not all would volunteer to be swabbed. Very great difficulty was encountered in obtaining permission to take nasal swabs from factory workers. This factor and the limited time available made it impossible to expand the numbers. At the outset of the experiment the two factories which provided the bulk of the swabs were matched for size and district but this was not possible for the remainder. Many more men than women were included in the survey.

RESULTS

It was not possible to demonstrate any consistent link between mercury resistance and any of the biological properties listed in the foregoing section up to and including 'survival in defibrinated blood'. These results are described in detail elsewhere (Hall, 1966).

Mersalyl at a dilution of 1/200 (0·2 mg./ml.) differentiated between the mercury-sensitive and -resistant control strains. It gave a zone of the usual size, 1·5 mm. for the sensitive, and no zone for the resistant. Dilutions below 1/200 gave zones for

both strains and 1/300 and above gave no zone. This behaviour was similar to that of strips impregnated with inorganic salts such as mercuric chloride, 1/500 (w/v).

No demonstrable inhibition of growth was found when serum from patients receiving Mersalyl was tested, but this was not surprising since a dilution of 1/200 Mersalyl is 100 times more concentrated than the level that could be expected in serum.

Eight mercury-sensitive strains were passaged daily in mercuric chloride-broth and tested with mercuric chloride strips and an agar-diffusion method. They developed resistance slowly and tolerance was acquired after 23–26 passages. Plate 1 shows both a naturally occurring sensitive and resistant strain and a strain which acquired tolerance before and after passage.

Nasal carriage of factory workers handling mercury compounds

Of the 157 people swabbed in the exposed population, 61 (39 %) yielded *Staph. aureus* and of the 217 controls, 60 (28 %) yielded *Staph. aureus*. These carrier rates are within normal limits. None of the positive carriers had been in hospital during the previous two years.

Table 1. *Distribution of mercury-resistant* Staphylococcus aureus *among exposed and control populations*

	Mercury-resistant	Partially mercury-resistant	Mercury-sensitive
Exposed	4	21	36
Controls	0	1	59

The *Staph. aureus* fell into three groups; those fully resistant, those fully sensitive and a partially resistant group whose zone was smaller than that of the Oxford Staphylococcus. When titrated in mercuric-chloride broth, 17 of these strains had a minimum inhibitory concentration of 1/160,000 and five strains had a minimum inhibitory concentration of 1/320,000 by comparison with the Oxford Staphylococcus of 1/640,000. These strains were bracketed together and classed as the partially resistant group.

Table 1 shows the distribution of the strains in the three categories. The χ^2 value for this table is 27·74, which is very highly significant since it exceeds the 0·1 % point of the χ^2 distribution on 2 degrees of freedom, namely 13·81. Although the number of individuals carrying fully resistant strains is small, the error so introduced in using the χ^2 will be small, since the calculated value of χ^2 is so large. Tetracycline resistance was not allied with mercury resistance; in the exposed group, one of the partially resistant strains and five of the fully sensitive strains were tetracycline-resistant. In the control group only one strain in the fully sensitive category was tetracycline-resistant.

DISCUSSION

It has been shown that the frequency with which hospital staphylococci are isolated from nasal carriers admitted to different departments varies (Hall, 1970). The prevalence of these cocci in these departments is related to the previous hospital history of the patients admitted and their average duration of stay (Stokes, Hall, Richards & Riley, 1965). In geriatric wards where almost all have had previous hospital history and a history of antibiotic therapy, and in which patients stay for a long time, the number of hospital staphylococci is high. In obstetric wards where patients seldom have been in hospital previously and seldom have received antibiotics and in which stay is short, few carriers are admitted. When the non-endemic strains from nasal carriers were studied it was found that geriatric patients carried the highest proportion of mercury-resistant strains.

The results from the clinical work indicate that medical and geriatric wards may often be the source of antibiotic-resistant and mercury-resistant staphylococci. It seems likely that a medical or geriatric patient will acquire a tetracycline-resistant strain in the nose, especially when on antibiotic treatment, and retain this for a certain period so that when readmitted he may still be carrying a resistant strain. On the basis of these observations the hypothesis may be put forward that medical wards act as the principal reservoirs for strains that are not only antibiotic-resistant but also mercury-resistant.

Originally the contention that contact with mercury salts might lead to the development of mercury resistance was considered but discarded (B. Moore, personal communication). Mercury is only rarely used in treatment in surgical wards where most studies on cross infection have been concentrated. Small quantities are employed as preservatives in some preparations for injection (British Pharmacopoeia). However, on closer examination it was found that medical and geriatric wards still use mercurial diuretics which contain a comparatively high concentration of mercury and moreover some patients receive them frequently and over long periods. Mersalyl ($C_{13}H_{16}HgNNaO_6$) was a commonly used diuretic in the past and a dose of one ampoule contains 40 mg. mercury; the usual dose is two ampoules given by injection. Other mercury-containing diuretics are Meralluride, Chlormerodrin, Mercuramide and Mercaptomerin.

Mersalyl is rapidly excreted by the kidney (85% appearing in the urine within 24 hr.) and is not easily broken down to inorganic compounds. When it is administered to elderly patients with some degree of renal failure the effective level of mercury present is prolonged.

It is proposed that mercury resistance indicates a strain which, having been exposed to the mercury ion, has developed resistance to it. This correlates with the known facts. Mersalyl is used in medical and geriatric wards and most patients receiving it are elderly and repeatedly admitted to hospital. Geriatric patients have the highest proportion of mercury-resistant strains and even when the local endemic strain is disregarded there is still a preponderance of mercury-resistant strains. In obstetric and ear, nose and throat patients, where mercurials are seldom used, mercury-resistant strains are less common (Hall, 1970). In the animal world,

mercury resistance is almost unknown among strains of bovine and canine origin (Meyer, 1966).

It has been shown in this paper that Mersalyl was inhibitory to mercury-sensitive strains *in vitro*. Paper strips impregnated with this compound behaved in the same manner as strips impregnated with inorganic salts when tested against sensitive and resistant controls. Furthermore, tolerance to mercury was acquired after many passages in medium containing mercuric chloride. In addition, data from factory workers suffering chance industrial exposure to mercury compounds indicate that these people are more likely to harbour mercury-resistant strains than is the general population. Exposure to mercury compounds may thus induce the emergence of mercury-resistant strains just as occurs with antibiotics, but it is unlikely that cocci come into contact with doses of mercury salts comparable with levels achieved in antibiotic therapy. With mercury compounds the concentration of available free mercury to act on staphylococci is unknown since the degree of ionization may vary, and furthermore the presence of protein in solution decreases the bacteriostatic action due to uptake of free mercuric ion by protein. It is known that large doses of mercury or its compounds cause profuse salivation and that mercury is concentrated in saliva and there may well be some accumulation in nasal secretions in addition. There may be a preferential excretion onto the skin although information on this point was difficult to ascertain.

It would be interesting to know the mercury reaction of strains isolated before the antibiotic era, since Mersalyl had been in use well before this time. It was introduced in the 1920's and indeed mercurials have been used in medicine for centuries. Unfortunately there is no large collection of cultures from the pre-antibiotic days in this country.

Moore claimed that mercury-resistant strains were more versatile and this would account for their presence in hospitals. He suggested that proneness to antibiotic resistance could be regarded as a reflexion of this versatility. However in the experiments described here they were not shown to spread or to survive drying better than mercury-sensitive strains. Moreover if his claim was correct mercury-resistant strains should be biologically dominant in the general population owing to their greater versatility.

Jessen *et al.* (1963) are of the opinion that, despite the lack of correlation between mercury resistance and virulence in the sense of causing a high mortality rate, production of α-lysin, hyaluronidase and egg-yolk reaction, the mercury reaction may still reflect an important metabolic or structural property of the strain. However, none of the properties investigated here were found to correlate with the mercury reaction.

Although it is claimed that there is a high correlation between penicillinase production and mercury resistance they are not always linked so that mercury resistance cannot be satisfactorily explained in these terms. It is recognized that multiple-antibiotic resistance is correlated with high penicillinase production and mercury resistance (Richmond, Parker, Jevons & John, 1964), while strains resistant to penicillin alone, which are now common in civilised populations, both in and out of hospitals, produce less penicillinase. Nevertheless, in strains tested

during the course of this investigation it was not uncommon to find mercury-resistant strains producing small amounts of penicillinase and mercury-sensitive strains producing large amounts. Richmond & John (1964) carry their contention a step further to say that mercury resistance is a marker of a high penicillinase producer. They were able to demonstrate co-transduction of these two properties, suggesting that the genes controlling penicillin synthesis and mercury resistance are often on the same plasmid. They do not claim an obligate connexion between the penicillinase genes and those responsible for mercury resistance; this would be the case if the mercury genes were actually involved in the synthesis or excretion of the penicillinase molecule. Paired strains were not tested for penicillinase production in this investigation because at the time co-transduction of penicillinase and mercury resistance had not been established. The alternative argument favoured here is that strains that have been circulating in hospitals for a time will be high penicillinase producers since these are the ones that will have been further selected by the environment. These are the strains, therefore, that are associated with hospitals, and mercury resistance may only be incidental.

Mercury resistance of staphylococci was discovered as an accident of investigation but it may not be a new phenomenon. Mercury resistance due to the use of mercury-containing compounds has probably ante-dated the phenomenon of antibiotic resistance although this cannot be stated categorically. If this be true, then in future years when mercurial diuretics are finally superseded, and a situation analogous to the withdrawal of antibiotics follows, mercury-sensitive strains should come to the fore and in time the association between antibiotic resistance, high penicillinase production and mercury resistance will no longer exist.

I would like to express my gratitude and appreciation to Dr E. Joan Stokes for her encouragement, guidance and support, and to Professor G. Belyavin for helpful criticism.

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REFERENCES

AKINLADE, N. D. (1962). A trial of mercuric chloride for the rapid identification of epidemic strains of *Staphylococcus pyogenes*. *West African Medical Journal* **11**, 35.

DUTHIE, E. S. (1955). The action of fibrinogen on certain pathogenic cocci. *Journal of General Microbiology* **13**, 383.

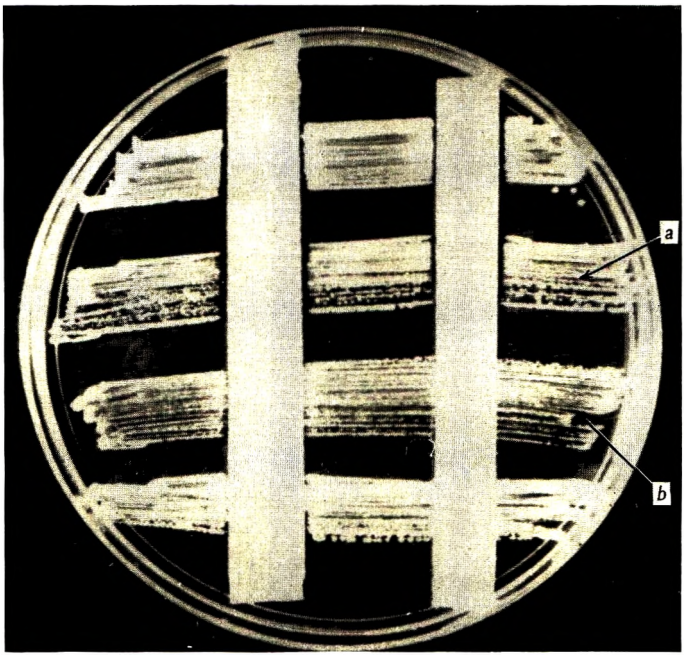
GOULD, J. C. (1958). Environmental penicillin and penicillin-resistant *Staphylococcus aureus*. *Lancet* i, 489.

HALL, B. M. (1966). A study of antibiotic-resistant *Staphylococcus pyogenes* in a hospital community with particular reference to Moore's mercury sensitivity test as a guide to strains of epidemic importance. Ph.D. Thesis, University of London.

HALL, B. M. (1970). Distribution of mercury resistance among *Staphylococcus aureus* isolated from a hospital community. *Journal of Hygiene* **68**, 111.

JESSEN, O., ROSENDAL, K., FABER, V., HOVE, K. & ERIKSON, K. R. (1963). Some properties of Staphylococcus aureus possibly related to pathogenicity. *Acta Pathologica et Microbiologica Scandinavica* **58**, 85.

LI, I. W., MUDD, S. & KAPRAL, F. A. (1963). Dissociation of phagocytosis and intracellular killing of *Staphylococcus aureus* by human blood leukocytes. *Journal of Immunology* **90**, 804.



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(*Facing p.* 129)

MEYER, W. (1966). Ergebnisse der Sublimatresistenzbestimmung bei Staphylococcus-aureus-Stämmen humaner und animaler Herkunft. *Zeitschrift für die gesamte Hygiene und ihre Grenzgebiete* **12**, 974.

MOORE, B. (1960). A new screen test and selective medium for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet* ii, 453.

RICHMOND, M. H. & JOHN, M. (1964). Co-transduction by a staphylococcal phage of the genes responsible for penicillinase synthesis and resistance to mercury salts. *Nature* **202**, 1360.

RICHMOND, M. H., PARKER, M. T., JEVONS, M. P. & JOHN, M. (1964). High penicillinase production correlated with multiple antibiotic resistance in *Staphylococcus aureus*. *Lancet* i, 293.

STOKES, E. J., HALL, B. M., RICHARDS, J. D. M. & RILEY, D. J. (1965). Control of hospital staphylococci. *Lancet* ii, 197.

TURNER, G. C. & WILLIS, A. T. (1962). Staphylococcal invasion of a new surgical ward. *Journal of Pathology and Bacteriology* **84**, 349.

EXPLANATION OF PLATE

A nutrient agar culture plate showing a mercuric chloride strip (left) and a Mersalyl strip (right). A naturally occurring mercury-sensitive strain (top) and mercury-resistant strain (bottom) are included and a strain is shown before (*a*) and after (*b*) it has developed tolerance to mercuric chloride *in vitro*.

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An investigation of the inhibitory properties of sodium thioglycollate in media for the recovery of clostridial spores

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SUMMARY

The effect of various concentrations of sodium thioglycollate (0–0·05 %) on the recovery of spores of four strains of clostridia was investigated. The results showed that thioglycollate at a concentration of 0·01 % can be inhibitory to some clostridia. However, the results also indicated that the inhibitory effect is dependent upon the composition of the growth medium. The presence of glucose appeared to be particularly important in reducing the inhibitory effect. These findings support the view that the use of sodium thioglycollate in sterility test media should be discontinued.

INTRODUCTION

Mossel & Beerens (1968) drew attention to the continued use of media containing sodium thioglycollate for the recovery of clostridial spores in the testing of the sterility of surgical apparatus, despite the demonstration by Hirsch & Grinsted (1954) and by Galesloot (1961) that under some circumstances sodium thioglycollate may inhibit the germination of the spores of some clostridia.

Mossel & Beerens (1968) studied the recovery of 14 strains of clostridia in five different media, including media containing sodium thioglycollate, and interpreted the reduced recovery in the presence of sodium thioglycollate as confirmation of the inhibitory effect of this compound on the germination of some clostridial spores. Their results do not, however, unequivocally support their conclusions. The recovery in thioglycollate agar USP (U.S. Pharmacopoeia, 1965) was not greatly different from the recovery in this medium when sodium thioglycollate was omitted. Recovery in Difco thioglycollate agar (Difco Manual, 1953) was better than that obtained in USP-thioglycollate agar and was further improved by the addition of glucose to this medium, although recovery was not as good as in a fifth medium of different basic composition which contained cysteine to reduce the redox potential. The effect of omitting sodium thioglycollate from the Difco medium or of adding it to the fifth medium was not investigated. Thus the results obtained by Mossel & Beerens (1968) supported the interpretation that the recovery of clostridia was dependent upon the nutritional state of the medium at least as well as the interpretation that the presence of sodium thioglycollate inhibited the recovery of clostridial spores.

In addition to their well established use in the sterility testing of surgical apparatus and pharmaceutical preparations (Pittman, 1946; Sykes, 1956; Report, 1960), media containing sodium thioglycollate are still used for the sterility testing of canned foods and for counting clostridial spores in foods (Sharf, 1966; Thatcher & Clark, 1968; Hersom & Hulland, 1969). In view of the relevance to the examination of foods of the comments of Mossel & Beerens (1968) in relation to surgical apparatus, and the nature of these workers' results, the possible adverse effect of sodium thioglycollate on the recovery of clostridia from spores was investigated further. This investigation was undertaken by determining the extent of recovery of several species of clostridia in several media in the presence of various concentrations of sodium thioglycollate. In addition, recourse was made to the results of a large series of sterility tests of canned meats in which liver broth and USP-thioglycollate medium were used in parallel.

MATERIALS AND METHODS

The clostridia examined were from the culture collection of the laboratories of the British Food Manufacturing Industries Research Association, Leatherhead, England, and consisted of one strain each of *Clostridium welchii*, *Cl. histolyticum*, *Cl. tertium* and *Cl. paraputrefaciens*.

Spore-bearing cultures of these organisms were obtained by culturing them in reinforced clostridial medium (RCM, Oxoid Manual, 1967) for 48 hr. at 37° C. and then storing the cultures at room temperature for 6 weeks. The cells were separated from the medium by centrifugation, washed three times in distilled water, and finally resuspended in distilled water. Immediately before use the suspensions were heated at 80° C. for 10 min. to kill vegetative organisms as in the investigation of Mossel & Beerens (1968).

The following media were used with varying concentrations of sodium thioglycollate up to 0·05 %.

Medium A. Reinforced clostridial agar (Oxoid).

Medium B. USP-thioglycollate agar with the omission of thioglycollate (U.S. Pharmacopoeia, 1965).

Medium C. Soya–peptone agar; Phytone, Baltimore Biological laboratories, 15 g./l.; yeast extract, 5 g./l.; cysteine-HCl, 0·5 g./l.; disodium phosphate, 2·5 g./l.; sodium chloride, 2·5 g./l.; agar 15 g./l.; distilled water to 1 l.: pH 7·2 ± 0·1. This medium was recommended for glycolytic clostridia (Mossel *et al.* 1965), in which category *Cl. welchii* comes, and was used as a reference medium by Mossel & Beerens (1968).

The recovery of the various strains of clostridia on these media was determined in duplicate using a surface-plate drop-count technique similar to that of Miles & Misra (1938) but with anaerobic incubation of the plates for 3 days at 37° C. after which the colonies were counted. This procedure has been shown to be satisfactory for colony counts of several species of clostridia (R. Spencer, unpublished results).

In the sterility testing of canned meats, a sample of meat was taken with aseptic precautions from a can and broken up in quarter strength Ringer solution con-

taining 0·1 % peptone and 0·1 % yeast extract. Duplicate portions of the suspension were inoculated into tubes of both liver broth and USP-thioglycollate broth. One tube of each medium was incubated at 55° C. and one tube of each medium at 37° C. After 6 days incubation the cultures were examined for growth by aerobic and anaerobic subculture.

RESULTS

Table 1 shows the extent to which the four clostridia were recovered on the three media at various concentrations of sodium thioglycollate.

Three of the clostridial cultures were inhibited by 0·01 % sodium thioglycollate in the soya-peptone medium; the fourth culture did not grow in this medium. It

Table 1. *The effect of increasing concentrations of sodium thioglycollate in different media on the recovery (as log_{10} counts) of four species of clostridia*

(The standard deviation of the differences between replicate log counts is 0·02, and the maximum deviation at the 95 % probability level is ± 0·04. RCM = reinforced clostridial medium; USP-thio = USP-thioglycollate; soya-pep. agar = soya-peptone agar.)

		Recovery counts (log_{10}) in media containing sodium thioglycollate (%)			
	Medium	0	0·01	0·03	0·05
Cl. welchii	RCM	6·06	6·24	6·25	6·17
	USP-thio.	4·96	4·98	5·05	4·97
	soya-pep. agar	5·12	< 2·44	< 2·44	< 2·44
Cl. paraputrefaciens	RCM	6·80	6·75	6·32	6·78
	USP-thio.	5·37	5·48	5·95	6·18
	soya-pep. agar	5·01	4·90	4·93	4·99
Cl. histolyticum	RCM	5·91	5·75	5·83	5·74
	USP-thio.	5·07	< 2·44	< 2·44	< 2·44
	soya-pep. agar	< 2·44	< 2·44	< 2·44	< 2·44
Cl. tertium	RCM	5·04	5·05	5·06	5·14
	USP-thio.	5·46	5·51	5·51	5·56
	soya-pep. agar	3·47	< 2·44	< 2·44	< 2·44

Table 2. *Influence of medium on isolation of clostridia from canned meat*

Number of samples positive in:		
Liver broth	USP-thioglycollate	Both liver broth and USP-thioglycollate
3	7	0

$\chi^2 = 1{\cdot}6$; d.f. = 1; $P > 0{\cdot}05$

should be noted that this medium was developed for use with glycolytic clostridia and that the culture which did not grow on it even in the absence of sodium thioglycollate, *Cl. histolyticum*, is a proteolytic clostridium. Mossel *et al.* (1965) showed that *Cl. histolyticum* is particularly sensitive to the composition of the medium used to recover it from spores.

One culture, *Cl. histolyticum*, was inhibited by 0·01 % sodium thioglycollate in the USP-thioglycollate agar while the recovery of the culture of *Cl. paraputrefaciens* was apparently increased by increasing concentrations of sodium thioglycollate in USP-thioglycollate agar.

No culture was inhibited by 0·05 % sodium thioglycollate in RCM.

Table 2 shows the extent to which clostridia were recovered from canned meats cultured in liver broth and in USP-thioglycollate medium. No attempt was made to identify the clostridia isolated. The recoveries in the two media were not significantly different ($P > 0·05$) and thus no evidence was forthcoming that the thioglycollate medium was inferior to liver broth in the recovery of clostridia from canned meats.

DISCUSSION

It seems that Clark (1943) first commented on the possible toxicity to thermophilic anaerobes of sodium thioglycollate, a substance recommended by Brewer (1940) for reducing the redox potential in anaerobic media. Hirsch & Grinsted (1954) observed that mesophilic clostridia were sensitive to sodium thioglycollate when it was incorporated in a medium at a concentration of 0·1 %, but not when it was present in the medium at 0·01 %. The effect of sodium thioglycollate at 0·03–0·05 %, as in USP-thioglycollate medium, was not determined. The results of Mossel & Beerens (1968), as pointed out above, whilst certainly providing evidence of inadequacies in the USP-thioglycollate medium so far as the recovery of clostridia from spores is concerned, do not demonstrate that sodium thioglycollate at a concentration of 0·03–0·05 % is inhibitory to clostridia.

The results of the present investigation demonstrate clearly that sodium thioglycollate at as low a concentration as 0·01 % can be inhibitory to some clostridia. Equally clearly the inhibitory properties of sodium thioglycollate depend very much upon the medium and were not apparent in RCM. Sodium thioglycollate at a concentration of 0·03–0·05 % in a nutritionally rich medium, and in particular in one containing glucose, may well be a satisfactory substance for reducing the redox potential of media for clostridia.

In contrast to these findings, there was no evidence that USP-thioglycollate medium was inhibitory when used in the sterility testing of canned meats. It is, however, known that thioglycollate can be neutralized by meat particles (Clark, 1943) which may account for the results obtained.

Although this investigation has been restricted to only four strains of clostridia, the results obtained substantiate previous reports that thioglycollate can exert an inhibitory effect against some clostridia. On the basis of these findings, and those of Mossel & Beerens (1968), USP-thioglycollate medium would appear to be inadequate as a sterility test medium.

The data on the recovery of clostridial spores from canned meats were provided by Brooke Bond Liebig Research Centre to whom we are obliged for permission to include these data in this report.

Much of the experimental work was carried out by Mr S. A. H. Razvi.

REFERENCES

BREWER, J. H. (1940). Clear liquid mediums for the 'aerobic' cultivation of anaerobes. *Journal of the American Medical Association* **115**, 598.

CLARK, F. M. (1943). Observations on growth of thermophilic anaerobes in Brewer's Medium. *Food Research* **8**, 327.

Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Chemical Laboratory Procedures (1953). 9th ed., p. 195. Detroit: Michigan.

GALESLOOT, TH. E. (1961). De anaerobe sporevormers die bedorf von smeltkass kunnen veroorzoken. *Nederlandsch Melk-en Zuiveltijdschrift* **15**, 263.

HERSOM, A. C. & HULLAND, E. C. (1969). *Canned foods: an introduction to their microbiology* (*Baumgartner*). 6th ed. London: Churchill.

HIRSCH, A. & GRINSTED, E. (1954). Methods for the growth and enumeration of anaerobic sporeformers from cheese, with observations on the effect of nisin. *Journal of Dairy Research* **21**, 101.

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

MOSSEL, D. A. A. & BEERENS, H. (1968). Studies on the inhibitory properties of sodium thioglycollate on the germination of wet spores of clostridia. *Journal of Hygiene* **66**, 269.

MOSSEL, D. A. A., BEERENS, H., TAHON-CASTEL, M., BARON, G., POLSPOEL, B. (1965). Etude des milieux utilizés pour le dénombrement des spores des bactéries anaérobies en microbiologie alimentaire. *Annales de l'Institut Pasteur de Lille* **16**, 147.

Oxoid Manual (1967). 3rd ed. London: Oxoid, Ltd.

Pharmacopoeia of the United States of America (1965). XVIII revision, p. 829.

PITTMAN, M. (1946). A study of fluid thioglycollate medium for the sterility test. *Journal of Bacteriology* **51**, 19.

SHARF, J. M. (1966). *Recommended methods for the microbiological examination of foods.* 2nd ed., p. 147. New York: American Public Health Assoc., Inc.

SYKES, G. (1956). The technique of sterility testing. *Journal of Pharmacy and Pharmacology* **8**, 573.

THATCHER, F. S. & CLARK, D. S. (1968). *Micro-organisms in foods, part III*, p. 169. Toronto: University of Toronto Press.

Report (1960). General requirements for the sterility of biological substances. *World Health Organization Technical Report Series no.* 200.

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Myxomatosis. The effect of age upon survival of wild and domestic rabbits (*Oryctolagus cuniculus*) with a degree of genetic resistance and unselected domestic rabbits infected with myxoma virus

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SUMMARY

The response of wild and domestic rabbits with a degree of genetic resistance to myxomatosis has been shown to be markedly affected by the age at which they were infected with a virulent strain of the virus. The response, in terms of mean survival time and percentage survival, fell with increasing age from 10 to 30 weeks with little change thereafter.

INTRODUCTION

Tests of the virulence of strains of myxoma virus and estimations of genetic resistance in populations of wild rabbits and domestic rabbits selected for resistance (Sobey, 1969) have been difficult to standardize owing to a large variation in survival time and case mortality, apparently caused by relatively minor environmental differences. One such source of variation is the age at which rabbits are infected. Fenner (1949) has shown that in mice infected with ectromelia virus, suckling mice and mice about a year old displayed a much higher mortality than young 8-week-old adults. Fenner also mentioned that there are many examples in human medicine where the age at which infection occurs has a marked bearing on the course of a disease. Studies on the effect of age upon the response of non-immune unselected domestic rabbits to infection with myxoma virus are reviewed by Fenner & Ratcliffe (1965). A limited range of ages was covered in these studies, 1–8 weeks and 17 weeks, over which period there was an increase in survival time and recovery rate.

In the present study observations were made on domestic rabbits selected for resistance to myxoma virus, unselected domestic rabbits and wild rabbits with a degree of genetic resistance; they were infected with a virulent myxoma virus, over the age range 10–50 weeks. Two groups of wild rabbits falling into two distinct age groups from other localities were also infected and observed.

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MATERIALS AND METHODS

Viruses

Three highly virulent strains and one moderately attenuated strain of myxoma virus cloned in our laboratory were used. Standard laboratory strain (SS) Strain no. 1, Glenfield (GV) Strain no. 5, Lausanne (Lu) Strain no. 7 and KM 13 Strain 30: strain numbers are according to Fenner & Marshall (1957).

Rabbits

(1) Unselected domestic rabbits were obtained from a randomly bred stock maintained by C.S.I.R.O. Division of Animal Genetics; this stock has never been exposed to myxomatosis. (2) Selected domestic rabbits: rabbits selected for resistance to myxomatosis (Sobey, 1969) with a mean grade of 6·5, equivalent to about six generations of selection, were used. (3) Wild rabbits from Lake Urana in New South Wales where populations have been subject to severe annual outbreaks of myxomatosis since 1951 (Marshall & Douglas, 1961). The rabbits used from this location were bred in Canberra by C.S.I.R.O. Division of Wildlife Research. Adult wild rabbits were caught, ear marked and screened for circulating antibodies to the soluble antigens of myxoma virus using the technique described by Sobey, Conolly & Adams (1966). Six does with and six does without circulating antibodies and four bucks without circulating antibodies were selected as parents. The does were housed in individual field enclosures and the bucks caged. Does were mated when receptive during the breeding season June to November 1968. Kittens were removed from the enclosures at 4 weeks of age, ear marked, flown to Sydney and caged. Eighty-two rabbits from four litter drops were used. (4) Wild rabbits from two localities, Natimuk and Seaspray in Victoria: Seaspray has been subject to annual epizootics since 1951 and Natimuk has failed to have an annual epizootic only during severe drought periods. Rabbits were captured in the field and screened for circulating antibodies as above, only those without antibodies to myxoma soluble antigens being kept for testing. These rabbits fell into two clearly defined age groups, subadult (10–16 weeks) and adult (greater than 25 weeks).

Testing conditions

At appropriate ages for testing the domestic rabbits and the wild rabbits originating from Urana were moved into a room carefully maintained at $75 \pm 2°$ F. Where possible sibs were tested over a range of ages. A dose of 3–10 lesion-forming units (L.F.U.) of SS virus was administered intradermally in the ear base. A large number of ampoules of virus were frozen at $-60°$ C. at the beginning of the experiment and repeated titrations during the course of the work revealed no change in the titre.

The wild rabbits from Natimuk and Seaspray were caged in a room maintained at $70 \pm 3°$ F. A number of animals from each age group from each locality was infected with either of three strains of virulent virus, SS, GV or Lu. Infection was made via the eye with virus dried on to an abrasive powder (D.E.P.), described by Sobey, Conolly & Adams (1967).

All rabbits were given a score equivalent to the number of days they survived after infection. Those rabbits which survived beyond 30 days were classed as recoveries and given a score of 30.

A pelleted food ration and water were supplied *ad. lib.*

RESULTS

Unselected domestic rabbits

The responses of animals infected with SS between the ages of 10 and 50 weeks, recorded separately for sex, are shown in Table 1. The mean survival times ($\bar{x}$ S.T.S) for both sexes are shown in Fig. 1A. There is no evidence of any difference in

Table 1. *The survival times, in days, of unselected domestic rabbits*

(Rabbits were inoculated intradermally in the ear base with 3–10 L.F.U. of SS strain of myxoma virus at ages between 10 and 50 weeks.)

Age tested in weeks	Males	Females	No.	$\bar{x}$ S.T.	%S
10	10, 11, N.R., N.R., N.R.*	11, 12, 13, N.R.			
11	10, 12, 14	11, 12			
12	11, 13, 14, 15	12, 12, 13			
13	16	13	19	12·4	—
14	11, 13	11, 12, 12			
15	—	—			
16	11, 13	11, 12, 12	10	11·8	—
17	—	—			
18	10, 11, 11, 11, 13, 13, 14	11, 11, 12, 12, 13, 13	13	11·9	—
19	—	—			
20	12, 12, 12	12, 13			
21	11	12, 12	8	12·0	—
22	11, 12, 12, 16, S†, N.R.	10, 11, 12, 12, 12, 12, 13, 16, S			
23	—	—	14	15·0	14
24	11, 12, N.R.	11, 12			
25	—	—	4	11·5	—
26	12, 13, N.R.	12, 13, 13			
27	—	—			
28	—	—			
30	10, 11, 12, 14, 15	11, 12, 13, 16, N.R.	14	12·7	—
31–40	14, 14, N.R.	11, 12, 12			
41–50	11, 12, 12	10, 13	10	12·1	—
Total			92	12·6	2

* N.R.: no reaction at site of injection, animal failed to contract myxomatosis.
† S: animal survived beyond 30 days after infection.

response between males and females. There is little evidence of an age effect except at about 22–23 weeks of age where two animals survived. The overall $\bar{x}$ S.T. of 12·6 days was higher than the 10·8 days found by Fenner & Marshall (1957) for SS, although the dose of virus administered was similar. Several minor differences in the method of testing may bear on this discrepancy in $\bar{x}$ S.T.; Fenner & Marshall inoculated in the shaved flank and held their rabbits at 70 ± 2° F., whereas

in the above work rabbits were inoculated in the ear base and held at 75 ± 2° F. Further, their unselected domestic rabbits and ours were from different stocks and may therefore have had some slight genetic divergence. In cloning the virus in our laboratory it is possible we may have emerged with a slightly different strain.

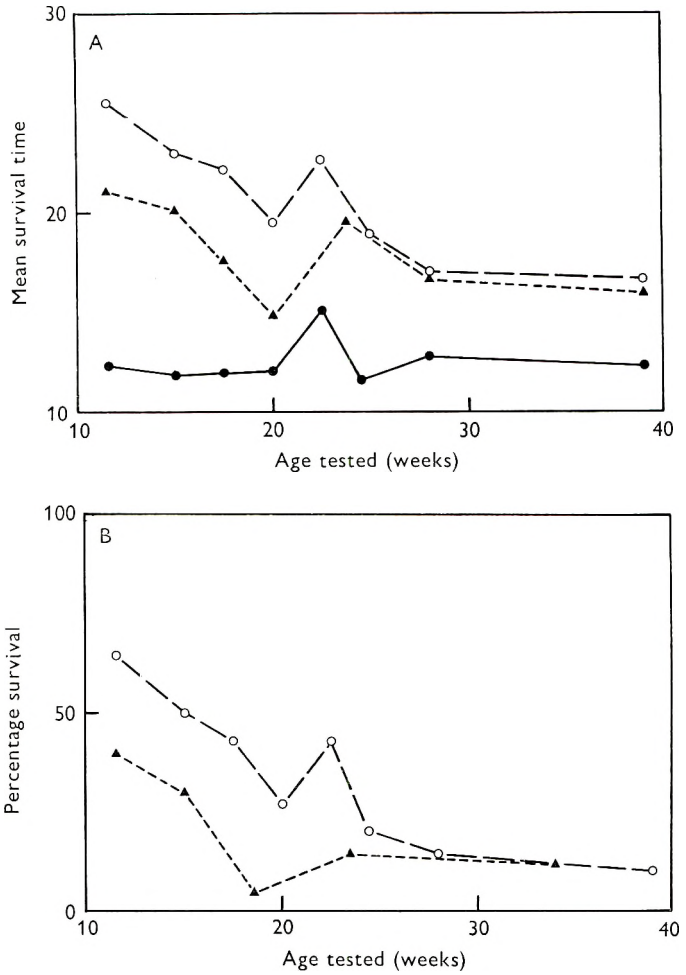

Fig. 1 (A). The mean survival times of groups of unselected and selected domestic rabbits and wild rabbits from Urana tested at different ages between 10 and 42 weeks. (B). The percentage survival of groups of selected domestic rabbits and wild rabbits from Urana tested at different ages between 10 and 42 weeks. ○——○, selected domestic; ▲- - -▲, wild Urana; ●— —●, unselected domestic.

Domestic rabbits selected for resistance to myxomatosis

The responses of animals infected with SS between the ages of 10 and 50 weeks whose dams had not been exposed to myxomatosis are shown in Table 2; and the responses of rabbits whose dams had survived the disease are shown in Table 3. The survival times of these two groups are plotted against age at testing in Fig. 2. When compared over the whole age range from 10 to 50 weeks at testing there is

no significant difference between the two groups in survival time. With respect to percentage survival however, there is a significant difference between the two groups in the age range 15 and 20 weeks ($\chi^2_{(1)}$ 4·5 $P < 0{\cdot}05$) suggesting that the fall in percentage surivival, with age at testing, was more rapid in animals whose dams

Table 2. *The survival times in days of domestic rabbits selected for resistance to myxomatosis for about six generations born to does which were not challenged with virus*

(Rabbits were inoculated intradermally in the ear base with 3–10 L.F.U. of SS strain of myxoma virus at ages between 10 and 50 weeks.)

Age tested in weeks	Male	Female	No.	$\bar{x}$ S.T.	%S
10	15, 18, S, S†	14, 21, S			
11	S	12, S, S			
12	14, S, S, S	S, S			
13	—	—	17	25	65
14	12, S, S, S	17, S, S, S, N.R.			
15	—	—			
16	11, 18, 25, S	12, 18, 22, S, S, S, S	19	24·5	58
17	14, 14, 17, S	14, S, S, S			
18	14, 14, 16, S, S, S, S	12, 13, 17, 21, 25, S, S, S, S	24	23·0	50
19	10, 12, 13	15, 17, S			
20	16, 29, S, S, S	14, 16, 18, 21			
21	13, 14, 14, S, S	16, S	22	20·4	32
22	10, 15, 16, 16, 17, 17, S, S, S	17, 20, 20, 24, S, S, N.R.			
23	14, 16, S, N.R., N.R.*	15, 19, 21, S, S	23	21·6	35
24	13, 16, 19, N.R.	13, 14, 20, S, S, S			
25	13, 13, 20, 28	15, 15, 20, S	17	20·0	24
26	19, 24	14, 16			
27	—	16			
28	12	11, 13			
30	13, 13, 16	S, N.R.	12	16·4	8
31–40	11, 14	12, 14			
41–50	13	N.R.	5	12·8	—
Total			139	19·2	39

* N.R.: no reaction at site of injection, animal failed to contract myxomatosis.
† S: Animal survived beyond 30 days after infection.

had recovered from myxomatosis than in the animals whose dams had not been exposed. The $\bar{x}$ S.T. and percentage survival for the combined data from both types of dam are plotted against time in Fig. 1 A, B respectively. Clearly there was a fall in the $\bar{x}$ S.T. and percentage survival over the whole age range from 10 to 40 weeks. Except for the elevated response at 22 to 23 weeks, which does not test statistically significant, the fall appears to be linear from 10 to about 30 weeks, after which it tends to plateau although the number of animals tested over the later age range was not large. It is interesting that the elevated resistance at 22–23 weeks corresponded to that found in the unselected domestic rabbits.

Unselected domestic rabbits seldom survive longer than 15 days after infection with SS. The percentage of animals which die before 15 days is inversely proportional to the percentage which survive infection, therefore, either estimate can be used as an index of the resistance of a group or class of animals to the disease. This is illustrated in Fig. 3 where an age effect in terms of percentage survival is mirrored by the percentage of animals which died before 15 days.

Table 3. *The survival times in days of domestic rabbits selected for resistance to myxomatosis for about six generations born to does which had recovered from the disease*

(Rabbits were inoculated intradermally in the ear base with 3–10 L.F.U. of SS strain of myxoma virus at ages between 10 and 50 weeks.)

Age tested in weeks	Male	Female	No.	$\bar{x}$ S.T.	%S
10	S†, N.R.*	S			
11	18, 26	13			
12	S, S	S			
13	—	—	8	27·1	63
14	13, 14, 20	13, 14, 17			
15	—	—			
16	14, 15, S, S	S, S, S, N.R.	13	20·8	39
17	S, S	22			
18	13, 14, 14, 16, 24	15	9	19·8	22
19	13, 16	S			
20	15, 16	14			
21	14, 15	14, 14, S	11	17·4	18
22	14, 15, S, N.R.	S, S			
23	23, S, S, N.R.	19, S	10	25·1	60
24	16, 17	14			
25	11, N.R.	—	4	14·5	—
26	—	12, 20			
27	—	—			
28	11, 13, 18, S	15, 16			
30	S	12	10	17·7	20
31–40	16, S	—			
41–50	—	17, 22	4	21·3	25
Total			69	20·4	33

* N.R.: no reaction at site of injection, animal failed to contract myxomatosis.
† S: animal survived beyond 30 days after infection.

Wild rabbits from Urana

The responses of the 82 animals infected with SS between the ages of 10 and 43 weeks are shown in Table 4 and plotted in Fig. 1 A, B. There was a fall in survival time between 10 and 20 weeks similar to that found in the selected domestic rabbits. Only two animals were tested in the 22–23 weeks age group but the elevated resistance between 22 and 25 weeks corresponded approximately with that found for both the selected and unselected domestic rabbits. No male rabbits older than 16 weeks survived the disease. When the data were grouped into two

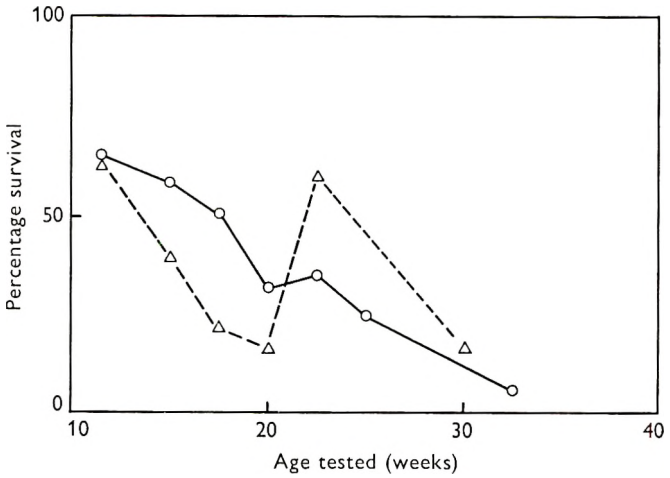

Fig. 2. The percentage survival of groups of rabbits born to does which had no contact with (unchallenged) and does which had recovered from (recovery) myxomatosis, tested at different ages between 10 and 42 weeks. ○——○, offspring from unchallenged does; △ - - - △, offspring from recovered does.

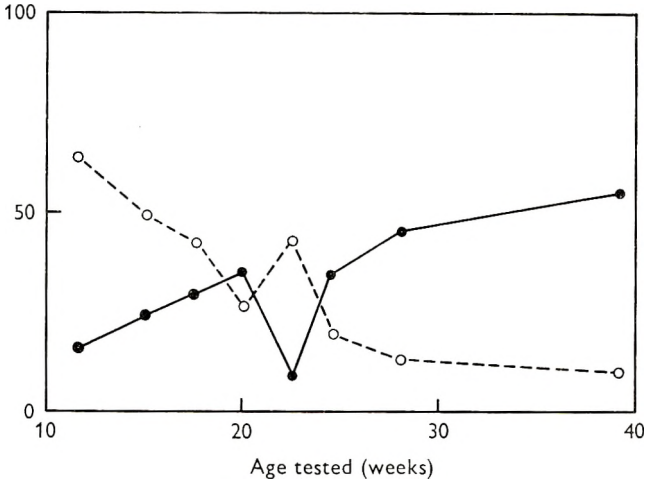

Fig. 3. The effect of age on the resistance of selected domestic rabbits as measured by the percentage which died before 15 days. ○- - -○, Percentage survival; ●——●, percentage surviving < 15 days.

age groups 10–19, and 20–42 weeks, as shown in Table 5, a partition of chi-square (Claringbold, 1961) showed a significant interaction between survival and age, there being more survivors in the 10–19 week group than in the 20–42 week age group, $\chi^2_{(1)} = 5{\cdot}01$ ($P < 0{\cdot}05$). The interaction of these factors with sex was highly significant $\chi^2_{(1)} = 7{\cdot}06$ ($P < 0{\cdot}01$) with a high male and low female survival in the 10–19 week age group and a low male and higher female survival in the 20–42 week age group. The resistance of animals from dams which had not been exposed to myxomatosis was not significantly different from that for animals whose dams had recovered from the disease although, as with the selected domestic rabbits, the

resistance of the latter was consistently lower than that of the former. Thus there is no suggestion that maternal antibodies confer any protective effect beyond 10 weeks of age to rabbits infected with SS virus.

Table 4. *The survival times in days of Urana wild rabbits born in enclosures to does which had recovered from myxomatosis and does which had never been infected*

(Rabbits were inoculated intradermally in the ear base with 3–10 L.F.U. of SS strain of myxoma virus at ages between 10 and 43 weeks.)

Age tested in weeks	Offspring from unchallenged does		Offspring from recovered does		No.	$\bar{x}$ S.T.	%S
	Male	Female	Male	Female			
10	S†	13, S	14, 14, 17	14, 16, 17			
11	—	—	—	—			
12	15, S, S	17	S, S, S	15, 16, N.R.			
13	—	—	—	—	18	21·0	39
14	15, 15, S	17, 17, 21	14, 16, S, S	15			
15	—	—	—	—			
16	S, S, N.R.*	12, N.R.	15, 20, S	13, 16, 16	20	20·1	30
17	—	—	—	—			
18	15, 16	14, S	13, 17, 21	12, 14, 25	10	17·7	
19	—	—	—	—			5
20	14, 20	15, 15	12, 16	17			
21	—	—	12, 14	13	10	14·8	
22	—	—	21	18			
23	—	—	—	—	2	19·5	14
24	—	—	—	20, S, N.R.			
25	11	—	13	23	5	19·4	
26	—	S	11	S			
27	13	13	—	—			
28	—	—	—	—			
30	—	15	13	11, 13	9	16·6	12
31–40	13	—	13, 13	14, 15, 16			
41–50	—	—	—	20, 22	8	15·8	
Total					82	18·5	21

* N.R. no reaction at site of injection, animal failed to contract myxomatosis.
† S: animal survived beyond 30 days after infection.

Table 5. *The percentage survival and mean survival times of male and female wild rabbits from Urana in the two age groups* 10–19 *and* 20–42 *weeks*

	Age in weeks					
	10–19			20–42		
Sex	No.	$\bar{x}$ S.T.	%S	No.	$\bar{x}$ S.T.	%S
Male	27	22·2	45	15	13·9	—
Female	21	17·2	9	19	18·5	16
Male and Female	48	20·0	29	34	16·5	9

%S: percentage survival. $\bar{x}$ S.T: Mean survival time in days.

Animals which showed no reaction at the site of injection and failed to contract myxomatosis (N.R.)

The three groups of animals described above, unselected domestic, selected domestic and wild rabbits from Urana were all tested under the same conditions and inoculated with the same stored batch of virus. A dose of ± 5 L.F.U. was selected to give the minimum dose that would ensure that of the order of 99 % of the inoculated animals would become infected (i.e. 1 % N.R.). The observed frequencies of % N.R. in the unselected domestic, selected domestic and wild Urana rabbits were 10, 6 and 5 % respectively, with no statistically significant difference between them. The mean % N.R. over the three groups was 6 % and although not significantly outside the expected range for the estimated 5 L.F.U. used, was higher

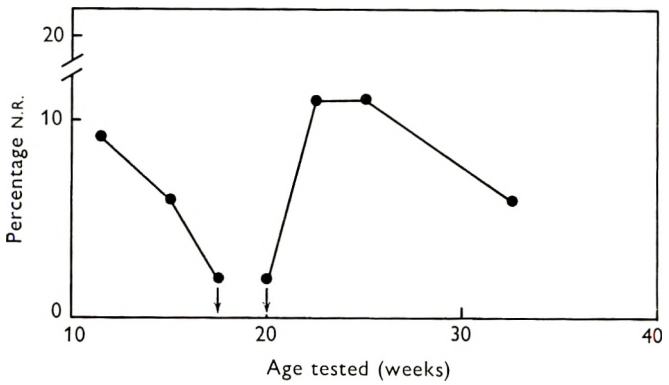

Fig. 4. The distribution, as a percentage, of rabbits which failed to show a reaction at the site of injection or to contract myxomatosis from the unselected and selected domestic rabbits and the wild rabbits from Urana tested at different ages between 10 and 42 weeks.

than expected. The distribution of N.R. over the age range 10–50 weeks was not random and is plotted for different age groups in Fig. 4. There appears to be a correlation between the % N.R. and $\bar{x}$S.T. over the age groups studied, with a fall from 10 weeks to a trough at 18–20 weeks, a sharp rise during 22–25 weeks and a subsequent falling off with increased age of testing.

Selected domestic rabbits infected with KM 13

During the early stages selection was based on animals which survived infection with attenuated viruses of field origin (Sobey, 1969). Most animals were inoculated with virus between 16 and 20 weeks of age, but because of batch testing a number of animals were tested outside of these age limits. A summary of the percentage survival of animals after two generations of selection, in different age groups, infected with KM 13 is given in Table 6. There are two interesting aspects to these data; the elevated response at 22–23 weeks and the absence of any fall-off in response between 14 and 21 weeks of age.

Wild rabbits from Natimuk and Seaspray

The data for the subadult and adult groups of rabbits from these two localities are shown in Table 7. With all three strains of virus used the subadult rabbits had a higher $\bar{x}$S.T. than the adults. The immune state of the parents of these animals was unknown. However, it seems unlikely that maternal antibodies were respon-

Table 6. *The percentage survival of domestic rabbits selected for resistance to myxomatosis for about two generations*

(Rabbits were infected intradermally in the ear base with *ca.* 500 L.F.U. KM13 strain of myxoma virus at ages between 14 and 28 weeks.)

Age tested in weeks	No.	%S*
14–17	293	10
18–21	328	11
22–23	137	28
24–28	45	13

* Animal survived beyond 60 days after infection.

Table 7. *The mean survival times of wild rabbits from Natimuk and Seaspray and unselected domestic rabbits infected with three virulent strains of virus GV, SS and Lu*

	Temperature during test	Method of infection	GV (strain 5)				SS (Strain 1)				LU (Strain 7)			
			Subadult		Adult		Subadult		Adult		Subadult		Adult	
Source of rabbits	(°F.)		No.	$\bar{x}$S.T.	No.	$\bar{x}$S.T.	No.	$\bar{x}$S.T.	No.	$\bar{x}$S.T.	No.	$\bar{x}$S.T.	No.	$\bar{x}$S.T.
Wild rabbits from Natimuk	70 ± 3	D.E.P.	6	11·0	14	9·8	10	14·0	15	11·1	5	13·6	13	11·7
Wild rabbits from Seaspray	70 ± 3	D.E.P.	6	11·0	16	9·8	9	11·4	16	10·3	3	15·4	7	12·9
Unselected domestic	70 ± 2	5 I.D.*	—	—	5	10·2	—	—	43	10·8	—	—	5	12·9
Unselected domestic	72 ± 3	D.E.P.	—	—	21	10·2	—	—	—	—	—	—	—	—

* Five infection doses (I.D.) given intradermally in the shaved flank (data from Fenner & Marshall, 1957). D.E.P., dried eye powder virus inoculated via the eye.

sible for the higher S.T.s of the subadult groups in view of the results given above where dams which had recovered from the disease did not confer any advantage on offspring 10 weeks or older; the most likely cause for the subadults having a higher $\bar{x}$S.T. than the adults appears to be the difference in age. It is interesting that the $\bar{x}$S.T.s of the adult groups were very similar to those found by Fenner & Marshall (1957) for unselected domestic rabbits for all three virus strains. Further the $\bar{x}$S.T. of unselected rabbits infected with eye powder virus was the same as that for rabbits infected intradermally with a low dose of virus where GV was used, suggesting that the method of infection was not responsible for lowering the $\bar{x}$S.T. of the wild rabbits.

DISCUSSION

In wild and domestic rabbits with a degree of genetic resistance, the age at which they were infected with the virulent SS strain of myxoma virus was found to influence the course of the disease. There was a fall in $\bar{x}$S.T. and percentage survival at between 10 and 30 weeks of age, after which age there appeared to be little change. Although not significant in any one group of rabbits there was a consistent elevation in survival at about 22–23 weeks of age. The percentage of animals which failed to become infected after inoculation also fell with age at infection with a rise in number at about 22–25 weeks of age. The domestic rabbits with only two generations of selection for resistance tested with KM13 did not show a fall in resistance over the age range 14–28 weeks, but they did show a rise in terms of percentage survival at 22–23 weeks.

When the data from the wild rabbits from Seaspray and Natimuk were collected it was difficult to explain why the $\bar{x}$S.T. of the subadult group was higher than that of the adult group. An advantage conferred by maternal antibodies could not be ruled out in view of the findings of Fenner & Marshall (1954) that the offspring from immune dams still had an advantage over the offspring from non-immune dams at 7–8 weeks of age when infected with SS. Further, the animals in these localities had been subjected to repeated epizootics over the years and selection for extended protection by maternal antibodies could be not ruled out. The findings described above show no advantage being conferred on the subadult group by maternal antibodies and suggest very strongly their extended $\bar{x}$S.T. was a result of their age *per se*.

The wild rabbits from Urana and the domestic rabbits with about six generations of selection for resistance were, on the average, very similar in their response over the whole range of ages tested. They differed, however, with regard to the responses of the two sexes. In the selected domestic rabbits there was no suggestion of a sex difference, whereas in the wild Urana rabbits males between the ages of 10 and 19 weeks had a better chance of survival than females of the same age, and the reverse was found with animals tested older than 20 weeks. If this interaction is indeed real then aberrant sex-ratios might be expected in the field depending on the age structure of the susceptible rabbits at the time of a myxomatosis epizootic.

The genetic resistance acquired by some wild rabbits since the introduction of myxoma virus into Australia in 1950 is shown by the high survival rate of the rabbits from Urana to SS; when first released standard strain had a case mortality of the order of 99·8% (Fenner, 1959). Similar findings have been reported by Marshall & Fenner (1958) and Marshall & Douglas (1961) for rabbits from this locality. The rabbits from Seaspray and Natimuk in Victoria showed an elevated $\bar{x}$S.T. in the subadult groups compared with unselected domestic rabbits; the numbers tested were too small to give an idea of the case mortality. Comparing wild rabbits with an unselected 'domestic baseline' is open to question. Vaughan & Vaughan (1968) found that wild rabbits from Skokholm Island with no history of exposure to myxomatosis had a higher 'baseline' in terms of $\bar{x}$S.T. and percentage survival than domestic New Zealand White rabbits. Wild rabbits without a history

of exposure to myxomatosis are no longer available on the Australian continent and the only 'wild baseline' available is that given by Fenner & Marshall (1957) who showed wild rabbits to have only a slightly larger $\bar{x}$ S.T. where viruses of high, moderate and low virulence were used. The parents of the wild rabbits used by Fenner & Marshall may have been exposed to some selection and it is quite possible that original Australian wild rabbits were very little different from unselected domestic rabbits in their response to myxomatosis.

The wild rabbits at Urana have been exposed to annual epizootics of myxomatosis since 1951 (K. Myers, personal communication). It is interesting that the degree of resistance achieved during this period appears lower than that achieved in domestic rabbits in six generation equivalents of selection. There is no *a priori* reason for believing that the heritability of resistance is different in wild and domestic rabbits, and the slower rate of genetic gain in the wild rabbit can be attributed to a lower selection differential. Selection in the wild rabbits has been based almost entirely on attenuated strains of virus whereas the selected domestic rabbits were exposed to three generations of selection based on the virulent SS virus. Myxomatosis epizootics occur at Urana in the late spring or early summer with elevated temperatures which would contribute to a greater survival expectancy than the caged domestic rabbits with a degree of temperature control (Marshall, 1959). Further, non-infected wild rabbits in any one year would contribute to the breeding in the following year, reducing the selection differential as compared with the selected domestic rabbits where the grading system (Sobey, 1969) compensated for any non-infected dams and where only recovered sires were used. The consequences of the immune status of the sire have not yet been determined and in this regard it should be noted that the wild rabbits from Urana in the above study were all sired by bucks which had never been infected, whereas all the selected domestic rabbits had sires which had recovered from myxomatosis.

Before the epidemiological consequences of the age effect can be evaluated it will be necessary to show that it occurs where more attenuated virus strains are used, since infection in the field at present is largely by strains of Grade III virulence (Fenner & Chapple, 1965). However, since selection in wild rabbits with attenuated strains of virus and in domestic rabbits with more virulent strains have both resulted in a similar age effect to SS it seems likely that this age effect will be expressed for virus strains other than SS.

We are indebted to Mr Clive Hale who bred the wild rabbits for earlier unpublished work which led to the present investigations.

REFERENCES

CLARINGBOLD, P. J. (1961). The use of orthogonal polynomials in the partition of Chi-square. *Australian Journal of Statistics* **3**, 48.

FENNER, F. (1949). Studies in mousepox (infectious ectromelia of mice). VII. The effect of the age of the host upon the response to infection. *Australian Journal of Experimental Biology and Medical Science* **27**, 45.

FENNER, F. (1959). Myxomatosis. *British Medical Bulletin* **15**, 240.

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. & MARSHALL, I. D. (1954). Passive immunity in myxomatosis of the European rabbit (Oryctolagus cuniculus); the protection conferred on kittens born by immune does. *Journal of Hygiene* **52**, 321.

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

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

MARSHALL, I. D. & DOUGLAS, G. W. (1961). Studies in the epidemiology of infectious myxomatosis of rabbits. VIII. Further observations on changes in the innate resistance of Australian wild rabbits exposed to myxomatosis. *Journal of Hygiene* **59**, 117.

MARSHALL, I. D. & FENNER, F. (1958). Studies in the epidemiology of infectious myxomatosis of rabbits. V. Changes in the innate resistance of Australian wild rabbits exposed to myxomatosis. *Journal of Hygiene* **56**, 288.

SOBEY, W. R. (1969). Selection for resistance to myxomatosis in domestic rabbits (Oryctolagus cuniculus). *Journal of Hygiene* **67**, 743.

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.

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

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The effects of UK 2054 on the multiplication of influenza viruses

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SUMMARY

The isoquinoline compound UK 2054 prevents the uptake of influenza virus by susceptible cells. Pre-incubation of virus particles with 500 μg./ml. UK 2054 at 37° C. for 2 hr. does not reduce virus infectivity. Host cells vary in their responsiveness to the inhibitory effect of UK 2054; virus multiplication is inhibited in chick allantoic cells by lower concentrations than those required to inhibit virus growth in chick embryo fibroblasts. The effectiveness of UK 2054 is reduced by the presence of serum.

It is concluded that inhibition of influenza virus multiplication by UK2054 might result from interaction of the inhibitor with both virus and cells. Any direct combination between inhibitor and virus is completely reversible.

INTRODUCTION

The compound 1-phenoxymethyl-3,4-dihydroisoquinoline (Pfizer compound UK 2054) is a weak, non-competitive inhibitor of bacterial and viral neuraminidases (Brammer, McDonald & Tute, 1968). This feature of the compound led to an investigation of its ability to inhibit virus multiplication in simple cell culture systems. It was found to have a spectrum of antiviral activity which includes those viruses which possess neuraminidase, namely, the influenza and parainfluenza viruses responsible for a large proportion of common respiratory infections. It is also effective however against many other viruses, such as rubella and respiratory syncytial viruses, which do not possess neuraminidase.

Toxicological studies indicate that UK 2054 and related isoquinolines are safe for administration to man (Brammer *et al.* 1968). It has been found that the closely related compound UK 2371 (1-(4-methoxyphenoxymethyl)-3,4-dihydroisoquinoline) is active against influenza type B infection in man (Beare, Bynoe & Tyrrell, 1968).

The mechanism by which isoquinolines inhibit virus multiplication is not understood. In the case of influenza viruses, these compounds are most effective when pre-incubated with virus before infection, and it has been concluded that they act by direct inactivation of the virus particle (Brammer *et al.*, 1968; Hobson, Flockton, & Gregory, 1969). In this paper some experiments are reported which suggest that direct inactivation of influenza virus by UK 2054 is reversible and may not be the only means by which the compound prevents infection.

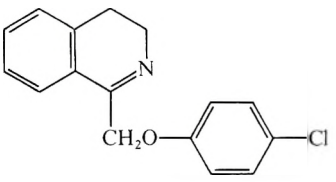
MATERIALS AND METHODS

Virus strains

Three strains of influenza virus were used in this study: influenza A/PR8/34, A2/Singapore/1/57 and fowl plague virus. These are egg-adapted strains which have been maintained in this laboratory for many years by serial egg passage. Virus-infected allantoic fluid was stored in glass ampoules at $-70°$ C. and thawed rapidly before use.

Anti-viral agents

UK2054 was prepared by the Chemical Research Division of the Pfizer Group, Sandwich, Kent, and was sent to us through the courtesy of Dr D. Jackson. The chemical structure of UK2054 is as follows:



Stock solutions were prepared in distilled water at a concentration of 1 mg./ml. and stored at 4° C. Actinomycin D was a gift from Merck, Sharp and Dohme.

Experimental system

Two cell systems were used to test the effect of UK2054 on influenza virus growth:

(1) 1 sq. cm. pieces of surviving allantois-on-shell, taken from 11-day-old fertile hens' eggs, were incubated in 0·7 ml. volumes of medium 199 dispensed into large Perspex trays (Fulton & Armitage, 1951). Full details of the quantitative aspects of this system have been described (Barry, 1961).

(2) Monolayer cultures of chick embryo fibroblasts were prepared from 11-day-old eggs and grown in 5 cm. plastic Petri dishes. The cultures were grown in medium 199 supplemented with 10% calf serum and incubated in an atmosphere of 5% CO_2. Cells were infected by adding 0·02 ml. volumes of the appropriate dilution of virus.

Virus titration

Unless otherwise stated, haemagglutination titrations were carried out 18 hr. after infection, according to the method of Fazekas de St Groth & Graham (1954). In all figures, except Fig. 1, haemagglutination (HA) titres are expressed as the $\log_2$ value per 0·25 ml. Virus infectivity was titrated by the method of Fazekas de St Groth & White (1958).

RESULTS

Degree of inhibition

Various doses of UK2054 ranging from 0 to 28 μg./ml. were added to egg pieces. Each piece was then infected with one of three strains of influenza virus at

an estimated multiplicity of 3 ID50/cell. The production of all strains was inhibited to a similar degree by increasing concentrations of UK 2054 (Fig. 1). The production of fowl plague virus and the PR 8 strain were not completely inhibited and in other experiments we frequently found that inhibition of the Asian strain was not complete. This effect is different from that found with inhibitors of DNA function such as actinomycin D, where the degree of inhibition varies directly with the dose of inhibitor and where virus production can be completely inhibited (Barry, Ives & Cruickshank, 1962).

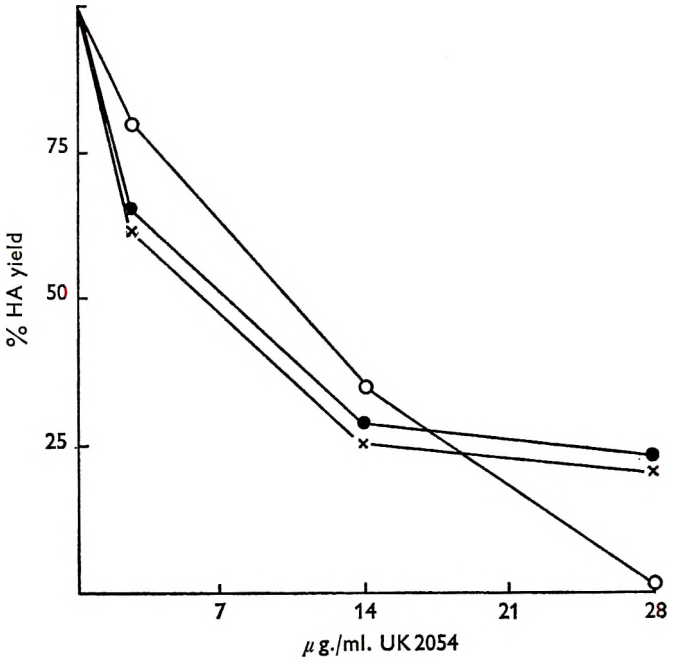


Fig. 1. Inhibition of influenza virus growth by UK 2054. UK 2054 present in culture medium throughout the experiment. ○, A2/Singapore/1/57 influenza virus; ●, fowl plague virus; ×, A/PR 8/34 influenza virus.

Nature of inhibition

To test whether UK 2054 has an all-or-none effect on virus production, the following experiment was carried out. One-step growth experiments, using the A 2/Singapore/1/57 strain at a multiplicity of 3 ID 50/cell, were performed in egg pieces in the presence or absence of 5 and 20 μg./ml. UK 2054. The results are shown in Fig. 2. In untreated egg pieces newly formed virus appears 5 to 6 hr. after infection and is released rapidly up to about 12 hr. after infection. In egg pieces treated with 5 μg./ml. UK 2054 the eclipse phase of the virus is 2 to 4 hr. longer and the rate of virus release is slower. Although there is no reduction in the 24 hr. yield, at 12 hr. after infection the inhibitor-treated cells have produced less than half the yield from control cells. The effect of 20 μg/ml. UK 2054 is to lengthen the eclipse phase and delay the release of virus still further.

This result is quite different from that obtained with actinomycin D, where the rate of virus release is normal but the final yield of virus reduced (Fig. 3). Thus,

UK 2054 does not have an all-or-none effect on virus production but delays the appearance of newly formed virus and its rate of release. Since this effect is similar to that seen when the multiplicity of virus infection is reduced, it seems likely that the inhibitor is reducing the effective multiplicity of infection by preventing the uptake of virus by host cells. If this is so, then the inhibitor should be effective only during the early stages of the virus growth cycle.

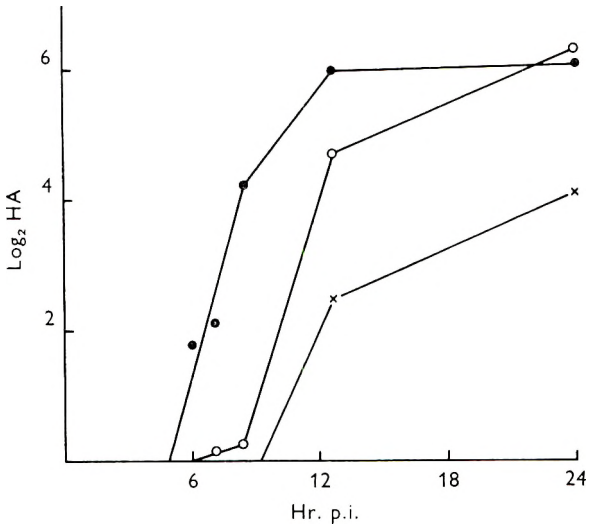


Fig. 2. The effect of UK 2054 on one-step growth curve of A 2/Singapore/1/57 influenza virus. ●, 0 μg./ml. UK 2054; ○, 5 μg./ml. UK 2054; ×, 20 μg./ml. UK 2054.

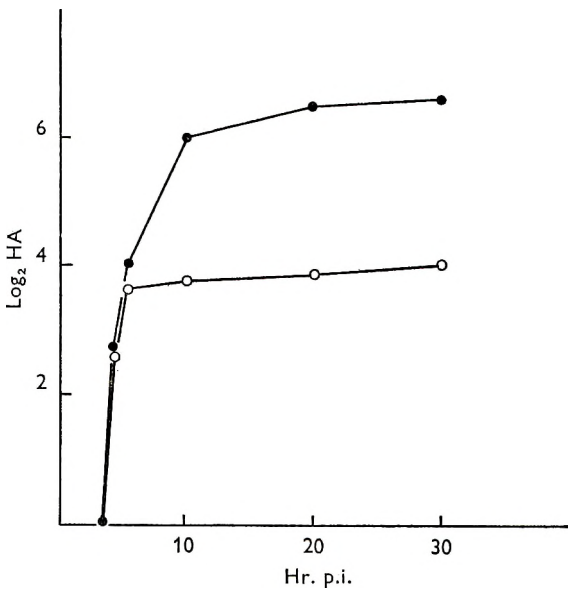


Fig. 3. The effect of actinomycin D on the one-step growth curve of A 2/Singapore/1/57 influenza virus. ●, 0 μg./ml. actinomycin D; ○, 5 μg./ml. actinomycin D.

Egg pieces were therefore infected with the A 2/Singapore/1/57 strain and treated with 20 μg./ml. UK 2054 either before or shortly after virus infection. The inhibitor was left on the egg pieces for periods of 30 min. and then removed by washing. Fig. 4 shows that the inhibitor is most effective when added before virus infection and that its effectiveness decreases as the eclipse phase proceeds. There is no inhibition of virus yield when UK 2054 is added later than 2 hr. after infection. This result is compatible with the idea that UK 2054 prevents virus uptake.

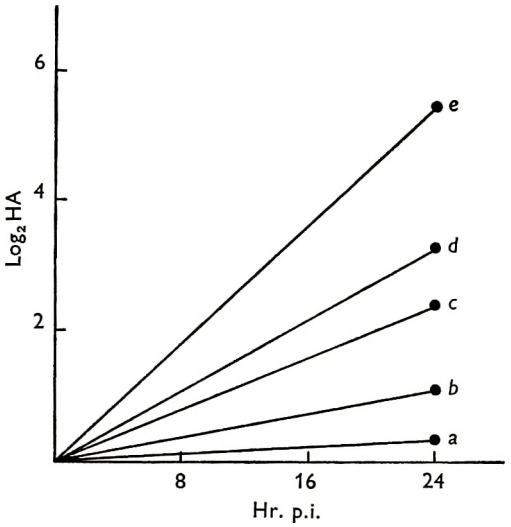


Fig. 4. The effect of UK 2054, at various times before and after virus infection, on the yield of A 2/Singapore/1/57 influenza virus from allantoic pieces. 20 μg./ml. UK 2054 were present from: (*a*) 30 min. before infection to time of infection; (*b*) 0 to 30 min. after infection; (*c*) 30 min. to 1 hr. after infection; (*d*) 1–2 hr. after infection; (*e*) No UK 2054.

TABLE 1. *The effect of UK 2054 (500 μg./ml.) on the infectivity of influenza A 2/Singapore/1/57*

Treatment	Titre (log_{10})/ml.
Virus + saline	7·85
*Virus, treated with and diluted in UK 2054	5·00
Virus, treated with UK 2054, diluted in saline	7·70

* Dilutions were prepared in 0·9 % saline containing 100 μg./ml. UK 2054.

Mechanism of inhibition

The mechanism involved in inhibition could be direct inactivation of the virus particle (Brammer *et al.* 1968), competition for cell surface receptors or interference with an early intracellular event.

Interaction of UK 2054 with influenza virus particles

The A 2/Singapore/1/57 strain was incubated for 2 hr. at 37° C. with 500 μg./ml.

UK 2054 and the resulting virus infectivity was measured in allantoic cells by finding the highest dilution of virus capable of infecting 50 % of the egg pieces. Serial tenfold dilutions of virus were made either in saline or in saline containing 100 μg./ml. UK 2054. The results are presented in Table 1. When dilutions are made in saline containing UK 2054, the infectivity of isoquinoline-treated virus is reduced one thousandfold. However, when dilutions are made in saline alone, the infectivity titres of treated and untreated virus preparations are indistinguishable. The fact that the inhibitory effect of UK 2054 is absent after dilution suggests that any interaction which may occur between virus and inhibitor is unstable.

Table 2. *The effect of UK 2054 on the production of fowl plague virus in chick embryo fibroblasts (C.E.F.) and chick allantoic cells (C.A.).*

	Host cell	
Inhibitor μg/ml.	C.E.F.	C.A.
0	7·2*	5·7
10	7·5	4·0
20	n.t.	3·2
40	7·2	n.t.

* Yields of virus are expressed as the haemagglutination titre ($\log_2$/ml.) determined 24 hr. after infection.

n.t. = not tested.

The inhibitory effect of UK 2054 in different host cells

If isoquinolines neutralize influenza viruses in much the same way as antibody (Hobson *et al.* 1969), then the degree of inhibition should be independent of the host cell. We therefore tested the effect of various concentrations of UK2054 on the production of fowl plague virus from two cell types. The results are presented in Table 2. UK 2054 at 10 μg./ml. inhibits the production of fowl plague in allantoic cells by about 65 %, whereas doses as high as 40 μg./ml. do not affect the yield of fowl plague from chick embryo fibroblasts. This difference in response suggests that inhibition is not simply due to the irreversible inactivation of virus particles by UK 2054. The chick embryo fibroblasts used in this experiment were infected in medium 199 in the absence of serum.

The effect of serum on inhibition

UK 2054 is bound readily by serum proteins (J. D. Coombes, personal communication). The presence of serum in any culture used to assay the effectiveness of anti-viral activity is therefore likely to complicate the results. An experiment demonstrating the effect of serum on inhibition by UK 2054 of fowl plague multiplication in chick fibroblasts is shown in Fig. 5. It is clear that doses of UK 2054 up to 40 μg./ml. have little or no effect on the yield of fowl plague virus from chick fibroblasts, whether or not serum is present. At higher doses, inhibition of virus growth is achieved only in the absence of serum.

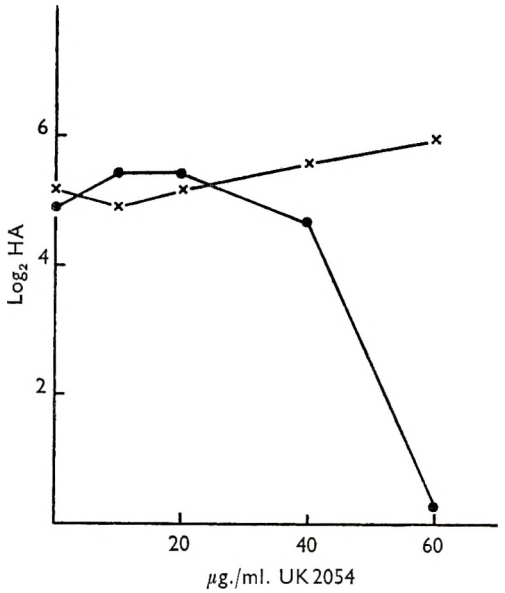


Fig. 5. The effect of serum on inhibition of fowl plague virus by UK 2054 in chick embryo fibroblasts. ×, 10% calf serum; ●, no serum.

DISCUSSION

It is claimed that isoquinoline inhibits the growth of neuraminidase-containing viruses by direct inactivation of the virus particle (Brammer *et al.* 1968; Hobson *et al.* 1969). Prolonged pre-incubation of virus–isoquinoline mixtures produces a greater degree of inhibition than brief pre-incubation, when the mixtures are tested subsequently for their ability to infect cells. The results presented above (Table 1) indicate that virus subjected to prolonged incubation with UK 2054 retains complete infectivity when diluted in saline which does not contain the compound. Consequently, any direct inactivation of virus by the inhibitor (mediated presumably by combination of inhibitor and virus neuraminidase) may be analogous to inactivation of virus by antibody, in that it is reversible when diluted.

The presence of inhibitor in the culture medium prevents virus uptake. However, host cells may vary in their sensitivity to similar mixtures of inhibitor and virus (Table 2). The simplest, but by no means the only, explanation of this phenomenon is that adsorption of inhibitor to the host cell can play a part in preventing virus adsorption. Thus, relatively low doses of UK 2054 may combine with allantoic cells, but not chick embryo fibroblasts, in such a way as to alter the response of the cells to the virus. Combination of inhibitor with host cell rather than with the virus itself could account for the effectiveness of isoquinolines against those viruses which do not contain neuraminidase. In support of the idea that UK 2054 exerts its effect on influenza viruses by combination with cells, as well as with virus, is the fact that concentrations of isoquinoline derivatives greater than 100 μg./ml. agglutinate chick red cells in a manner indistinguishable from that produced by

influenza viruses (unpublished observations). This suggests that isoquinolines may compete for the same cell receptors as influenza viruses.

The effects of UK 2054 on the multiplication of influenza viruses differ from those of amantadine hydrochloride. The effectiveness of amantadine is not dependent on the host cell system used to propagate the virus, and it appears to prevent some step in the replication cycle later than adsorption (Hoffman, Neumayer, Haff & Goldsby, 1965; Neumayer, Haff & Hoffman, 1965). The ability of serum to neutralize the effects of UK 2054 may prove to be a serious drawback to the therapeutic usefulness of isoquinoline derivatives.

REFERENCES

BARRY, R. D. (1961). The multiplication of influenza virus. I. The formation of incomplete virus. *Virology* **14**, 389.

BARRY, R. D., IVES, D. R. & CRUICKSHANK, J. G. (1962). Participation of deoxyribonucleic acid in the multiplication of influenza virus. *Nature, London* **194**, 1139.

BEARE, A. S., BYNOE, M. L. & TYRRELL, D. A. J. (1968). Prophylaxis of influenza with a synthetic isoquinoline. *Lancet* i, 843.

BRAMMER, K. W., MCDONALD, C. R. & TUTE, M. S. (1968). Antiviral properties of 1-Phenoxymethyl-3,4-Dihydro and 1,2,3,4-Tetrahydroisoquinolines. *Nature, London* **219**, 515.

FAZEKAS DE ST GROTH, S. & GRAHAM, D. M. (1954). The production of incomplete virus particles among influenza strains: experiments in eggs. *British Journal of Experimental Pathology* **35**, 60.

FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958). An improved assay for the infectivity of influenza viruses. *Journal of Hygiene* **56**, 151.

FULTON, F. & ARMITAGE, P. (1951). Surviving tissue suspensions for influenza virus titrations. *Journal of Hygiene* **49**, 247.

HOBSON, D., FLOCKTON, H. I. & GREGORY, M. G. (1969). The inhibitory activity of an isoquinoline derivative on growth of an influenza A virus in tissue culture. *British Journal of Experimental Pathology* **50**, 494.

HOFFMANN, C. E., NEUMAYER, E. M., HAFF, R. F. & GOLDSBY, R. A. (1965). Mode of action of the antiviral activity of amantadine in tissue culture. *Journal of Bacteriology* **90**, 623.

NEUMAYER, E. M., HAFF, R. T. & HOFFMAN, C. E. (1965). Antiviral activity of amantadine hydrochloride in tissue culture and in ovo. *Proceedings of the Society for Experimental Biology and Medicine* **119**, 393.

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