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Frequency of rubella antibodies among adult population in Greece

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(*Received* 23 *August* 1968)

INTRODUCTION

Rubella was considered of little clinical and epidemiological significance until 1941 when Gregg (1941) discovered the relationship between maternal rubella and congenital defects. The isolation of rubella virus and the application of laboratory techniques in the identification of the disease made possible the study of various aspects of the epidemic of 1964 (Rubella Symposium, 1965). These recent epidemiological studies have contributed to the recognition of rubella as a major Public Health problem and have resulted in a more realistic estimate of the risks of maternal rubella. Great interest in producing an effective vaccine against rubella followed the above discoveries and various groups are now actively working on it (Parkman, Meyer, Kirschtein & Hopps, 1966; Plotkin, Farquhar, Katz & Ingalls, 1967). In view of this fact the determination of the immune status of Greek adults on a national scale was undertaken, as an attempt to estimate the risk of contracting rubella during pregnancy. This would greatly simplify the initiation of any future prophylactic programme against rubella. The results of this study are reported in the present paper.

MATERIAL AND METHODS

It was shown in previous studies (Papaevangelou, 1967) that no significant difference in the prevalence of rubella antibodies existed among the various age groups of young adults at the reproductive period of their life. The immunity status of the age group 20–25 was determined in this study. A preliminary comparison of the sex incidence of immunity to rubella of this age group was undertaken. The sample consisted of 144 males and 120 females born and living in Greater Athens area and 50 males and 52 females from the rural Department of Corinthia. An attempt was made to match them for age, social class, and geographical location.

The prevalence of immunity to rubella was then studied in a representative sample of males 20–25 years old. This consisted of 1200 recruits born and living in every district of Greece. Each district was represented proportionally to its population.

Serum specimens were drawn under aseptic conditions and were kept frozen until tested. The immunity was determined by the detection of the neutralizing

rubella antibodies. The 'Judith' strain of the rubella virus and the technique described by Leehrøy (1966) with stationary instead of rolled tube cultures of the rabbit cornea cell line (S.I.R.C.)* were used. The highest dilution of serum that protected 50 % or more of the cultures from rubella virus cytopathic effect was taken as the neutralizing titre. The presence of neutralizing antibodies in a titre of 1/4 or higher was considered as indicating immunity to rubella.

RESULTS

Table 1 shows the results of the study on the sex incidence of immunity to rubella. No difference between the sexes was found in the Greater Athens area or in the Department of Corinthia.

Table 1. *Comparative study of the sex incidence of immunity to rubella in Greater Athens area and the Department of Corinthia*

	Greater Athens area		Department of Corinthia	
Sex	No. examined	No. with antibody titre < 1/4	No. examined	No. with antibody titre < 1/4
M	144	10 (6·9)	50	8 (16)
F	120	9 (7·5)	52	9 (17·3)
Total	264	19 (7·2)	104	17 (16·3)

Figures in parentheses indicate percentages.

Table 2. *Frequency of rubella antibodies among males 20–25 years old*

Place of birth	No. examined	No. with antibody titre < 1/4
Greater Athens	266	21 (7·9)
Rest of Central Greece and Euboea	139	32 (23·0)
Peloponnesos	158	16 (10·1)
Ionian Islands	30	8 (26·7)
Epirus	50	10 (20·0)
Thessaly	99	39 (39·4)
Thessaloniki	74	3 (4·1)
Rest of Macedonia	196	23 (11·7)
Thrace	51	16 (31·4)
Aegean Islands	68	18 (26·5)
Crete	69	15 (21·7)
Total	1200	201 (16·8)

Numbers in parentheses indicate percentages.

The immunity status of the representative sample of young adult males, aged 20–25, is shown in Table 2. In 201 (16·8 %) neutralizing antibodies were absent. The percentage of susceptible men among the various districts ranged from 4·1 % for Thessaloniki to 39·4 % for the district of Thessaly.

The existence of any difference in the proportion of susceptibles between rural

* The 'Judith' strain as well as the rabbit cornea cell line (S.I.R.C.) were kindly provided by Dr J. Leehrøy, Enterovirus Dept., Statens Serum Institut, Copenhagen, Denmark.

and urban areas was studied among 674 recruits, who had never moved from their place of birth. It was shown (Table 3) that the proportion of susceptibles was higher for the rural population (25·8 %) than for the urban population (14·0 %).

Table 3. *Comparative study of the prevalence of rubella antibodies between urban and rural populations*

	Urban		Rural	
Place of birth and permanent home	No. examined	No. with antibody titre < 1/4	No. examined	No. with antibody titre < 1/4
Greater Athens	144	10 (6·9)	—	—
Rest of Central Greece and Euboea	23	2 (8·7)	72	17 (23·6)
Peloponnesos	25	2 (8·0)	51	6 (11·8)
Ionian Islands	10	2 (20·0)	30	10 (33·3)
Epirus	18	4 (22·2)	8	0
Thessaly	30	10 (33·3)	19	11 (57·9)
Thessaloniki	32	1 (3·1)	—	—
Rest of Macedonia	12	3 (15·8)	28	4 (14·3)
Thrace	7	1 (14·3)	13	6 (46·1)
Aegean Islands	55	14 (25·5)	54	17 (31·5)
Crete	24	5 (20·8)	12	3 (25·0)
Total	387	54 (14·0)	287	74 (25·8)

Numbers in parentheses indicate percentages.

DISCUSSION

Rubella is generally accepted as a mild infectious disease of infancy. It is important, however, in view of the established teratogenic potential, that rubella is unique among the common infectious diseases in that a significant proportion of young adults has yet to experience infection. This is in contrast to measles and other communicable diseases of childhood from which few persons even in the remotest part of the country escape exposure. In the Naval Training Center of Great Lakes, Ill., U.S.A., rubella is the most common exanthematous disease with an attack rate amongst recruits in a 9-week training period of 3–5 %, which is much higher than measles (Miller *et al.* 1967). This difference is attributed to the lower infectiousness of the virus and to its other poorly understood, special characteristics, which produce epidemic waves at longer intervals. The disease is followed by life-long immunity even though second attacks have been described. Most authors believe, however, that such cases are attributed to wrong clinical or laboratory diagnosis rather than to true second attacks (Brody, 1966). In any case there is no doubt that the immunity lasts at least 20 years (Brody *et al.* 1965). It is obvious therefore that the frequency of antibodies among recruits provides an almost completely accurate picture of cumulative exposure of a population of these ages.

Serological surveys on a national scale, based on serum samples collected from recruits, have been conducted very widely recently. Such a sample is representative of the same age group in the general population; it is almost always available,

and is easy to work with because the individuals are collected together in one place. It was decided to use such a sample for the study of the frequency of antibodies among adults, after it was shown that there was no significant difference in the sex incidence of the immunity (Table 1). Both in Greater Athens area and the department of Corinthia the percentage of susceptible females was slightly higher, but the difference was without any statistical significance ($P > 0{\cdot}5$).

According to our results 83·2 % of Greek adults at the reproductive age were found immune to rubella. This is consistent with the findings of most workers (Schiff & Sever, 1966; Field, 1967; Rawls *et al.* 1967). Pierce (1967) found that 30 % of the recruits at the Naval Training Center, Great Lakes, Ill., were susceptible to rubella. A study involving 600 pregnant women, ages 14–44, from various States of the U.S.A. estimated the over-all percentage of susceptibles as 17·5 % (Sever, Schiff & Huebner, 1964). In the same study a great variation of the percentage of susceptibles was found in relation to geographic location. We found a

Table 4. *Correlation between the degree of immunity to rubella and the percentage of rural population in the sample examined in each district*

Place of birth	No. examined	Rural (%)	Antibody titre < 1/4 (%)
Greater Athens	266	0	7·9
Rest of Central Greece and Euboea	139	75·5	23·0
Peloponnesos	158	67·0	10·1
Ionian Islands	30	76·6	26·7
Epirus	50	32·0	16·0
Thessaly	99	39·4	39·4
Thessaloniki	74	0	4·1
Rest of Macedonia	196	55·1	11·7
Thrace	51	60·8	31·4
Ionian Islands	68	48·5	26·5
Crete	69	33·3	21·7
Total	1200	40·3	16·8

range from 4·1 % to 39·4 % for the various districts. The lower proportion was found in the conurbations of Thessaloniki (4·1 %) and Greater Athens (7·9 %). In recent years a number of surveys have shown that many factors determine the relative incidence of infectious diseases in different areas. The urban-to-rural proportion of the population is considered as an important factor in the development of the herd immunity. Such a correlation was obvious from our results (Table 4), even though this was not found statistically highly significant (Kendall's S Statistic, $P = 0{\cdot}11$). This was attributed to the residential mobility of the sample examined. It was found that 319 (26·6 %) out of the 1200 studied did not continue to live in their place of birth. Most of them (297 or 93 %) had moved to the conurbations of Athens and Thessaloniki.

The study of the susceptibility among those that had never moved from their place of birth (Table 3) showed a highly significant ($P < 0{\cdot}001$) difference between urban (25·8 %) and rural (14·0 %) population.

The factors responsible for the degree and the characteristics of immunity of a population to an infectious disease are not determined easily. A highly significant correlation between age and proportion with antibody to rubella has been established (Sever *et al.* 1964; Papaevangelou, 1967). In the present study the importance of the urban to rural proportion of the population of an area to its degree of immunity has been shown. It is general knowledge, however, that other factors are responsible too. Sever *et al.* (1964) reported a significant difference between Whites and Negroes. There are also indications (Papaevangelou, 1967) that the communications and easy access to conurbations, as well as the socio-economic conditions and the habits of various population groups, are important for the relative prevalence of immunity.

SUMMARY

No significant sex difference in the incidence of immunity to rubella at the ages of 20–25 was found in a comparative study of young adults from the Greater Athens area as well as from the rural department of Corinthia. The immunity to rubella of Greek adults on a national scale was then studied in a representative sample of 1200 males 20–25 years old. In 83·2 % of them neutralizing antibodies to rubella were detected. A statistically higher proportion of immunes was found among those from urban areas. The factors responsible for the relative prevalence of immunity to rubella in various population groups are discussed.

REFERENCES

Brody, J. A., Sever, J. L., McAlister, R., Schiff, G. M. & Cutting, R. (1965). Rubella epidemic on St Paul Island in the Pribilofs, 1963. Epidemiologic, clinical and serologic findings. *J. Am. med. Ass.* **191**, 619.

Brody, J. A. (1966). The infectiousness of rubella and the possibility of reinfection. *Am. J. publ. Hlth* **56**, 1082.

Field, A. M. (1967). The occurrence of neutralizing and complement fixing antibodies in rubella. *J. Hyg., Camb.* **65**, 409.

Gregg, N. M. (1941). Congenital cataract following German measles in the mother. *Trans. ophthal. Soc. Aust.* **3**, 35.

Leehrøy, J. (1966). Neutralization of rubella virus in a rabbit cornea cell line (SIRC). *Acta path. microbiol. scand* **67**, 158.

Miller, C. H., Dowd, J. M., Rytel, M. W., Miller, L. F. & Pierce, W. E. (1967). Prevention of rubella with γ-globulin. *J. Am. med. Ass.* **201**, 560.

Papaevangelou, G. J. (1967). The serologically detected immunity to rubella among Greek adults. *Aesculapious, Athens.*

Parkman, P. D., Meyer, H. M., Kirschstein, R. L. & Hopps, H. E. (1966). Attenuated rubella virus. *New Engl. J. Med.* **275**, 569.

Pierce, W. E. (1967). In Miller *et al.* (1967).

Plotkin, S. A., Farquhar, J., Katz, M. & Ingalls, T. H. (1967). A new attenuated rubella virus grown in human fibroblasts: evidence for reduced nasopharyngeal excretion. *Am. J. Epidem.* **86**, 468.

Rawls, W. E. *et al.* (1967). WHO Collaborative study on the seroepidemiology of rubella. *Bull. Wld Hlth Org.* **37**, 79.

Rubella Symposium (1965). *Am. J. Dis. Child.* **110**, no. 4.

Sever, J. L., Schiff, G. M. & Huebner, R. J. (1964). Frequency of rubella antibody among pregnant women and other human and animal populations. *Obstet. Gynec., N.Y.* **23**, 153.

Schiff, G. M. & Sever, J. L. (1966). Rubella: recent laboratory and clinical advances. *Prog. med. Virol.* **8**, 30.

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A selective motility medium for routine isolation of *Salmonella*

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(*Received* 26 *August* 1968)

Craigie (1931) described a technique for the selection of motile organisms from a poorly motile culture by passing them through semi-solid nutrient agar in a tube containing a narrower, open-ended tube so that, after inoculation on the agar surface in the inner tube, the motile organisms moved through twice the depth of the medium to reach the outer surface. Semi-solid agar media have been used by several workers for the isolation of salmonellas by selective motility. Jones & Handley (1945) used a semi-solid medium containing cacotheline as a selective medium after initial culture of suspected material in a broth containing hydroquinone. Ino & Graber (1955) used passage through semi-solid medium in a U tube to recover *Salmonella* from cultures contaminated with *Pseudomonas aeruginosa*. Stuart & Pivnick (1965) used a modification of Rappaport's medium in 0·6 % agar to isolate salmonella from faeces. They found Craigie tubes unsuitable and devised a modified U tube in which a small-bore side arm was attached to the base of a test-tube. This technique gave about one-fifth more positive cultures than did standard methods of isolation. The average time required for the spread of salmonellas other than *Salmonella typhi* through the medium, 5 cm. deep, was 1·5 days. Harvey, Mahabir & Price (1966) and Harvey & Price (1967) used passage through semi-solid nutrient agar as a method of secondary enrichment for salmonellas after culture in selenite F broth and on selective agar media. By this method, isolations from animal feeding stuffs were more than doubled.

Harper (1968) showed that salmonellas, which spread more quickly through semi-solid nutrient agar than do other faecal organisms, can be isolated from mixed cultures or from faeces by inoculation into coiled tubes, 80 cm. long, containing semi-solid nutrient agar (1·5 %). There are several disadvantages in the use of these tubes in routine work: care is required in filling the tubes to avoid introducing air bubbles; the tubes are fragile; they are space-consuming in an incubator; the passage of the organism takes 2 days.

Modifications of Shigella–Salmonella (SS) medium (Rose & Kolodny, 1942), in various depths, were tested to try to devise a medium which could be used in shorter tubes and so avoid these disadvantages. A successful medium should select the organism after passage through a much shorter length of tube; the passage of salmonella should be complete after overnight culture; the passage of other

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organisms should be retarded and salmonella should be obtained in pure culture for several days after inoculation.

MATERIALS AND METHODS

Craigie tubes

Media were prepared containing 0·2 % agar (Oxoid no. 3) and distributed in Craigie tubes consisting of $\frac{3}{4} \times 6$ in. test-tubes with a central open-ended tube, of 5 mm. internal diameter, projecting about 2 cm. above the surface of the medium. The upper end of the central tube was widened so that it could be inoculated easily with little risk of contamination of the outer surface. The central tube was inoculated and, when the culture was seen to have spread to the outer surface, subcultures from this were made on to MacConkey agar plates.

Media

Selenite F medium

This was prepared as described by Leifson (1936).

Shigella–Salmonella (SS) medium

For direct plating of specimens, this was prepared as described by Rose & Kolodny (1942), except that ferric ammonium citrate was substituted for ferric citrate, the former being more easily soluble.

For use in Craigie tubes certain modifications were made as a result of extensive trials. Twenty to fifty tubes of each batch of medium prepared were inoculated with normal faeces and with faeces contaminated with *Salmonella* by adding a drop of broth culture of *S. typhimurium* to 5 ml. of emulsified faeces, or with mixtures of *Escherichia coli* and *S. typhimurium*. Faeces known to contain salmonella were also used when available. Several strains of *E. coli* and two strains of *S. typhimurium* were used in these experiments.

Modifications of SS medium

Lactose. This was omitted to avoid bubble formation in the medium. Neutral red was therefore unnecessary, and was also omitted.

Brilliant green. An increase of the brilliant green content retarded the spread of all organisms.

The presence or absence of the normal quantity of brilliant green in the medium made no difference to the speed of spread of salmonella or of *E. coli* through the medium. Brilliant green was therefore omitted.

Bile salts. The presence of bile salts in the medium retarded the spread of both *E. coli* and salmonella but this effect was greater on *E. coli*. Doubling the normal quantity of bile salts considerably retarded the spread of *E. coli* while only slightly affecting that of salmonella. The inclusion of four times the normal amount of bile salts retarded the spread of salmonella. Three commercial brands of bile salts were used in parallel: Difco, Oxoid no. 3 and B.B.L. The results using Oxoid no. 3 bile salts were more satisfactory than those using the Difco product as salmonella was more reliably isolated from overnight cultures. Difco bile salts retarded the spread of other organisms more efficiently, but also tended to retard the spread of

salmonella. B.B.L. bile salts retarded the spread of all organisms, including salmonella, to such an extent that this organism did not spread in 1 day through medium 5 cm. deep. In the final medium, therefore, 17 g./l. (twice the normal quantity) of Oxoid no. 3 bile salts was used.

Depth of medium. The standard depth was 10 cm. This was reduced to 8 and 5 cm. to try to decrease the time of incubation necessary to isolate salmonella. In tubes of these lesser depths, pure cultures of *E. coli* or organisms from inoculated normal faeces reached the outer surface within 2 days. When salmonella was isolated from mixed cultures it did not usually remain in pure culture on the outer surface for more than 1 day and often *E. coli* was found within 1 day. In tubes 10 cm. deep, *E. coli* took at least 3 days to complete the passage and in mixed cultures often failed to do so in 7 days. Salmonella usually completed the passage in 1 day. In the final medium a depth of 10 cm. was used.

Storage of medium. When the medium was stored in the refrigerator for a few hours after its preparation, it appeared to be firm, but when it was inoculated on the same day, the inoculum could often be seen to sink down through the inner tube. In effect, therefore, the organisms had to spread through a shorter length of the medium to reach the outer surface and organisms other than salmonella completed the passage in a shorter time. When the tubes had been stored overnight in the refrigerator the inoculum remained on the surface of the inner tube. The medium was not therefore used until it had been stored for at least one night.

Final composition of SS medium for Craigie tubes

The final medium contained: beef extract (Lab Lemco paste) 5 g., proteose peptone (Oxoid) 5 g., sodium citrate 8·5 g., sodium thiosulphate 8·5 g., ferric ammonium citrate 1 g., bile salts (Oxoid No. 3) 17 g., agar (Oxoid No. 3) 2 g., distilled water to make 1 l. The instructions for its preparation were as follows: Dissolve all the ingredients, except agar, in about 500 ml. of distilled water. Cool and add sufficient distilled water to make 1 l. Adjust pH to 7·0. Add agar and bring to the boil, with constant stirring to prevent charring. Simmer with gas turned off for 1–2 min. Stir well. Dispense in Craigie tubes in 10 cm. depth. Insert wool plugs firmly. With the tubes in racks, cover the tops with a metal tray, to minimize wetting of the plugs. Autoclave at 10 lb. pressure for 10 min. Allow autoclave to cool slowly to prevent bubbling and soiling of the plugs. Store in the refrigerator for at least one night.

Appearance of cultures

The spread of a culture of salmonella through these tubes usually causes blackening of the medium, due to H_2S production. The edge of the culture can be seen advancing 3–4 cm. ahead of the blackening. Cultures of other organisms which produce blackening of the medium usually spread more slowly and there is often little or no space between the edge of the spreading culture and the blackened part of the medium. Most faecal organisms spread through the medium without causing blackening, or blackening only the upper part of the inner tube.

Occasionally salmonella strains fail to cause blackening in this medium although they produce H_2S in Kligler's medium (Kligler, 1918).

Some strains and serotypes do not produce H_2S.

RESULTS

This medium has been used in the routine examination of faeces and rectal specimens in this general hospital laboratory for about 3 years.

Rectal specimens were obtained by means of a hollow glass tube 12–13 cm. long, with an external diameter of about 4 mm., closed and rounded at one end, with an opening about 2 mm. in diameter, 2 cm. from the closed end. The rounded end is inserted into the rectum, withdrawn and placed in a test-tube containing about 2 ml. of nutrient broth. The specimen is sent as soon as possible to the laboratory, and the broth in the test-tube used as the inoculum for culture.

Specimens were examined by three methods. (1) Direct inoculation on SS plates; subculture of two pale colonies, if present, on Kligler's medium; subculture into urea medium if Kligler results suggest possible *Salmonella*; subculture for biochemical reactions and slide agglutinations if urea negative. (2) Inoculation into selenite-F medium; subculture on SS plate; procedure as above. (3) Inoculation of a few drops of emulsified faeces or of broth from a rectal specimen into the inner tube of the selective motility medium; subculture from the outer surface on MacConkey's medium; examination of pale colonies for biochemical reactions and slide agglutination. If the passage is not quite completed in 1 day, further overnight incubation can be avoided by withdrawing a drop from the advancing edge of the culture with a long Pasteur pipette.

Salmonella, other than *S. typhi*, was isolated from 300 specimens which had been fully examined by all these three methods. The specimens were obtained from 117 patients, five of whom had double infections with two salmonella serotypes. The specimens were mostly faeces and rectal specimens, but also included three pus swabs and two urines. The 122 organisms isolated from 117 patients included twenty-four serotypes. Fifty-five (45%) were *S. typhimurium*. Other serotypes isolated from more than two patients were: *S. adelaide* (6), *S. anatum* (3), *S. birkenhead* (5), *S. bovismorbificans* (7), *S. chester* (10), *S. derby* (3), *S. give* (3), *S. muenchen* (5) *S. newington* (3), *S. potsdam* (4), *S. saintpaul* (3).

The isolations of salmonellas by the three methods used are shown in Table 1. Table 2 shows the number of successful isolations by each method. While salmonellas were isolated from nearly 90% of the specimens by means of the motility medium, the other methods were successful with less than half the specimens and the combined use of these two methods isolated the organism from less than two-thirds of the specimens. By use of the selective motility medium, salmonellas were isolated from 123 specimens from which it was not isolated by either of the other methods. However, salmonellas were isolated from thirty-one specimens by one or both of the other methods when the motility tube method failed.

Isolation of *Salmonella* from faeces by passage through long tubes of semi-solid nutrient agar is not reliable when the patient has received antibiotic treatment

(Harper, 1968). This limitation does not apply to the selective motility medium. The thirty-one specimens from which salmonellas were not isolated in this medium were not all from treated patients. Some were first specimens from untreated patients. Others were from patients whose later specimens yielded salmonellas from this medium. At least half of the positive specimens were from patients who had received antibiotic treatment. One patient continued to excrete *Salmonella* for 4 months after its first isolation and had several courses of antibiotic treatment during this time. Of twenty-eight positive specimens from this patient, only one failed to yield the organism from the Craigie tube.

Table 1. *Isolation of* Salmonella *by three methods*

(The figures in parentheses are percentages of all positive specimens.)

SS agar	Selenite F broth	Craigie tube	No. of specimens	Combined use of SS agar and selenite broth	Craigie tube	No. of specimens
—	—	+	123	—	+	123 (41·0)
+	+	+	67			
+	−	+	52	+	+	146 (48·7)
−	+	+	27			
−	+	−	20			
+	−	−	6	+	−	31 (10·3)
+	+	−	5			
		Total	300		Total	300

Table 2. *Efficiency of three methods of isolation*

(Total positive specimens, 300. The figures in parentheses are percentages of all positive specimens.)

	Positive	Negative
SS agar	130 (43·3)	170
Selenite F broth	119 (39·7)	181
Combined use of SS agar and selenite F broth	177 (59·0)	123
Craigie tube	269 (89·7)	31

S. typhi differs from other motile salmonellas in that cultures of this organism spread much more slowly through semi-solid nutrient agar (Harper, 1968). Spread through the selective medium is also slow and the organism cannot be isolated by this method from mixed cultures of *S. typhi* and *E. coli*. During the time this medium was in routine use, *S. typhi* was isolated nineteen times from a carrier and once each from two other patients. In no case was it isolated from the motility medium.

DISCUSSION

Motile salmonellas other than *S. typhi* can be isolated from mixed cultures inoculated into Craigie tubes containing a modified Shigella–Salmonella medium in 0·2 % agar.

In routine use, in this laboratory, this medium has proved to be a valuable addition to the methods used in examination of faeces for the presence of *Salmonella*.

The method is simple and involves few manipulations. Pure cultures of salmonella are usually obtained by subculture from the Craigie tubes on MacConkey agar plates after incubation for 1 day. Pure cultures can usually still be obtained after incubation for several days. This fact is useful, as results still can be obtained if the tubes are left in the incubator for an extra day owing to pressure of other work over week-ends and holidays. In the absence of salmonellas, other faecal organisms do not usually spread through the medium to the outer surface within 3 days.

The medium has been successfully used in the examination of specimens from individual patients thought to be suffering from gastro-intestinal infections.

It seems that it would also be useful as a method of screening large numbers of specimens of faeces or other material for the presence of salmonellas.

SUMMARY

A modified Shigella–Salmonella medium in semi-solid agar was shown to inhibit the spread of most faecal organisms through a Craigie tube, while selectively allowing the passage of *Salmonella*.

A medium for routine use is described.

Over a period of 3 years, in this laboratory, 90% of specimens from which salmonellas were isolated gave positive results with this medium. Over 40% of these specimens would have been reported as negative if the method had not been in use. However, 10% would have been missed if this method alone had been used. It should therefore be used in conjunction with other culture methods, of which direct plating on SS agar was the most successful in this series.

REFERENCES

CRAIGIE, J. (1931). Studies on serological reactions of flagella of *B. typhosus*. *J. Immun.* **21**, 417.

HARPER, J. (1968). Semi-solid agar as a selective medium for salmonella. *J. Path. Bact.* **95**, 550.

HARVEY, R. W. S., MAHABIR, D. E. & PRICE, T. H. (1966). A method of secondary enrichment for salmonellas independent of selectively toxic chemicals. *J. Hyg., Camb.* **64**, 361.

HARVEY, R. W. S. & PRICE, T. H. (1967). The isolation of salmonellas from animal feeding stuffs. *J. Hyg., Camb.* **65**, 237.

INO, J. & GRABER, C. D. (1955). Recovery of salmonella from contaminated cultures. *U.S. arm. Forces med. J.* **6**, 586.

JONES, R. E. & HANDLEY, W. R. C. (1945). A selective medium for the isolation of salmonella bacilli from heavily contaminated material. *Mon. Bull. Minist. Hlth*, **4**, 107.

KLIGLER, I. J. (1918). Modification of culture media used in the isolation and differentiation of typhoid, dysentery and allied bacilli. *J. exp. Med.* **28**, 319.

LIEFSON, E. (1936). New selenite enrichment media for the isolation of typhoid and paratyphoid (salmonella) bacilli. *Am. J. Hyg.* **24**, 423.

ROSE, H. M. & KOLODNY, M. H. (1942). The use of SS (Shigella–Salmonella) agar for the isolation of Flexner dysentery bacilli from the feces. *J. Lab. clin. Med.* **27**, 1081.

STUART, P. F. & PIVNICK, H. (1965). Isolation of salmonellae by selective motility systems. *Appl. Microbiol.* **13**, 365.

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Salmonellas, shigellas and enteropathogenic *Escherichia coli* in uncooked food*

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INTRODUCTION

Salmonella infections due to consumption of contaminated fruit or vegetables have been very rarely reported. Gayler, MacCready, Reardson & McKernan (1955) described an outbreak of infection by *Salmonella miami* involving 17 persons in five families, all of whom had eaten water-melon bought from the same supermarket. This was the only food eaten in common by all the affected persons, and which had not been eaten by any symptomless members of the five families. *S. miami* was grown from the faeces of all the affected persons, and from slices of water-melon still left in their homes, but not from slices of water-melon from the supermarket. It was, however, grown from the shelf in the supermarket on which the knife used for slicing water-melon was stored. Some experiments which these authors carried out showed clearly that *S. miami* multiplied greatly overnight at room temperature in the interior of water-melon. They also mentioned, in an addendum, an unpublished outbreak in which there were six cases of *S. bareilly* infection in two families, and the infecting organism was grown from remaining slices of water-melon.

Apart from these two well-documented outbreaks, occasional outbreaks and sporadic cases of salmonella infection associated with eating fruit or fresh vegetables have been reported in England (Report, 1950, 1954, 1955, 1956). In these reports, however, no distinction is made between outbreaks or cases where the association is proved by isolation of the infecting organism from the food, and those where the association is presumed on purely epidemiological evidence without confirmation by culture from the food.

A preliminary note on the incidence of salmonellas in vegetables and fruits in Ceylon was communicated by Falisevac, Padley & Gulasekharam (1959). As this work was incomplete, a reinvestigation was started on 1 April 1967 and was completed on 15 August 1967, and the results are presented in this paper.

MATERIALS AND METHODS

Fresh vegetables and fruits sold at one of the markets in Colombo and those supplied by the Marketing Department to the Lady Ridgeway Hospital for children were examined. The Marketing Department is a Government organization that

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supplies provisions to some of the hospitals. Deliveries of these to the market and to the Marketing Department were made within 24 hr. of collection from different parts of Ceylon where these were grown. The market and the Marketing Department usually disposed of them within 3–4 days. The vegetables and the fruits from the market were bought at random from different stalls.

Each sample was wrapped separately in sterile kraft paper, and reached the laboratory within $\frac{1}{2}$ hr. There they were put into sterile beakers of 500 ml. capacity and 40 ml. of saline was added. Ash pumpkin, cucumber, pumpkin, snake gourd and papaw are big fruits and the whole fruits could not be introduced into the beakers, so small sliced pieces of these were obtained from the dealers. These fruits were sometimes sold as pieces. The rest of the fruits and vegetables were introduced into the beakers without being cut. Each beaker was gently rotated so that the contents were well mixed. The liquid was transferred into McCartney bottles and centrifuged at 3,000 rev./min. for 30 min. The supernatant was discarded. In the case of the vegetables two loopfuls from the the deposit of each sample, and in the case of the fruits three loopfuls, were plated on S.S. deoxycholate-citrate and MacConkey agar and finally the remaining deposit was put into tetrathionate and selenite broths. These were incubated at 37°C for 18–24 hr. The procedure for isolation and identification of pathogens were described by Velaudapillai, Mendis & Niles (1966).

There were 12 varieties of leaves and shoots (cabbage, celery, gotukola, kankum, kohila, leeks, lettuce, norkoal, nivithi, rhubarb, sarana, spinach), 11 of vegetable fruits (breadfruit, brinjal, butter beans, capsicum chilli, drumstick, elabattu, green beans, green chilli, ladies' finger, long beans, tomatoes), six of gourds (ash pumpkin, bottle gourd, bitter gourd, cucumber, pumpkin, snake gourd), ten of roots and tubers (beet root, carrot, king jam, lotus roots, onions (small), onions (big), potatoes, radish, sweet potatoes and yam), 15 of fruits (ambarella, ash plantain, gaduguda, lavalu, lovi, mango, mangosteen, orange, plantain, passion fruit, papaw, rambuttan, veralu, wood apple, wild olive) and two of pulses (dhal and green grams).

RESULTS

The total numbers of vegetables and fruits that were examined are shown in Table 1, with positive findings. In this table salmonellas, shigellas and enteropathogenic *E. coli* are not separated. As 54 different varieties of vegetables and fruits were examined, they are grouped together according to Nicholls (1961). The distribution of serotypes of *Salmonella*, *Shigella* and *E. coli* among the different foodstuffs is shown in Table 2.

DISCUSSION

Out of a total of 392 fruits examined, only one was positive. The fruits were gathered from trees that grew to a height of over 5 ft.

Vegetables were infected with *Salmonella*, *Shigella* or pathogenic *E. coli* to the extent of 1·3%. The isolation of pathogenic *E. coli* and *Shigella* does not appear to have been reported so far. The vegetables from the market harboured almost an

Table 1. *Results of bacteriological examination of uncooked food*

	Source of food						
	Marketing Department		Market		Total		
	Number		Number		Number		
	examined	positive	examined	positive	examined	positive	% positive
Leaves and shoots (12 varieties)	111	1	317	5	428	6	1·4
Vegetable fruits (11 varieties)	120	2	272	2	392	4	1·0
Gourds (6 varieties)	36	0	78	2	114	2	1·8
Roots and tubers (10 varieties)	155	1	204	4	359	5	1·4
Fruits (15 varieties)	10	0	382	1	392	1	0·3
Pulses (2 varieties)	124	1	0	0	124	1	0·8
Total (56 varieties)	556	5	1253	14	1809	19	1·1

Table 2. *Pathogens isolated from fruits and vegetables obtained from the Lady Ridgeway Hospital (supplied by the Marketing Department) or from the Market*

Fruit or vegetable		Pathogens isolated	
		Hospital	Market
Cabbage	*Brassica cleracea capitata*	—	*Sh. flexneri 2*
Celery	*Apium grabeolens rapaceum*	—	*S. waycross*
Kankum	*Ipomoea reptans*	—	*Sh. flexneri 2*
Leeks	*Allium porrum*	*S. bareilly*	*S. typhimurium* *Sh. flexneri 4*
Lettuce	*Lactuca sativa*	—	*S. inverness*
Spinach	*Spinacia oleracea*	*S. newport*	*Sh. flexneri 4*
Butter beans	*Phaseolus lonatus*	*Sh. flexneri 4*	—
Elabattu	*Solanum xanthocarpum*	—	*S. riogrande*
Green beans	*Phaseolus vulgaris*	*S. bareilly*	—
Pumpkin	*Cucurbita moschata*	—	*Sh. sonnei* *Sh. boydii 4*
Carrot	*Daucus carota*	—	*S. enteritidis* *Sh. boydii 11*
Ash plantain	*Musa paradisiaca*	—	*E. coli* 026:K60
Wild olive	*Eleocarpus oblongus*	—	*S. sandiego*
Dhal	*Lens esculenta*	*S. waycross*	—

equal number of salmonellas and shigellas while those from the hospital had more salmonellas.

The vegetables, unlike the water-melon, are not cut into pieces before sale. Since they do not have sufficient water and sugar to support growth of pathogenic organisms, vegetables probably merely play a role of mechanical carriers.

The number of each individual vegetable and fruit examined was not high enough for statistical analysis. However, they were arbitarily grouped according to the height to which they grew. On this basis, it was found that 24 varieties of vegetables grew up to a height of about 1 ft. and of these eight were infected while two out of the 18 taller groups were infected. The rate of infection appeared to be inversely related to the height of the plants, probably because of irrigation which wets roots and the leaves of stumpy plants. The water used for this purpose was from canals without bunds or embankments, and shallow wells lacking properly constructed walls, which were subject to human and animal faecal pollution. The possibility of manure, which is chiefly of animal origin, as a contributory factor must be borne in mind.

Out of a total of 556 different fruits and vegetables that were examined from the hospital five (0·9 %) were positive for salmonellas. The corresponding figures for the market were 1253 and six (0·5 %). There were therefore more salmonellas from the hospital vegetables than from the market. Velaudapillai, Jayasundera & Nagaratnam (1966) isolated more salmonellas than shigellas from the same hospital. They also found the incidence of *S. bareilly* to be high. In this study, *S. bareilly* was isolated three times. It is quite possible that some of these and also some of the other salmonella serotypes might have originated from contamination by carriers or the kitchen staff or through aerial contamination. Velaudapillai & Sabanathan (1966) ascribed an outbreak of infantile diarrhoea caused by *E. coli* 0119:K69:H6 in a premature-baby unit to aerial contamination.

The reason why more shigella contamination is found in vegetables from the market than in those supplied by the marketing department is that the former are exposed for a longer time and are subjected to more handling. Moreover, from time to time the dealers sprinkle them with their hands with water from a bucket. The water is not suspect, being the city supply, but the dealers' hands are not above suspicion. This is supported by the isolation of *Shigella flexneri 4* from leeks and spinach from the same stall on consecutive days. Salmonella infection may also be spread in this way. Since lettuce, kankum and wild olive, all of which are eaten raw, and carrot which may be eaten raw, were all found to be contaminated by pathogens, it is of the utmost importance that such vegetables and fruits should be well washed before they are eaten. The dhal might have been contaminated recently as it would not support the growth of organisms in the dry state.

SUMMARY

Centrifuged deposits of washings of vegetables and fruits were plated on S.S. deoxycholate citrate and MacConkey agar for evidence of contamination.

Vegetables like beans, carrots, cabbage, celery, elabattu, leeks, kankum, pump-

kin and spinach were found to be contaminated with *Salmonella*, *Shigella* and enteropathogenic *E. coli*. The extent of contamination of the vegetables which grew to a height of about 1 ft. above the ground was greater than that of the taller varieties. The rate of contamination among fruits was almost negligible.

The contamination might have originated from the water used for irrigation, from manure, or from the handlers of the vegetables.

REFERENCES

FALISEVAC, J., PADLEY, R. & GULASEKHARAM, J. (1959). Salmonella infections in men. *Ceylon med. J.* **5**, 72–81.

GAYLER, G. E., MACCREADY, R. A., REARDSON, J. P. & MCKERNAN, B. F. (1955). An outbreak of salmonellosis traced to watermelon. *Publ. Hlth Rep., Wash.* **70**, 311–3.

NICHOLLS, L. (1961). *Tropical Nutrition and Dietetics*, 4th ed., pp. 212, 385, 398. London, Baillière, Tindall and Cox.

REPORT (1950). Food poisoning in England and Wales, 1949. *Mon. Bull. Minist. Hlth* **9**, 254–68.

REPORT (1954). Food poisoning in England and Wales, 1951 and 1952. *Mon. Bull. Minist. Hlth* **13**, 12–24.

REPORT (1955). Food poisoning in England and Wales, 1953. *Mon. Bull. Minist. Hlth* **14**, 34–42.

REPORT (1956). Food poisoning in England and Wales, 1955. *Mon. Bull. Minist. Hlth* **15**, 263–74.

VELAUDAPILLAI, T., JAYASUNDERA, L. B. & NAGARATNAM, W. (1966). Comparison of the organisms causing diarrhoea in a children's hospital and in estates. *Ceylon J. med. Sci.* **15**, 45–54.

VELAUDAPILLAI, T., MENDIS, O. & NILES, G. R. (1966). Enteropathogenic *E. coli* among children. *Ceylon J. med. Sci.* **15**, 1–6.

VELAUDAPILLAI, T. & SABANATHAN, S. (1966). Ausbruch von Diarrhoe in einer Frühgeburtenabteilung infolge Infektion mit *E. coli* 0119:K69:H6. *Arch. Hyg. Bakt.* **150**, 186–8.

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Immunity in experimentally induced enzootic pneumonia of pigs

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For many years the main research objective in the study of enzootic pneumonia of pigs was to establish the nature of the causal agent. Now that this has been shown to be a mycoplasma (Goodwin, Pomeroy & Whittlestone, 1965) differing from a wide range of other mycoplasmas (Goodwin, Pomeroy & Whittlestone, 1967), work in several laboratories is being concentrated on the serological and immunological aspects of this disease. Various tests are available for studying mycoplasmal antibodies in pig sera, and some of these have already been examined: indirect haemagglutination (Ross & Switzer, 1963), agar-gel precipitation and immuno-fluorescence (Goodwin *et al.* 1967), metabolic inhibition (Goiš, 1968) and complement fixation (Roberts, 1968). The broad history of serological tests, however, shows that the mere demonstration of titres is of limited value; too often, such tests have been shown later to be non-specific, or to have such a high rate of falsely positive or falsely negative results that they are of little practical use in diagnosis or for the measurement of immune status. Before investigating sera from the field, therefore, we decided to study the merits of different serological tests using the sera from hysterectomy-produced pigs that had been infected at known times with enzootic pneumonia. A proportion of these pigs were subsequently challenged with the infection and killed to observe their immune status; sera collected before and after challenge in these animals were similarly evaluated.

Although Betts, Whittlestone & Beveridge (1955) said that there was no field evidence to show that a natural attack of enzootic pneumonia produced any appreciable degree of immunity, Whittlestone (1957) subsequently commented on the fact that young pigs were generally more obviously affected than older stock, partly because the latter had usually experienced the infection previously. The general weight of field evidence, however, suggests that a good immunity develops in this disease: clinical signs are rare in adult stock, and are usually seen in such animals only when the disease first enters one of the few herds that are free from enzootic pneumonia. Goodwin (1965) observed that the disease was clinically suppressed in the litters from older sows, even though the causal agent was probably present; he felt that one explanation for this suppression could be that passive immunity was transferred in the colostrum, and he postulated that, because they did not themselves develop active, substantial lung lesions of

pneumonia, litters protected in this way might not develop a sufficiently strong immunity to protect their own first litters via the colostrum in due course.

Lannek & Börnfors (1957) showed that a strong immunity developed in experimentally induced enzootic pneumonia. Pigs were infected, allowed to recover (as judged by radiological examinations) and then challenged 118 days after the primary infection: all the challenged pigs were free from enzootic pneumonia at slaughter, whereas all the positive controls, which were the same age, had pneumonia. Some of the challenged animals were housed with the positive controls for 28 days, and were thus probably exposed to a natural challenge in addition to the artificial challenge. Although the authors wrote that neutralizing antibodies had not been demonstrated by then in pig sera, it seems that they did not examine the sera of these particular immunized pigs.

MATERIALS AND METHODS

Pig inoculations

All the pigs were hysterectomy-produced, colostrum-deprived animals, kept under conditions of strict isolation as previously described (Goodwin *et al.* 1967). They were inoculated intranasally with ground suspensions in broth of lung affected with enzootic pneumonia, the dose varying between 5 and 15 ml. of dilutions that ranged from 1/5 to 1/10. The term 'challenge' applies only to the attempts to re-infect pigs that had previously been inoculated in this way. All the pigs that started as a group remained as a group while they were subject to the same procedures; because of this, the inoculated pigs were, on some occasions, also incidentally exposed to their infected litter-mates. However, whenever a pig was treated differently from the other animals in its group it was removed to a separate isolation cubicle beforehand.

The diagnosis of enzootic pneumonia was based on the examination of touch preparations for organisms with the morphology of *Mycoplasma suipneumoniae* (Whittlestone, 1967) and on the nature of the gross lesions and the histological picture. The lesions described in the text as early ones were pale creamy-pink in colour with ill-defined edges; when cut, they exuded oedematous or slightly cloudy fluid. Histologically, they showed alveolar-cell pneumonia, catarrhal or purulent bronchiolitis, perivascular and peribronchiolar mononuclear-cell accumulations, and varying degrees of peribronchiolar lympho-reticular hyperplasia. The lesions referred to as late ones had the gross appearance of small areas of collapse with pin-head lymphoid-like nodules; when cut, the tissue was dry. Histologically, there was hyperplasia of the peribronchiolar lympho-reticular tissue which sometimes invaded the lamina propria, causing partial obliteration of the bronchiolar lumina, and was associated with compression collapse of the surrounding alveoli; apart from an occasional area of alveolar-cell pneumonia, affecting a few alveoli, there was usually no cellular exudate. Where lungs were recorded as being free from abnormalities, there were no macroscopic or microscopic changes, and no mycoplasmas were seen in the touch preparations.

Culture medium

The liquid medium consisted of Hanks's balanced salt solution (39 %), Hartley's broth (30 %), inactivated pig serum (20 %), 5 % lactalbumin hydrolysate (10 %), penicillin solution containing 40,000 units/ml. (0·5 %), yeast extract (0·5 %), and thallium acetate (1/8000). The pig serum was obtained from a herd free from enzootic pneumonia. The yeast extract was prepared as described by Herderscheê (1963). The Hanks solution, the Hartley's broth and the lactalbumin hydrolysate were autoclaved; the pig serum, the yeast extract and the thallium acetate were sterilized by Millipore filtration. The complete medium was stored at about − 20° C.

Strains of Mycoplasma

Two strains of *M. suipneumoniae* were used: our type strain (J) and strain CZ (Goodwin, Pomeroy & Whittlestone, 1968). The source of *Mycoplasma hyorhinis* was mycoplasma 603 (Goodwin *et al.* 1967) and the sources of *Mycoplasma pneumoniae*, mycoplasma B 3, and *Mycoplasma granularum* were as shown in the same publication; *Mycoplasma gallisepticum* was supplied by Dr D. Taylor-Robinson. All these mycoplasmas were checked with specific antisera when used: the serum for *M. pneumoniae* was supplied by Dr R. H. Leach (the Wellcome Research Laboratories) and the serum (514) for *M. gallisepticum* was supplied by Dr H. P. Chu; the remaining mycoplasmas were checked before use against rabbit sera prepared in this laboratory against the same strains of antigen as used in this work.

Serological techniques

Serum samples were stored at about − 20° C.

Metabolic inhibition (MI)

The metabolic-inhibition test for acid-producing mycoplasmas (Taylor-Robinson, Purcell, Wong & Chanock, 1966) was performed in 3 × ½ in. glass tubes with metal caps (Oxoid) sealed with Parafilm (Gallenkamp). The serum dilutions were made in two series in standard liquid medium, and 0·5 ml. of mycoplasma culture, diluted as required in the same medium, was added to 0·5 ml. of each serum dilution, to give final serum dilutions of 1/3, 1/6, 1/12, etc., and also 1/4, 1/8, 1/16, etc. Medium and organism controls were included with each test, and the tests were read when the pH of the organism control was 0·5 lower than the medium control. The culture of *M. suipneumoniae* normally used in this test had been cloned by five consecutive single-colony subcultures on solid medium, but a few comparisons were made using both cloned and uncloned antigen. The serum samples that were heat-treated were held at 56° C. for 30 min.

In fifty consecutive tests where various serum samples were examined in duplicate, 50 % showed no difference in the titre of the samples, 30 % differed by only one dilution, and a further 16 % (to make a total of 96 %) did not vary by more than two dilutions. (As two series of dilutions were made, a difference of one dilution in this work is less than the more conventional difference of one doubling dilution.) The reproducibility of the test was then observed by examining one

serum sample on six different days, the same bottle of liquid medium being used throughout: the greatest difference in all the titres was two dilutions. This test was then repeated six times in duplicate on three different days, using six different batches of liquid medium: the maximum variation in titre was two dilutions, except for one result which showed a difference of three dilutions.

The effect of varying the mycoplasma concentration was also observed. Six sera were tested, using standard ampoules of the organism: the ampoule contents were diluted in tenfold stages from 10^{-2} to 10^{-6}, and between the dilutions of 10^{-3} and 10^{-5}, inclusive, the titres obtained for each serum did not vary by more than two dilutions, but at either end of the scale (that is, at 10^{-2} and 10^{-6}) there was sometimes a greater variability. The organism dilution most usually used in the work described later was 10^{-3}.

Indirect (passive) haemagglutination (IHA)

The stabilizing solution, referred to as 1 % protein, was normal rabbit serum, inactivated at 56° C. for 30 min., and diluted 1/100 in phosphate buffered saline (PBS) pH 7·2, after being absorbed at 37° C. for 1 hr. with an equal volume of washed, packed, sheep erythrocytes. Pig sera for testing were inactivated and absorbed in the same way.

Sheep erythrocytes in Alsever's solution were washed three times with PBS, pH 7·2, adjusted to a final concentration of 2·5 % and mixed with an equal volume of freshly prepared 1/120,000 tannic acid in PBS, pH 7·2. After 15 min. at 4° C., the cells were centrifuged, washed twice in PBS, pH 7·2, and resuspended to the original volume and concentration in PBS, pH 6·4.

The mycoplasma antigens (uncloned) were prepared by centrifuging liquid-medium cultures, previously incubated until the pH had changed from 7·4 to 6·9, at 53,700*g* for 30 min. The deposit was washed twice in PBS, pH 7·2, resuspended in one-hundredth of the original volume, and deep frozen until required. The opacity of the *M. suipneumoniae* antigen suspension corresponded to Brown's tube no. 4.

To sensitize the tanned red cells, the appropriate dilution of antigen (1/30 in PBS, pH 6·4) was mixed with an equal volume of cells and incubated at 37° C. for 15 min. in a water bath. The red cells were then centrifuged, washed three times in 1 % protein and resuspended in the same solution to the original volume of antigen. The optimum concentration of antigen for sensitization had been found by block titration with specific rabbit antiserum. In case these sera contained antibodies against liquid-medium constituents contaminating the antigens used to immunize the rabbits, the rabbit sera were absorbed with 100 mg./ml. of freeze-dried liquid medium, in addition to being absorbed with washed, packed, sheep erythrocytes.

The tests were made in 12 × 75 mm. Pyrex tubes. Doubling serum dilutions, starting at 1/5, were made in 1 % protein in 0·5 ml. volumes. To each tube 0·05 ml. of sensitized cells was added. After thorough shaking, the tubes were incubated at 37° C. for 2 hr.; the results were then noted, the tubes re-shaken and left at room temperature overnight before making the final reading. The end-point was taken as the highest serum dilution to give a clearly positive agglutination; red-cell

patterns intermediate between this and the controls were taken as negative. All tests on unknown sera had the following controls: the first four dilutions of each test serum with tanned, unsensitized, red cells; sensitized cells plus diluent (antigen control); a positive control consisting of a complete titration of a specific rabbit antiserum; and a negative control using a pig serum that was known to be free from antibody by this test. In reproducibility experiments the titres for the same serum samples tested on different days did not vary by more than one doubling dilution.

The specificity of the test was demonstrated by adding 0·05 ml. of antigen to one set of paired serum dilutions, and 0·05 ml. of 1 % protein to the other. After being held overnight at 4° C., 0·05 ml. of sensitized cells was added. After the usual incubation, the tubes were read for inhibition of the haemagglutination. Both the rabbit serum and high-titred pig serum were inhibited by dilutions of the *M. suipneumoniae* antigen, but not by an antigen prepared in similar fashion from *M. hyorhinis*. Also, significant titres were not obtained when the rabbit and pig sera were titrated against tanned red cells sensitized with *M. hyorhinis*, mycoplasma strain B 3, *M. granularum* or *M. gallisepticum* antigen. Non-specificity of a low order, however, was observed with a few sera, but in no instance did this extend beyond a dilution of 1/40.

Complement fixation (CF)

The technique of Bradstreet & Taylor (1962) was used, except that the haemolytic system was prepared just before use and, as suggested by Roberts (1968), the sera were not inactivated. The tests were made in 12 × 75 mm. Pyrex tubes. The optimum concentration of antigen (uncloned J strain of *M. suipneumoniae*) was established by block titration against a positive control serum: it was found that the antigen concentration was rather critical, and the highest concentration that was not anti-complementary was used. Two series of doubling dilutions of each pig serum were made, starting at 1/10 and 1/15, respectively. The test was read visually immediately after incubation and the titres quoted refer to fixation of 75 % or more. A standard, known positive pig serum was included with each test and, when the titre for this serum varied by more than half of one doubling dilution, the results were discarded.

A few serum samples were exchanged with another laboratory (Pfizer Ltd) and these were titrated blind in each case: the sera yielding titres of less than 1/10 in each laboratory gave the same result in the other; but the titres for positive sera were notably higher in this laboratory.

RESULTS

Infection and challenge of pigs

Four main immunization experiments were made.

Experiment 1. Three of four litter-mates aged 24½ weeks were infected. After 18 days one of the infected pigs (2803) was killed, when it had extensive, early lesions of enzootic pneumonia; from this it was presumed that all three infected

animals had probably developed lesions of enzootic pneumonia. The uninfected control (2806) was killed at the same time and had no abnormalities in the lung.

The two surviving pigs (2858 and 2859) were left until 16 weeks after their initial infection; they were then challenged with a lung suspension which was made from their litter-mate (2803) killed earlier and which, as described below, was shown to be capable of inducing enzootic pneumonia. After a further 20 days both pigs were killed and in each there were only extremely small, late lesions of enzootic pneumonia at the tips of the cardiac lobes; no mycoplasmas were seen in the touch preparations prepared from these lesions.

The same dose of the challenge inoculum was given to two pigs (2856 and 2857) aged 17½ weeks, two of their litter-mates remaining as uninfected controls (2854 and 2855). These four pigs were killed on the same day as the two challenged pigs: the two infected animals had moderate and extensive early lesions, respectively, of enzootic pneumonia, while the uninfected controls showed no abnormalities in the lungs.

Experiment 2. Four of eight litter-mates aged 24 weeks were infected. After 18 days one of the infected group (2805) was killed: it had moderately extensive, early lesions of enzootic pneumonia and from this it was presumed that the whole group had been satisfactorily infected. Two uninfected controls (2807 and 2809) killed at the same time showed histological changes not suggestive of enzootic pneumonia and no mycoplasmas were seen in touch preparations made from their lungs.

As in Expt. 1, the survivors were left until 16 weeks after the initial infection, when all three infected animals were challenged and one of the two remaining controls received the same dose of the challenge inoculum. When killed 22 days later the control pig (2860) had extensive, early lesions of enzootic pneumonia, whereas a challenged pig (2861), killed at the same time, had only a very small, late lesion of enzootic pneumonia in the right cardiac lobe; two organisms that were probably mycoplasmas were seen in one area of the touch preparation made from this lesion.

The two remaining challenged pigs in the infected group were re-challenged twice more, 20 weeks 3 days and 21 weeks 2 days after the initial infection. The inoculum used for the second of these challenges was given in parallel to the remaining uninfected control, and all three animals were killed 20 days later. The control animal (2878) showed extensive, early lesions of enzootic pneumonia, whereas one of the challenged pigs (2877) had no abnormalities in the lung, apart from some increased cellularity around the bronchial tree, and the other (2876) showed only fairly small, late lesions of enzootic pneumonia. No mycoplasmas were seen in the touch preparations made from the latter lesions. All the lung material used to provide the challenge inocula in this experiment came from the pig that was killed first in the infected group.

Expts. 1 and 2 were started together, but in separate accommodation, in November 1965; both experiments were completed by April 1966. They indicated a strong natural immunity to challenge, but the longest time between initial

infection and killing after a single challenge was 19 weeks, at which time there were very small lesions in the lungs of the challenged animals. To establish whether such lesions were residual ones from the initial infection, and to observe whether a naturally induced immunity might still protect pigs from a substantial subsequent challenge after a much longer interval than 19 weeks, two further experiments (3 and 4) were made.

Experiments 3 *and* 4

In these later experiments the pigs were under 1 month old when infected; this was to see whether young pigs would develop as protective an immunity as the older ones in Expts. 1 and 2. A single inoculum was used to initiate both Expts. 3 and 4: as it was made from a combination of lesions from one positive control from each of the first two experiments, there was a connexion between the first two and the last two experiments. But as the latter were not begun until July 1966 there was a break of 3 months in the use of the animal accommodation.

The general plan of Expts. 3 and 4 was identical, and all infections, challenges, bleedings and killings were performed in parallel on the same dates. The only difference between the experiments was that in Expt. 3 the pigs were 16 days old, while in Expt. 4 they were 29 days old, when first infected.

Experiment 3. Five pigs were infected and three litter-mates were kept as controls. After 12 days one of the infected group (2895) was killed: it had extensive, early lesions of enzootic pneumonia, and from this it was assumed that the whole group had been satisfactorily infected. Four months after the primary infection a second pig (2928) was killed, in order to see whether the pneumonic lesions had resolved: this animal had virtually normal lungs macroscopically; microscopically, there was some evidence of late enzootic pneumonia but no mycoplasmas were seen in the touch preparations.

At 19 weeks after infection one of the three survivors (2943) was challenged and one control animal (2944) received the same dose of the challenge inoculum: both pigs were killed after a further 20 days. The control had moderately extensive, early lesions of enzootic pneumonia, whereas the challenged pig had only minute, late lesions and no mycoplasmas were seen in the touch preparations.

A second, similar challenge was made 39 weeks after primary infection and, as before, the two pigs were killed 20 days later. The control (2958) had extensive, early lesions of enzootic pneumonia, whereas the challenged pig (2960) showed only histological evidence of enzootic pneumonia and no mycoplasmas were found in the touch preparations.

A third, similar challenge was made 60 weeks after primary infection, the pigs again being killed 20 days later. The control (3024) had extensive, early lesions of enzootic pneumonia, whereas the challenged pig (3025) showed only minimal histological evidence of late enzootic pneumonia and no mycoplasmas were seen in the touch preparations.

Experiment 4. Five pigs were infected as in Expt. 3, but only two litter-mates were available as controls. The pig that was killed after 12 days in the infected group (2896) had moderately extensive lesions of enzootic pneumonia. The pig

that was killed after 4 months (2929) had almost healed, late lesions and no mycoplasmas were found in the touch preparations.

When the first challenge was made at 19 weeks, the control pig (2945) and the challenged pig (2942) showed essentially the same picture as the corresponding animals in Expt. 3.

This was likewise the case after the second challenge, in which the control was pig 2959 and the challenged animal was pig 2961.

None of the control pigs remained by the time of the third challenge, but as the inoculum was the same for both Expts. 3 and 4, the control pig for Expt. 3 (3024) indicated that the challenge dose was capable of initiating the disease in a non-immune animal. The challenged pig (3026) had very small consolidated areas which, histologically, proved to be a macrophage and giant-cell pneumonia with giant-cell granulomata; no mycoplasmas were seen in the touch preparations.

Examination of sera by different methods

In Expts. 1 and 2 all the 16 pigs involved were bled for serum samples just before entering the experiment, but the pigs in Expts. 3 and 4 were not. In all four experiments, serum was collected when each pig was killed. Various other serum samples were taken at intervening stages, as follows. In Expt. 1, pig 2858 was bled before it was challenged. In Expt. 2, pigs 2861, 2876 and 2877 were bled before their first challenge. In Expt. 3, pigs 2960 and 3025 were bled before challenge, and their respective positive controls (2958 and 3024) were bled before being infected. In Expt. 4, pigs 2961 and 3026 were likewise bled before challenge, and their respective positive controls (2959, and 3024 from Expt. 3) were also bled before being infected. Whenever antibody titres are quoted in the Tables in this section, the essential data about the sera are given; there is thus no need to deduce this information from the previous section on infection and challenge.

Metabolic-inhibition test

All the serum samples referred to above were examined using the MI test. The highest titre obtained was 1/24, and nearly all the titres were less than 1/12. It became apparent, however, that the titres did not correlate with the immune status of the pigs; for some of the animals that were shown by challenge to be strongly immune had low titres, while titres of up to 1/16 were obtained with over one third of the serum samples (10 out of 23) taken from uninfected controls or from pigs that had not yet been exposed to infection. For this reason, the results are not presented in detail. We wished to know, however, what the MI test might be measuring in these sera, and to study this question further the following experiments were undertaken. Before proceeding to these, however, the sera that appeared to give falsely positive titres were re-tested: in every case they continued to give positive results.

The sera from four pigs that had not been exposed to infection but which nevertheless had titres against *M. suipneumoniae*, the sera from five pigs that had been infected with ground lung, and the sera from two pigs (3014 and 3016) that had been injected with *M. suipneumoniae* in other work, were examined by the

MI test against various mycoplasmas; the results are summarized in Table 1. Whenever a comparison was made between unheated and inactivated serum, the titrations were performed in parallel on the same day; in addition, the titrations for each serum against *M. suipneumoniae* and *M. hyorhinis* were performed in parallel.

It can be seen that, apart from pig 3014, whenever an unheated serum gave an MI titre against *M. suipneumoniae*, it also gave a titre against the other mycoplasmas against which it was tested (*M. hyorhinis*, *M. pneumoniae* and *M. gallisepticum*). Sometimes these titres were reduced by the inactivation method, but often they were not. From these results it appears that many of the pig sera listed contained one or more metabolic-inhibitory substances that were non-specific (as judged by tests using four different mycoplasmas of porcine, human and avian origin) and were still active after heating at 56° C. for 30 min. Secondly, some of the pig sera contained one or more metabolic–inhibitory substances that were likewise non-specific, but were either inactivated or partly inactivated by the same heat treatment. Because of the different combinations of these substances that could be present in the various sera, and because the metabolic–inhibitory substances appeared to operate at different levels against the different mycoplasmas, it is difficult to analyse these results further.

In order to see whether the titre obtained might vary with the strain of organism used, sera from six pigs were titrated against the cloned J strain, the uncloned J strain and the CZ strain of *M. suipneumoniae*: with each serum, there was no significant difference between the results.

Indirect-haemagglutination test

A total of 21 serum samples were examined from 19 pigs that had not been exposed to infection in the four immunization experiments. All but one of these had an IHA titre of less than 1/5; the exception was the pre-infection sample from pig 2858 in Expt. 1, where the titre was 1/160.

The sera from 12 pigs in the four experiments that were killed either 12 (one), 18 (two), 20 (eight) or 22 (one) days after infection were tested. All these animals had substantial lesions of enzootic pneumonia, but all the sera had IHA titres of less than 1/5.

However, in constrast to the above findings, high IHA titres were obtained with sera from all the pigs infected 16 or more weeks previously, and with sera from all the challenged animals, as shown in Table 2. The second main conclusion from this Table is that the titres were usually as high before challenge as after challenge; most of the animals were killed so soon after challenge, however, that a slow increase in titre, as occurred after the primary infections would not have been detected. It is nevertheless possible that the IHA titre did not increase much further after challenge, because the titres in pigs 2876 and 2877, 20 days after their third challenge were no higher than in pig 2861, 22 days after its single challenge. With pig 2858, however, the titre had increased by 20 days after challenge, although at least part of this variation might be a reflexion of the variability of the test.

Table 1. *Titres obtained in the metabolic-inhibition test with various pig sera against different mycoplasmas*

Pig	Status when sampled	Organism used in titration: *M. suipneumoniae* Unheated serum		*M. suipneumoniae* Inactivated serum		*M. hyorhinis* Unheated serum		*M. hyorhinis* Inactivated serum		*M. pneumoniae* Unheated serum		*M. pneumoniae* Inactivated serum		*M. gallisepticum* Unheated serum		*M. gallisepticum* Inactivated serum	
2807	Control	8	16	4	4	8	12	3	6	—		—		—		—	
2854	Control	8	16	8	12	4	6	< 3	< 3	—		—		—		—	
2958N	Control	4	4	4	4	4	6	3	3	—		—		—		—	
3080*	Control	4		6		8		6		24		6		⩾ 32		3	
3014	Doubly injected†	12	16	3	4	< 3	< 3	< 3	< 3	—		—		—		—	
3016	Doubly injected†	8	12	6	8	6	12	< 3	< 3	—		—		—		—	
2928	18 weeks after infection	⩾ 32	⩾ 32	8	12	12	12	12	12	—		—		—		—	
2861‡	22 days after challenge (immune)	6		6		8		6		⩾ 32		12		12		4	
2860‡	22 days after infection (not immune)	3		4		4		3		⩾ 32		8		8		< 3	
2858‡	22 days after challenge (immune)	12	12	12	16	8	12	12	16	⩾ 32	⩾ 32	16	16	16	16	6	6
2803‡	18 days after infection (not immune)	4	4	3	4	8	8	3	3	8	12	4	4	8	8	< 3	< 3

NOTE. The figures given are the reciprocal of the serum dilution. The paired figures are the results of duplicate tests on the same day.

* This pig was in an experiment not described in this paper.

† These pigs were injected with *M. suipneumoniae*, either twice intramuscularly (3014), or intradermally and then intravenously (3016).

‡ Pigs 2861 and 2860 had pre-infection titres of < 3 against *M. suipneumoniae*, whereas pigs 2858 and 2803 had pre-infection titres of 3 and 6 respectively against the same organism.

Complement-fixation test

In general, we have not found the test easy to work with. Different batches of antigen varied both in their activity and anti-complementary effect; even with an apparently satisfactory batch of antigen, the test was occasionally nullified by an unexpected anti-complementary effect.

Table 2. *Titres obtained in the indirect-haemagglutination (IHA) test and the complement-fixation (CF) test with pig sera taken after infection and after challenge with enzootic pneumonia*

	Pre-challenge			Post-challenge*		
	Weeks after	Titre†		Days after	Titre†	
Pig no.	infection	IHA	CF	challenge	IHA	CF
2858	16	5,120	80	20	20,480	160
2859	16	N.D.	N.D.	20	10,240	120
2861	16	2,560	640	22	5,120	480
2876	16	1,280	160	20‡	1,280	40
2877	16	1,280	160	20‡	1,280	60
2928	18	20,480	640		Pig not challenged	
2929	18	40,960	N.D.		Pig not challenged	
2942	—	N.D.	N.D.	20§	20,480	60
2943	—	N.D.	N.D.	20§	10,240	480
2960	39	10,240	240	20	10,240	160
2961	39	20,480	320	20	20,480	240
3025	60	10,240	20	20	10,240	< 10
3026	60	10,240	20	20	20,480	30

* All the challenged pigs were shown to be immune.
† The figures given are the reciprocal of the serum dilution.
‡ This was the third challenge.
§ Challenged 19 weeks after initial infection. N.D. = Not done.

Nine sera from uninfected pigs were examined by the CF test: all had a titre of less than 1/10. Ten sera, taken 12–22 days after infection, were likewise examined: four of these had titres of less than 1/10, one had a titre of 1/20 and the remainder (50 %) had titres ranging from 1/40 to 1/120. There was no correlation between either the extent of the pneumonic lesions or the time of sampling and the titre obtained; thus, the pig sampled earliest (12 days after infection) had a titre of 1/40. The remaining titres are shown in Table 2: these refer to serum samples taken at longer times after infection and also after challenge, and they can be directly compared with the IHA titres for the same serum samples. It can be seen that all the seven samples taken 16–39 weeks after infection had titres between 1/80 and 1/640, but the two pigs bled 60 weeks after infection had low titres.

In general, there was no increase in CF titre after challenge: the mean titres for the eight pigs sampled both before and after challenge were about 1/200 and 1/150, respectively, which indicates that possibly, on the contrary, the titre was falling with time, and this idea is supported by the low titres obtained 60 weeks after infection.

DISCUSSION

There is reason to believe that we were working with a relatively pure system microbiologically in the animal experiments; for the pigs had been produced by hysterectomy and kept in strict isolation throughout the whole course of the experiments and, also, the J strain of *M. suipneumoniae* used to infect the pigs was one link in a long chain of serial passages in pigs, during which there has been no evidence to suggest that any other mycoplasma, and in particular *M. hyorhinis*, was concurrently present. More specifically, the pneumonias from the following pigs in these experiments were cultured on solid medium and in liquid medium: 2856 and 2857 (Expt. 1), 2805, 2860 and 2878 (Expt. 2), and 2895, 2896, 2958 and 2959 (Expts. 3 and 4). No isolations of *M. hyorhinis* were made and *M. suipneumoniae*, which was the only mycoplasma recovered, was isolated from every case (Goodwin *et al.* 1968, table 1). It is known that this solid medium is able to support the growth of a wide range of mycoplasmas (Goodwin *et al.* 1967). Therefore, although the primary infections and challenges in these experiments were not made with cultures of *M. suipneumoniae*, it seems very likely that no other mycoplasma was involved.

It has been said earlier that, because one pig in each of the four main infected groups in the pig experiments had enzootic pneumonia when killed 12 or 18 days after infection, it was concluded that all the litter-mates in these groups had probably developed the disease when first infected. This conclusion is supported by the fact that from the 11 pigs that remained, nine were challenged once and killed: all these nine were found to be solidly immune. Secondly, there were nine positive controls in these experiments, and 18 similar pigs in other experiments immediately before or after the four described here: all these 27 animals developed enzootic pneumonia when inoculated in the same way. Thus, altogether, 31 pigs were inoculated about this time and killed shortly afterwards; as all these animals developed enzootic pneumonia, it seems highly probable that the remaining 13 in the present experiments, which were not killed early, did so also.

The animal experiments showed that all but one of the 11 challenged pigs had virtually no lung lesions at slaughter; the exception was pig 2876 in Expt. 2, which had been challenged three times, but even in this animal the lesions were fairly small in extent, dry, recovering macroscopically, and late histologically. As these animals resisted challenges that produced extensive, early lesions in their respective controls, they were—as judged by pulmonary evidence—strongly immune. This was the case when the pigs were as young as 16 days at first infection, and over a year old (60 weeks) at first challenge. It is interesting that such a strong, long-lasting immunity should develop in first-generation, hysterectomy-produced pigs, and it is unlikely that the immunity after natural infection would be any less in normally born pigs in the field.

Taylor-Robinson, Shirai, Soběslavský & Chanock (1966) concluded that the titres given by the tetrazolium-reduction inhibition test in man correlated with resistance to febrile illness caused by *M. pneumoniae*; they also believed that this test was measuring antibody, as the proportion of positive sera in a population increased with age. On the other hand, Davies & Hudson (1968) could find no

correlation between antibodies to *Mycoplasma mycoides*, as measured by growth inhibition in liquid medium, and immunity to contagious bovine pleuro-pneumonia. In our work there was likewise no such correlation; furthermore, the test frequently measured non-specific inhibitory substances. These substances might not have been specifically associated with the sera because, from the nature of the MI test, coupled with the fact that *M. suipneumoniae* does not grow as readily as some other mycoplasmas in our present media, errors in technique will tend to give a falsely positive result. But this seems unlikely, however, as we would not expect such errors to be reproducible; also, some sera have recently been re-examined by another worker in this laboratory who obtained results similar to the original ones. It is more probable, therefore, that the inhibitory substances were actually in the pig sera but, if so, they were not at constant levels; indeed, a high titre was sometimes obtained with one sample and a negative result with a subsequent sample from the same animal. At the moment, therefore, we can see no way in which the MI test in our hands can give intelligible results. Nor can much light be thrown on the nature of the non-specific inhibitory substances: they could be dietary in origin, however, and vary in type and concentration in the serum according to the nature of the diet and the time interval between feeding and bleeding.

The indirect-haemagglutination test gave much more coherent results: apart from one pig, all the pre-infection serum samples, or sera from uninfected pigs, had a titre of less than 1/5, and in every case high titres eventually developed after infection. It is not known how soon after infection they would be readily apparent, however, because no serum samples were taken between the first month and 16 weeks after infection, by which time the titres were substantial. Although all the pigs that were immune to challenge had high IHA titres, it cannot be assumed that such titres correlate with the degree of immunity. Only further work, in which there is variability in immunity among the animals, might elucidate this point further. However, these experiments have shown that paired sera taken before and after experimental infection with enzootic pneumonia show a marked change in reaction in the IHA test.

The results obtained with the complement-fixation test showed a consistent change from a titre of less than 1/10 before infection to a titre of 1/80 or more by 16–39 weeks after infection. In this sense, the results of the CF test were comparable with those of the IHA test. CF titres of 1/40 or more, however, had commonly appeared by 12–22 days after infection, whereas the IHA titres obtained at this stage of the disease were always less than 1/5. On the other hand, it seems likely that the CF titres declined earlier because they were very low at 60 weeks after infection, whereas the IHA titres were still high at this time. It could be, of course, that the two pigs bled as late as this were peculiar, and had never developed substantial CF titres, but it seems more probable, because their littermates had high titres at 39 weeks, that they themselves had had higher titres earlier. If the CF titre does decline almost to extinction point around one year of age, then this test would be of little use in the field for detecting whether older animals might have been exposed to the disease. Further work is clearly necessary

on this point, and also to see for how long the IHA titres are detectable in pigs that have had the disease.

It has already been mentioned that the IHA titres may not be a measure of immunity, but it seems more certain that there was little correlation between the CF titres and immune status; for pigs 3025 and 3026, which were powerfully immune 60 weeks after infection, both had CF titres of only 1/20. Davies & Hudson (1968) described a similar situation in contagious bovine pleuro-pneumonia. It is not known what aspect of the post-infection response these two serological tests were assessing but it is clear that they were measuring different factors, because the CF titres appeared before the IHA titres and, secondly, the two pigs just mentioned had at one stage high IHA titres and very low CF titres.

SUMMARY

Hysterectomy-produced, colostrum-deprived pigs, reared in special isolation accommodation, were infected with enzootic pneumonia and later challenged with the same strain of the disease. Both the original infections and the subsequent challenges were made with intranasal inoculations of suspensions of ground pneumonic lung, but there was no evidence to suggest that any mycoplasma other than the J strain of *Mycoplasma suipneumoniae* was involved.

Pigs that had recovered from the disease were strongly immune to challenge, in that they developed virtually no lung lesions when inoculated with lung suspensions that produced extensive lesions of enzootic pneumonia in control animals. This was the case, even when the pigs were as young as 16 days old when first infected and were not challenged until up to 60 weeks later.

Sera from these pigs taken before infection, about 2–3 weeks after infection, at various times after natural recovery, and before and after challenge were examined using the metabolic-inhibition test, the indirect-haemagglutination test and the complement-fixation test.

The metabolic-inhibition test proved of little value, because non-specific inhibitory substances were present in the sera of some pigs both before and after infection: these substances inhibited the growth of *Mycoplasma hyorhinis*, *Mycoplasma pneumoniae* and *Mycoplasma gallisepticum* as effectively as *M. suipneumoniae*. Sometimes the non-specific inhibition was reduced by heating the sera at 56° C. for 30 min., but at other times it was not, which suggests that at least two types of non-specific inhibitors were present.

Apart from one pig, all the sera that were expected to be negative for antibodies against *M. suipneumoniae* proved to be so by the indirect-haemagglutination test. Titres of less than 1/5 were obtained in this test using the sera from pigs killed 12–22 days after infection, but high titres were obtained 16–60 weeks after infection. It was not possible to say whether these titres correlated with immunity.

All the pre-infection sera when examined by the complement-fixation test had titres of less than 1/10, but by 12–22 days after infection over half the serum samples had titres of 1/40 or more, and titres of 1/80–1/640 were obtained at 4 and 9 months after infection. There was some evidence to show that these titres

declined more rapidly than the titres obtained in the indirect-haemagglutination test; for they were very low at 60 weeks after infection, at which time the indirect-haemagglutination titres were still high.

It seemed, therefore, that these two serological tests were measuring different aspects of the post-infection response. Also, because the complement-fixation titres were very low in two pigs that were shown to be powerfully immune, these titres did not appear to correlate with immunity.

Our work with the metabolic-inhibition test and the complement-fixation test has benefited from discussions with Dr D. Taylor-Robinson and Mr A. S. Wallis, respectively. We are grateful to Drs H. P. Chu, R. H. Leach and D. Taylor-Robinson for the reference sera and the culture mentioned in the text. Most of the expenses of this work, including the salary of two of the authors (R.G.H. and R.L.W.), were met by a grant from the Agricultural Research Council.

ADDENDUM

The results of Boulanger & L'Ecuyer (1968) appeared while this paper was in the Press. In the complement-fixation test for enzootic pneumonia, these authors inactivated their pig sera and used unheated calf serum as a supplementary factor; by this method, they obtained a rise and fall of post-infection CF titres broadly similar to those published here.

More recently, Takatori Huhn & Switzer (1968), using essentially the technique of Boulanger & L'Ecuyer (1968), likewise found that CF titres first appeared 2–3 weeks after the experimental production of enzootic pneumonia.

REFERENCES

Betts, A. O., Whittlestone, P. & Beveridge, W. I. B. (1955). Investigations on the control of virus pneumonia of pigs (v.p.p.) in the field. *Vet. Rec.* **67**, 685.

Boulanger, P. & L'Ecuyer, C. (1968). Enzootic pneumonia of pigs: complement-fixation tests for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. *Can. J. comp. Med.* **32**, 547.

Bradstreet, C. M. P. & Taylor, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus diseases. *Mon. Bull. Minist. Hlth* **21**, 96.

Davies, G. & Hudson, J. R. (1968). The relationship between growth-inhibition and immunity in contagious bovine pleuro-pneumonia. *Vet. Rec.* **83**, 256.

Goiš, M. (1968). Serological response of pigs to infection with mycoplasma. Demonstration of growth and metabolism-inhibiting antibodies in sera of pigs inoculated intranasally with *Mycoplasma hyorhinis*. *Zentbl. VetMed.* **15** (B), 382.

Goodwin, R. F. W. (1965). The phenomenon of suppressed respiratory disease in the litters of older sows. *Vet. Rec.* **77**, 383.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Vet. Rec.* **77**, 1247.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *J. Hyg., Camb.* **65**, 85.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1968). Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. *J. Hyg., Camb.* **66**, 595.

HERDERSCHEE, D. (1963). An improved medium for the cultivation of the Eaton agent. *Antonie van Leeuwenhoek* **29**, 154.

LANNEK, N. & BÖRNFORS, S. (1957). Immunity to enzootic pneumonia in pigs following recovery from the disease. *Nord. VetMed.* **9**, 91.

ROBERTS, D. H. (1968). Serological diagnosis of *Mycoplasma hyopneumoniae* infection in pigs. *Vet. Rec.* **82**, 362.

ROSS, R. F. & SWITZER, W. P. (1963). Comparison of isolates of *Mycoplasma hyorhinis* by indirect haemagglutination. *Am. J. vet. Res.* **24**, 622.

TAKATORI, I., HUHN, R. G. & SWITZER, W. P. (1968). Demonstration of complement-fixation antibody against *Mycoplasma hyopneumoniae* in the sera of pigs infected with swine enzootic pneumonia. *Natn. Inst. Anim. Hlth Q., Tokyo* **8**, 195.

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

TAYLOR-ROBINSON, D., SHIRAI, A., SOBĚSLAVSKÝ, O. & CHANOCK, R. M. (1966). Serological response to *Mycoplasma pneumoniae* infection II. Significance of antibody measured by different techniques. *Am. J. Epidem.* **84**, 301.

WHITTLESTONE, P. (1957). Some respiratory diseases of pigs. *Vet. Rec.* **69**, 1354.

WHITTLESTONE, P. (1967). Mycoplasma in enzootic pneumonia of pigs. *Ann. N.Y. Acad. Sci.* **143**, Art. 1, 271.

J. Hyg., Camb. (1969), **67**, 209
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An appraisal of sewage pollution along a section of the Natal coast

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INTRODUCTION

Rapid industrial and municipal expansion taking place on the Natal Coast has focused attention on the ever-increasing quantities of waste ejected into the sea, and on the effects of this practice upon recreational waters adjoining the beaches. For Natal, though swiftly assuming the stature of an industrial giant, continues in its rôle of prime holiday resort and playground for Southern Africa.

At a local level, a determined attack on sewage- and waterborne-waste disposal problems has been initiated by certain Natal municipalities and industries. This has involved waste-water reclamation projects, increased industrial re-use of water, larger and more efficient treatment plants to keep pace with expansion, and, for the immediate future, three submarine pipelines to carry presently irretrievable water in the form of waste, far out to and under the sea thereby utilizing the ocean for effective dilution and dispersal of pollution.

The South African Council for Scientific and Industrial Research has been vitally concerned with many facets of these submarine pipelines, and problems ranging from outfall design to the collection of physico-oceanographic data are being investigated off this section of the coast.

In particular, the National Institute for Water Research of the South African Council for Scientific and Industrial Research has, for a number of years, been measuring environmental phenomena (on a selective basis) in this region in order to determine the degree of pollution, to pinpoint the sources and to establish standards for subsequent monitoring of the fully operational outfalls. The broad aspects of this have already been dealt with (Stander, Oliff & Livingstone, 1967), and the detailed chemical and faunal picture has been fully documented (Oliff *et al.* 1967*a*, *b*).

The present text is a digest, offering the salient features of detailed bacteriological work on the region.

Various factors in pollution of the sea were studied from a number of sources, ranging from 'clean' beaches, through various levels of contamination, to grossly polluted areas. From the distribution and occurrence of micro-organisms, and other data, a bacteriological standard for classifying these and similar waters was formulated. Such a method of appraisal should be of value in monitoring changes in the future.

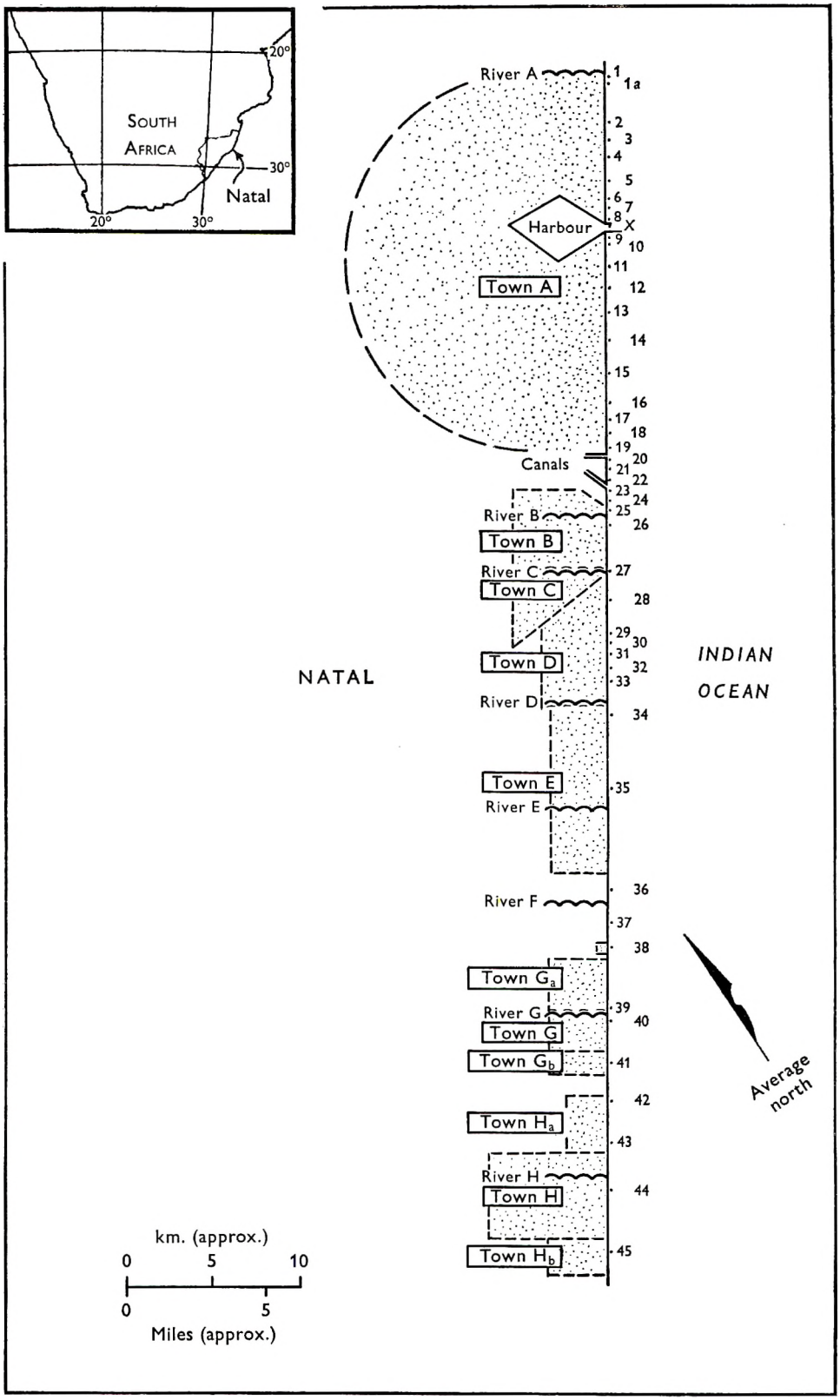

Fig. 1. Section of Natal coastline (schematic) showing sampling stations and main towns and rivers.

DESCRIPTION OF THE SURVEY AREA

Natal is 36,000 square miles in extent, and constitutes about 7½ % of the total area of the Republic of South Africa. The province supports a population of 3,000,000. Climate ranges from the tropical to the subtropical in the east, with temperate mountain ranges in the west. For the particular coastal region under survey, shown in Fig. 1, the following annual averages apply:

Rainfall	874·4 mm.	Humidity	76 %
Temperature	19·9° C.	Evaporation	67·9 in.

Prevailing winds blow roughly parallel to the coast, alternately from the north-east and south-west, with almost equal frequency. In the sea, nearshore currents flow parallel to the coast and reverse direction every 1–2·5 days; their average velocity is between 0·1 and 0·6 ft./sec. Onshore currents occur 10–33 % of the time. Wave height is normally 3–6 ft., and wave approach is somewhat oblique about 50 % of the time. Rip currents occur on about 40 % of occasions; longshore currents flow about 10 % of the time, their usual coefficient of diffusion being of the order of 60 sq. ft./min. (Stander *et al.* 1967).

On the section of coastline examined, certain relevant background data were assembled. These primarily referred to the years 1965 and 1966, and included population, area, and important disposal, sanitary or polluting features of the various towns. Broadly speaking, these aspects, and coastal topographical features, dictated the siting of sampling stations.

By extrapolation, it is reasonable to suggest that, given parallel conditions of climate and similar features for some other region, equivalent bacteriological indices would probably occur in its neighbouring sea. Conversely, given a clear bacteriological picture of the sea-water of a region, a fair estimate of the factors contributing to its pollution potential can be made.

Extension of the area-typing was borne out by a short survey on a sector of the Cape coastline. Annual climatic averages for this temperate Cape sector are as follows:

Rainfall	486·3 mm.	Humidity	72 %
Temperature	17·3° C.	Evaporation	69·2 in.

SELECTION OF INDICATORS

Bacteriological assessment of these waters required a fairly broad approach. Certain indicators were considered and rejected as being of little if any practical value in the work. (Their use in some other context is often of undoubted importance.) These included *Streptococcus faecalis*, *Clostridium perfringens*, the bacteriophage of *Salmonella typhi*, and *Mycobacterium tuberculosis*, among others. The following indicators were finally selected.

Total coliforms

This count provides a fair non-specific indication of terrigenous pollution generally. Obviously, it is not specific enough when faecal pollution is under consideration.

Presumptive Escherichia coli

This group provides a more accurate picture of faecal pollution and is in common use throughout most parts of the world. However, the numbers include, indiscriminately, *E. coli* I which is of faecal origin, Irregular II, of doubtful habitat, and Irregular VI, usually of non-faecal origin and capable of proliferation in jute, rags, hemp, etc. (Wilson & Miles, 1964) and, at least in Natal, in the marginal vegetation of rivers. As an example of the differentiation applied to the present survey, a paper mill on River A, producing paper from rags imported from Japan and ejecting waste into the river, showed the following typical analysis on one of its felt-base effluents:

Total presumptive *E. coli*	233,450,000 per 100 ml.
E. coli I	Nil per 100 ml.
Irregular II	150,070,000 per 100 ml.
Irregular VI	83,380,000 per 100 ml.

Escherichia coli *I*

This organism is an indicator of definite and recent faecal pollution.

Parasite units

These were measured as numbers of ova of *Taenia* species and *Ascaris* species, and proved valuable in the work of plume tracking; their importance as a quantitative measure of the grosser degrees of sewage pollution ensured their inclusion in this particular survey. They can remain in sea-water for several hours before becoming unrecognizable.

Coagulase positive, mannitol positive staphylococci

This organism was recorded on a presence- or absence-in-the-sample basis. However, its presence in local sea-water, despite its predilection for NaCl, was rare in comparison to its occurrence in local inland waters, and it would appear to indicate very recent specific pollution possibly of a non-faecal nature. Though common in the respiratory tract of man, it is also to be found in the faeces of about 25 % of normal individuals. Some significance may or may not be attached to its recovery near to relatively stagnant or stored sea-water, i.e. a harbour mouth, a canal mouth experiencing tidal damming effects, river mouths, unchlorinated tidal swimming pools, etc. These staphylococci are present, however, in appreciable numbers in polluted rivers examined by ourselves and others (Brand, Kemp, Pretorius & Schoonbee, 1967). The organism has been reported as succumbing fairly rapidly in polluted sea-water. (Anon., 1956.) For these reasons its recovery was regarded as having some local significance.

Salmonella typhi, *salmonellas and shigellas*

Isolation of salmonellas from polluted waters proved a relatively straightforward and speedy process, and was therefore extensively used in this survey. The organisms probably do not live for more than a few days at most in sea-water,

and many of the claims to the contrary may be due to the use of old laboratory cultures rather than freshly isolated strains. Coetzee & Fourie (1965), using naturally occurring typhoid bacilli in the form of faeces from a typhoid carrier, found a T-90 (the time taken for a 90 % reduction in the viable count) in the region of 4 hr. in sea-water. Shigellas, which are known to be less hardy than salmonellas, probably survive for even shorter periods, and consequently shigella isolations from sea-water, uncommon though they are, can be regarded as of great importance in evaluating degrees of pollution.

Salinity

This single measure of a physical nature was included in order to indicate the degree of dilution of the saline medium by fresh water.

MATERIALS AND METHODS

Sampling

Water samples were collected in sterile containers 6 ft. from banks of rivers and canals, the side of a boat or, in the surf zone, 6 ft. from the shoremost lip of a just broken wave. An ordinary sample-stick with bottle-holding attachment was used. Pipe and drain effluents were sampled direct.

For all surface investigations, the water layer between the surface and 6 in. deep was sampled. In depth work and sediments various patent sampling bottles and dredges were used.

Total coliforms

Two 100 ml. samples of water, or of appropriate dilutions in sterile distilled water, were membrane-filtered. The membranes were resuscitated for 1 hr. at 37° C. on pads impregnated with resuscitation broth (Oxoid), then transferred to pads impregnated with MacConkey membrane broth (Oxoid) for a further 17 hr. (total incubation, 18 hr.). A selection of yellow colonies were Gram-stained for microscopy.

All yellow colonies were recorded, after adjustment for dilution, as total coliforms, average per 100 ml.

Total presumptive Escherichia coli

Two 100 ml. samples of water, or of appropriate dilutions in sterile distilled water, were membrane-filtered. The membranes were resuscitated for 2 hr. at 37° C. on pads impregnated with resuscitation broth (Oxoid), then for a further 16 hr. at 44·5° C. in a water bath (total incubation, 18 hr.) on pads impregnated with MacConkey membrane broth (Oxoid).

A selection of yellow colonies were Gram-stained for microscopy. All yellow colonies were recorded, after adjustment for dilution, as total presumptive *E. coli*, average per 100 ml.

Escherichia coli *I, Irregular II and Irregular VI*

A representative number of yellow colonies from the membranes from the presumptive *E. coli* test (see above) were subcultured into tryptone broth (Difco) and incubated at 44·5° C. for 24 hr. From these tubes, subcultures were made in Koser's citrate medium (Difco) with 0·5 % of brom thymol blue indicator solution,* and incubated at 44·5° C. for 96 hr. The tryptone broth cultures were tested for indole production with Kovacs's reagent. From these results a differential *E. coli* I, Irregular II and Irregular VI count per 100 ml. was calculated. (References to the above methods: Difco Laboratories, 1953, 1962; Ministry of Health, 1956; Taylor, 1958; American Public Health Association, 1960; Oxoid Division, 1961; Wilson & Miles, 1964.)

Parasite units

Originally 1 l. of sea-water, and, later in the survey, 250 ml., was used. Increased clarity of the microscopic films with the smaller sample afforded less chance of ova present escaping notice; this appeared to compensate for the difference in the total volume examined. The sample was allowed to stand for 30 min. and the supernatant, except the last $\frac{1}{2}$–1 in., was carefully drawn off with a small water-vacuum pump and discarded. The retained portion was shaken well, and centrifuged in 50 ml. tubes at 3000 rev./min. for 3 min. All *Ascaris* and *Taenia* ova in the whole deposit were counted under the microscope, and the numbers recorded.

Coagulase positive, mannitol positive staphylococci

Two 25 ml. samples were membrane-filtered. Membranes were cultured on *Staphylococcus* medium no. 110 (Difco) at 37° C. for 43 hr. A selection of the yellow and orange colonies were Gram-stained for microscopic checking. A further selection were subcultured on plates of the same medium, and growth was tested for coagulase production, using diagnostic plasma (dehydrated) (Warner-Chilcott). On the area of medium from which growth was removed, a few drops of brom-cresol purple indicator were placed to detect mannitol fermentation (Difco Laboratories, 1953). Results were recorded as presence or absence per 50 ml.

Salmonella typhi, *and* Salmonella, Shigella, Proteus *and* Pseudomonas *groups*

The method used was developed from that of Livingstone (1965). Originally, 2 l. of sea-water was filtered through a sterile cotton wool plug and the plug placed in 250 ml. of freshly prepared selenite brilliant green broth (Difco) (SBG). Later, 250 ml. of the actual sample was added to 6 g. of SBG powder; the broth so formed was split into two 125 ml. subsamples and to one of these about 0·6 g. (i.e. about 0·5 %) dulcitol was added. Increased sensitivity obtained in some cases from the dulcitol-containing portion, and possibly a lessening of the effects of logarithmic growth of unwanted organisms crowding out any salmonellas present

* Brom thymol blue, 1·6 g., N-NaOH, 1·3 ml., absolute alcohol, 20 ml., distilled water to make 50 ml.

in the smaller samples, appeared to compensate for the difference in total volume of the sample examined.

For further testing, a modified SS (Difco) agar was used, in which the lactose was increased to 1·5 %, and 1·5 % of saccharose, not normally present, was added. Both halves of the sample were incubated at 37° C. for 20 hr., and each was then subcultured on two plates of the modified SS agar. One of each pair was incubated at 37° C. and the other at 40° C. for 20 hr. A selection from the clear colonies on these plates were then inoculated on triple-sugar-iron (BBL) agar slopes and in urea broth (Oxoid). Growth not showing the characteristics of *Proteus* or *Pseudomonas* was 'purified' on modified SS agar and tested against appropriate polyvalent antisera (Burroughs-Wellcome). All salmonellas and shigellas were submitted elsewhere (Dr H. W. Botes, Onderstepoort Veterinary Research Laboratories, Transvaal; and Dr J. H. McCoy, Public Health Laboratory, Hull Royal Infirmary, Yorkshire, England) for independent confirmation and serotyping. (*S. typhi* isolated were sent for phage-typing to Dr C. G. Crocker, Institute for Pathology, Pretoria).

Results were recorded as presence or absence of the various organisms per 250 ml.

Salinity

Salinity readings were made (courtesy local National Physical Research Laboratory) on an electrical conductivity salinometer.

RESULTS AND DISCUSSION

Judging from the plethora of bacterial candidates offered in the literature, there would appear to be no perfect indicator of sewage pollution. Criteria for 'safe' or 'ideal' recreational waters at the seaside range from the absence of 'a sewage nuisance' on frankly aesthetic grounds (Moore, 1954*a*, *b*) to the absence of *E. coli* (Yotakis, 1959); the most popular criterion apparently being the 1000 coliforms per 100 ml. standard (McKee & Wolf, 1963). However, the main concern here was the need to measure local water quality effectively in order to assess future changes in that quality.

In Natal, a feature of the coastline is the large number of rivers threading their way to the sea. The mouths of about a quarter of these are closed by a sandbar for 40–50 weeks in the year. Others perennially flow into the sea, and at times considerable flooding occurs when all river mouths burst wide, and the sea is discoloured for miles. Here, coliforms or presumptive *E. coli* are comparatively valueless, and if one's standard is based on *E. coli* I, care must be exercised to ensure that it is indeed this particular organism of faecal pollution that is being evaluated and not Irregular VI, the 44·5° C. positive coliform, which is not necessarily of faecal origin (Wilson & Miles, 1964), and which is capable of proliferation around marginal vegetation, at least in Natal rivers.

As *E. coli* I is able to survive more than 24 hr. in local sea-water and as some offshore currents can attain speeds of up to 16–24 miles/24 hr. (F. P. Anderson, personal communication) the finding of this indicator off the 'clean' beaches is not surprising.

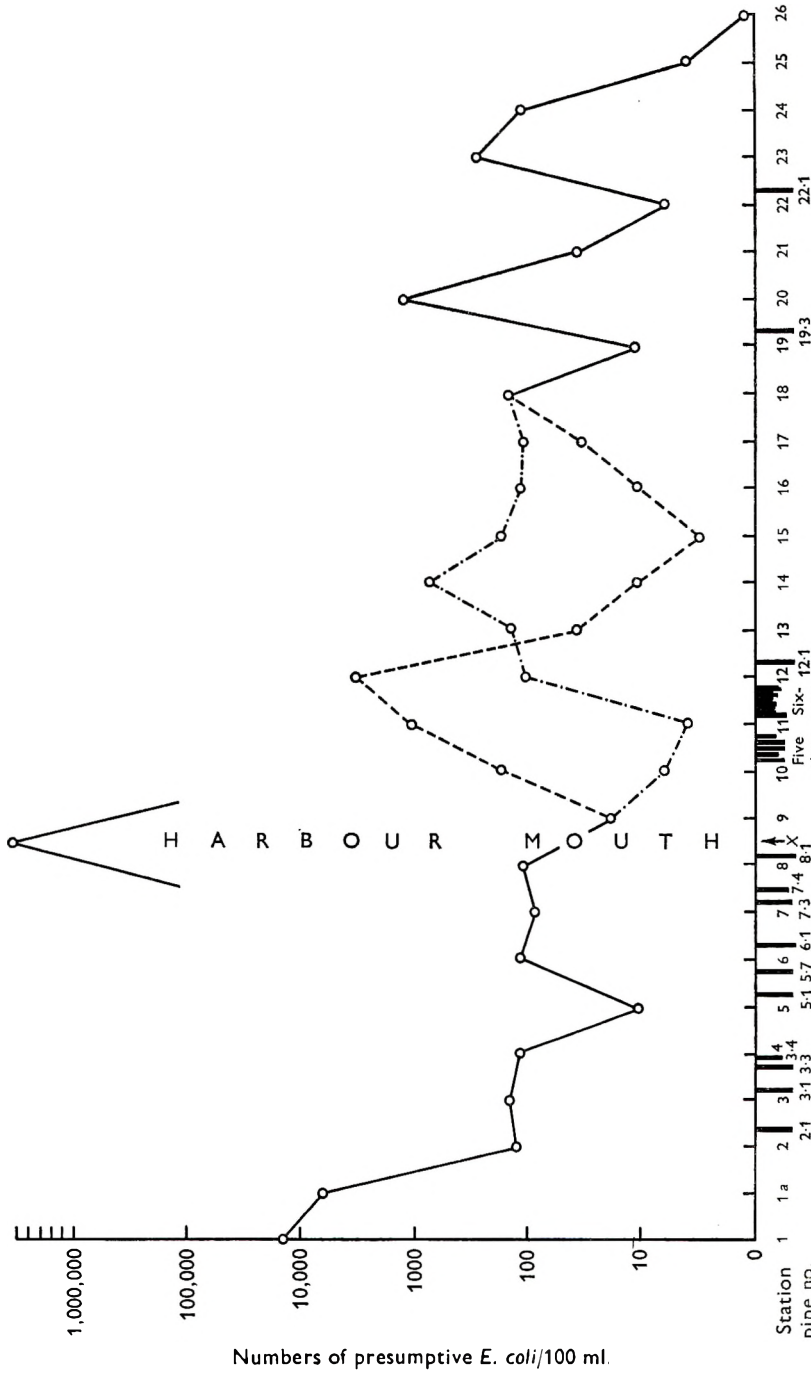

Fig. 2. Numbers of presumptive *E. coli* recovered from the surf sampling stations, showing the positions of pipes, drains and canals discharging sewage. Between stations 9 and 18: ← – – north-going current; – · → south-going current.

In fact, the complete and consistent absence of *E. coli* I from any particular beach in this area is a near impossibility. Figure 2 shows graphically the numbers of presumptive *E. coli* and the position of various pipes, drains and canals between Stations 1 and 26 ejecting sewage, and provides some indication of the influence of currents.

It is apparent that the use of *E. coli* I alone as an indicator would be of no value to those concerned in monitoring the effects of the diversion of sewage through the new submarine outfalls.

A means of measuring local water quality was therefore evolved employing a graded process whereby any improvement or worsening of the nearshore sea-water as regards sewage pollution could be assessed.

Water quality gradation

In order to cover every possible local contingency of sewage affecting neighbouring bathing beaches the present work was necessarily diversified in approach; and a planned system of adverse scoring was adopted. Each indicator was selected

Table 1. *Evaluation of indicators*

Indicator	Degree	Value
E. coli I per 100 ml.	0–10	1
	11–100	2
	101–1000	4
	>1000	8
Parasite units per 250 ml.	1–7	4
	>7	8
Coagulase and mannitol positive staphylococci per 50 ml.	Present (+)	4
Salmonellas per 250 ml.	Present (+)	4
Salmonella typhi per 250 ml.*	Present (+)	4
Shigellas per 250 ml.	Present (+)	4
Salinity, in ‰	<34‰	4

* *S. typhi* if present would therefore contribute a total value of 8, scoring 4 under salmonellas and 4 under *S. typhi*.

Table 2. *A system of classifying sea-waters by indicator values*

Indicator values	Class
1–4	I
5–8	II
9–16	III
>16	IV

and scored basically on a value of 4 (the figure for 101–1000 *E. coli* I per 100 ml.). Certain indicators regarded as of special pollution significance were given greater weight by being accorded high values; the design of the system ensured higher scoring for these, if their numbers or infrequency of occurrence were thought to warrant this. This system of scoring is shown in Table 1. Table 2 shows how the

total indicator scores from Table 1 were used to divide the waters into four classes I to IV.

Such a system ensured that the quantity of sewage effluents discharged, often extremely variable in the case of pipes, drains and canals, made little impact on the overall system and could be dispensed with in the data processing. Only direct measurements on the quality of the medium were involved. These measurements were based broadly enough to ensure that no random momentary upsurge or lessening of any single factor was possible whereby gradation was altered to the extent of calling for major reclassification of the sea-water. Any important change would involve most of the factors.

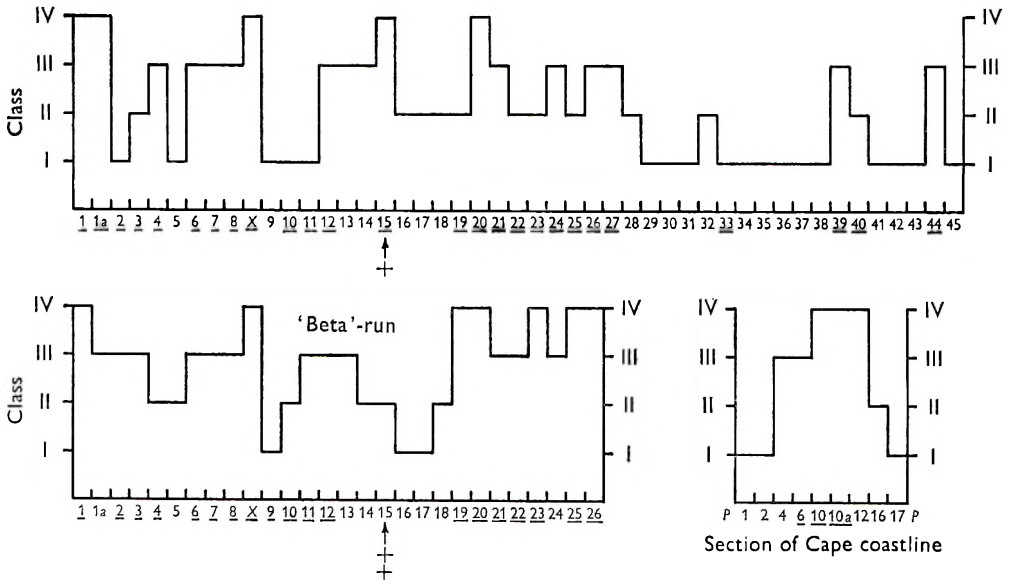

Fig. 3. Gradation of surf-waters by indicator values. Station numbers underlined indicate proximity to known sources of pollution. +, Tidal bath before chlorination; ‡ tidal bath later, chlorinated.

Moreover, as can be seen from the 'Beta' run classification in Fig. 3, a single series of samples down the coast afforded a very close approximation of conditions obtaining in the region, provided sanitary conditions upon the neighbouring land remained unchanged. This occurred despite the great restlessness and turbulence of wave, wind and current in the region.

The evolution and application of indicator values to a system of water gradation could involve nearly insuperable problems regarding objectivity. However, in the present survey, such a potential weakness was, it is thought, fairly obviated by firmly relating the classification of the sampling stations, arrived at from bacteriological findings, with their onshore sanitary features and conditions. Some indication of the more significant stations is shown in Table 3 and Fig. 3.

Classification of every sampling station examined in the light of this system appears in Table 3. The classification is again presented in Fig. 3 in schematic form.

Table 3. *Gradation of surf-waters by indication values*

Average of 3 runs									Actual or potential polluting features in the vicinity	Beta run								
Station no.	*E. coli* I	Parasite index	C + M + staph.	*S. typhi*	Salmonellae/ Shigellae	Salinity	Total	Class		Station no.	*E. coli* I	Parasite index	C + M + staph.	*S. typhi*	Salmonellae/ Shigellae	Salinity	Total	Class
									River A mouth: domestic and industrial discharges									
1	8	4	.	4	4	4	24	IV	.	1	8	4	4	.	4	4	24	IV
1*a*	8	4	.	.	4	4	20	IV	.	1*a*	8	.	.	.	4	.	12	III
2	2	.	.	.	.	.	2	I	Pipe 2.1: sewage	2	8	.	.	.	4	.	12	III
3	4	.	.	.	4	.	8	II	Pipes 3.1, 3.3, 3.4: sewage	3	8	.	.	.	4	.	12	III
4	4	4	.	4	4	.	16	III	.	4	8	.	.	.	.	.	8	II
5	1	.	.	.	.	.	1	I	Pipes 5.1, 5.7: sewage	5	8	.	.	.	.	.	8	II
6	4	4	.	.	4	.	12	III	Pipe 6.1: sewage	6	8	4	.	.	.	.	12	III
7	2	4	.	.	4	.	10	III	Pipes 7.3, 7.4: sewage	7	8	4	.	.	.	.	12	III
8	4	4	.	.	4	.	12	III	Pipe 8.1: sewage	8	8	.	.	.	4	.	12	III
X	8	8	4	4	8	4	36	IV	Main harbour discharge at *X*: approx. 20 m.g.d. sewage and industrial effluent	*X*	8	8	4	.	4	4	28	IV
9	2	.	.	.	.	.	2	I		9	1	.	.	.	.	.	1	I
10	4	.	.	.	.	.	4	I	Pipes 10.5, 10.6, 10.7, 10.9, 10.10: sewage	10	4	4	.	.	.	.	8	II
11	4	.	.	.	.	.	4	I	Pipes 11.2, 11.3, 11.5–11.7, 11.9–11.15, 11.17, 11.20, 11.21, 11.23: sewage and whaling effluents	11	8	4	.	.	4	.	16	III
12	8	4	.	.	.	.	12	III	Pipe 12.1: sewage	12	8	4	.	.	4	.	16	III
13	2	4	.	.	4	.	10	III	.	13	8	4	.	.	.	.	12	III
14	4	4	.	.	4	.	12	III	.	14	4	4	.	.	.	.	8	II
15	4	4	4	4	4	.	20	IV	Unchlorinated tidal swimming bath; chlorinated later	15	4	.	.	.	4	.	8	II
16	2	.	.	.	4	.	6	II	.	16	2	.	.	.	.	.	2	I
17	2	.	.	.	4	.	6	II	.	17	1	.	.	.	.	.	1	I
18	4	.	.	.	4	.	8	II	.	18	1	.	4	.	.	.	5	II
19	1	.	.	.	4	.	5	II	Canal 19.3: domestic and industrial discharges	19	8	4	4	.	4	4	24	IV
20	8	4	4	4	4	4	28	IV	.	20	8	4	.	.	4	4	20	IV
21	2	4	.	.	4	.	10	III	.	21	8	4	.	.	4	.	16	III
22	1	.	.	.	4	.	5	II	Canal 22.1: domestic and (largely petroleum) industrial discharges	22	8	.	4	.	.	4	16	III
23	2	.	.	.	4	.	6	II	.	23	8	4	4	.	4	4	24	IV
24	2	4	.	.	4	.	10	III	.	24	8	.	.	.	.	4	12	III
25	1	.	.	.	4	.	5	II	.	25	8	4	4	.	4	4	24	IV

Table 3 (*cont.*)

Station no.	Average of 3 runs: *E. coli* I	Parasite index	C+M+staph.	*S. typhi*	Salmonellae/ Shigellae	Salinity	Total	Class	Actual or potential polluting features in the vicinity
									River B mouth. Unchlorinated tidal swimming bath
26	1	.	.	.	8	.	9	III	.
27	2	.	4	.	4	4	14	III	.
									River C mouth
28	2	.	4	.	.	.	6	II	.
29	1	.	.	.	.	.	1	I	.
30	2	.	.	.	.	.	2	I	.
31	1	.	.	.	.	.	1	I	.
32	1	.	.	.	4	.	5	II	.
33	1	.	.	.	.	.	1	I	Chlorinated tidal swimming bath
									River D mouth (usually closed)
34	1	.	.	.	.	.	1	I	.
35	2	.	.	.	.	.	2	I	2 unchlorinated tidal swimming baths
									River E mouth
36	2	.	.	.	.	.	2	I	.
									River F mouth (limited flow)
37	2	.	.	.	.	.	2	I	.
38	2	.	.	.	.	.	2	I	.
39	4	.	4	.	.	4	12	III	.
									River G mouth: domestic and industrial discharges
40	4	.	4	.	.	.	8	II	Unchlorinated tidal swimming bath
41	2	.	.	.	.	.	2	I	.
42	2	.	.	.	.	.	2	I	.
43	2	.	.	.	.	.	2	I	
									River H mouth: domestic and industrial discharges
44	8	.	.	.	.	4	12	III	Tidal swimming bath: chlorinated
45	4	.	.	.	.	.	4	I	Tidal swimming bath: 'usually chlorinated'

Station co.	'Beta' run: *E. coli* I	Parasite index	C+M+staph.	*S. typhi*	Salmonellae/ Shigellae	Salinity	Total	Class
26	8	4	4	.	4	4	24	IV

Cape section								
P1	1	.	.	.	.	.	1	I
P2	1	.	.	.	.	.	1	I
P4	8	.	.	.	4	.	12	III
P6	8	.	4	.	4	.	16	III
P10	8	4	4	.	4	.	20	IV
P10*a*	8	8	4	.	.	.	20	IV
P12	8	8	4	.	.	.	20	IV
P16	2	.	.	.	4	.	6	II
P17	1	.	.	.	.	.	1	I

(P6, near a wool washery discharge; P10 and 10*a*, main sewage outfall; P12, two waste pipes, from a cannery and a tannery.)

Future water quality

A submarine outfall, carrying sulphite waste from a cellulose processing plant has been established recently between Stations 39 and 40, subsequent to the present work. This effluent, formerly discharged at River G (see Fig. 1), was found to be toxic to coliforms. Consequently, in this case, some deterioration of water quality, measured on bacteriological grounds alone is expected in the sea-water in the vicinity of River G when this outfall is fully operational.

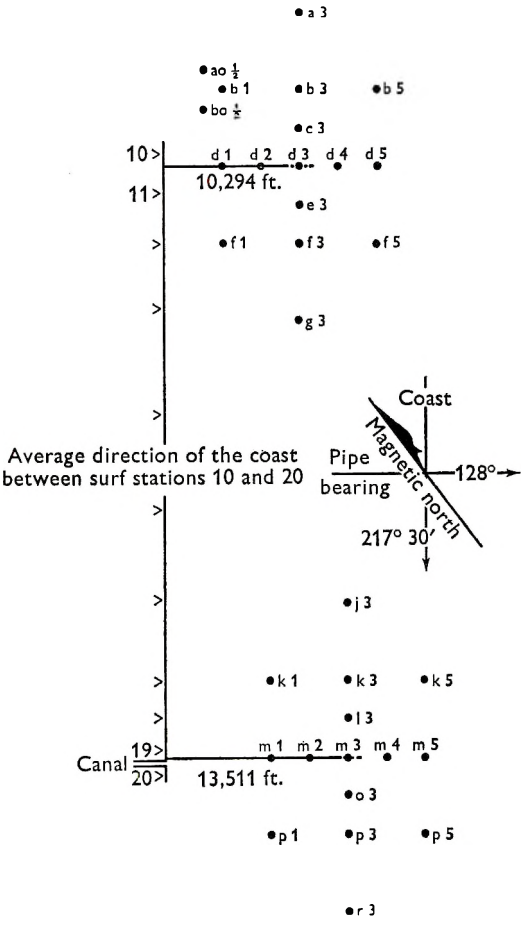

Fig. 4. Section of coast showing forthcoming submarine outfall lines with sea monitoring stations (arbitrary grid system: ½-mile units). Scale, 1:80,000.

Most of the sewage entering the sea between Stations 1 and 26 was untreated during this survey, apart from coarse meshing and primary settling in tanks near Station X while awaiting discharge with the outgoing tide. Two major submarine outfalls are planned for the region, near Stations 10 and 19. Occultation, that is, removal of the organisms surveyed here to and below the lowest levels of present-day detection and enumeration methods through dilution, dispersal, sedimentation and loss of viability, will obviously produce a steady and measurable bacterial

improvement of the water quality, as all the major and most of the minor discharges are collected and diverted to central treatment plants for pumping and dispersal under the sea. Sea-sampling stations were established in the pipeline area (Fig. 4) and the nature of their backgrounds established in preparation for the forthcoming monitoring programme. It is expected that many of the present Class IV and III surf-sampling stations will be promoted to the altogether more desirable grades of Class II and even Class I, when these pipelines are fully operational.

SUMMARY

A bacteriological survey was made on the distribution and occurrence of coliforms and pathogenic indicators of pollution within the surf-zone and near-shore waters along a section of the Natal Coast, prior to the use of submarine outfalls. The distance covered measured approximately 47 miles. The waters sampled and assessed ranged from 'clean' beaches to heavily polluted areas; a single short run off an Eastern Cape coastal region was included for comparative purposes. In all cases, the bacteriological picture was related to sanitary features on the shore. The method is based on *Escherichia coli* I counts, parasite units, staphylococci, salmonellas and salinity, and provides an objective approach to the assessment of any future changes in water quality consequent on development.

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REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION, AMERICAN WATER WORKS ASSOCIATION AND WATER POLLUTION CONTROL FEDERATION (1960). *Standard Methods for the Examination of Water and Waste Water*, 11th edn. New York: American Public Health Association, Inc.

ANON (1956). *An Investigation of the Efficacy of Submarine Outfall Disposal of Sewage and Sludge*, Publication no. 14. California: State Water Pollution Control Board.

BRAND, P. A. J., KEMP, P. H., PRETORIUS, S. J. & SCHOONBEE, H. J. (1967). Water quality and abatement of pollution in Natal rivers. *Natal Town and Regional Planning Report*, Vol XIII, pt. 1 *et seq.*

COETZEE, O. J. & FOURIE, N. A. (1965). The efficiency of conventional sewage purification works, stabilization ponds and maturation ponds with respect to the survival of pathogenic bacteria and indicator organisms. *J. Proc. Inst. Sew. Purif.* pt 3.

DIFCO LABORATORIES (1953). *Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Products*, 9th ed. (reprinted 1963). Detroit: Difco Laboratories.

DIFCO LABORATORIES (1962). *Difco Supplementary Literature*. Detroit: Difco Laboratories.

LIVINGSTONE, D. J. (1965). An improved method for isolating salmonellae from polluted waters. *Publ. Hlth, Johannesburg* **65**, 87.

MCKEE, S. E. & WOLF, H. W. (1963). *Water Quality Criteria*. Publication 3-A. California: State Water Quality Control Board.

MINISTRY OF HEALTH (1956). The bacteriological examination of water supplies. *Rep. publ. Hlth med. Subj., Lond.* no. 71.

MOORE, B. (1954*a*). A survey of beach pollution at a seaside resort. *J. Hyg., Camb.* **52**, 71.

MOORE, B. (1954*b*). Sewage contamination of coastal bathing waters. *Bull. Hyg., Lond.* **29**, 689.

OLIFF, W. D., BERRISFORD, C. D., TURNER, W. D., BALLARD, J. A. & MCWILLIAM, D. C. (1967*a*). The ecology and chemistry of sandy beaches and nearshore submarine sediments of Natal. I. Pollution criteria for sandy beaches in Natal. *Water Res.* **1**, 115.

OLIFF, W. D., BERRISFORD, C. D., TURNER, W. D., BALLARD, J. A. & MCWILLIAM, D. C. (1967*b*). The ecology and chemistry of sandy beaches and nearshore submarine sediments of Natal. II. Pollution criteria for nearshore sediments of the Natal coast. *Water Res.* **1**, 131.

STANDER, G. J., OLIFF, W. D. & LIVINGSTONE, D. J. (1967). Problems in using the sea as part of a waste disposal system. Conference Paper no. 3. The Institute of Water Pollution Control: Annual Conference, June, 1967.

TAYLOR, E. W. (1958). *The Examination of Waters and Water Supplies* (Thresh, Beale and Suckling), 7th ed. London: J. A. Churchill Ltd.

THE OXOID DIVISION (1961). *The Oxoid Manual of Culture Media, including Ingredients and and other Laboratory Services*. 2nd ed. London: The Oxoid Division of Oxo Ltd.

WILSON, G. S. & MILES, A. A. (1964). *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th ed. London: Edward Arnold Ltd.

YOTAKIS, L. (1959). The bacteriology of bathing beaches and the counting of *Esch. coli* and *Strep. faecalis* in sea-water (Trans.). *Int. J. prophyl. Med. Soc. Hyg., Wien* **3**, 159.

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Air-movement control for treatment and isolation rooms

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A supply of filtered air has been shown to be effective in the reduction of airborne cross-infection in operating theatres (Shooter, Taylor, Ellis & Ross, 1956; Blowers, Mason, Wallace & Walton, 1955) and dressing rooms for burns (Lowbury, 1954). The provision of air conditioning, or even mechanical ventilation, in ward areas has not yet been generally accepted in Britain, but the ventilation of more specialized ward areas such as isolation and treatment rooms is becoming more common. In the case of isolation rooms, it is often thought necessary to keep patients in a bacteriologically clean environment and to prevent the movement of pathogenic micro-organisms to or from the isolation room. The isolation of transplant and other patients with low resistance to infection has received much attention lately, and Woodruff, Nolan, Bowie & Gould (1968) described an isolation unit designed for this type of patient. The same bacteriological requirements can be said to hold true for treatment rooms where the patients' wounds or infections can be dressed or inspected at a central point away from the rest of the ward activities.

The following series of tests were undertaken to assess and measure the factors which influence the control of air movement in these areas.

MATERIALS AND METHODS

The treatment room and isolation rooms were those at the Hairmyres Experimental Ward Unit, which is described in detail elsewhere (Report, 1968).

The treatment area

This area (as shown in Fig. 1) consisted of a treatment room, an air lock and a dirty utility room. The quality and quantity of the air supplied to and extracted from each room in the area could be accurately controlled. Normally, the treatment room had an air supply of 390 ft.^{3}/min. and the air lock 560 ft.^{3}/min., both being supplied through ceiling diffusers. The dirty utility room had an air extraction of 340 ft.^{3}/min. but no supply of air. A pass-through damper was fitted in the wall between the treatment room and the air lock; this could be opened or closed as required. Pressure relief flap dampers were fitted in the sliding doors between the air lock and the ward corridor.

Extract grilles were fitted at low level in the treatment room and the air lock. By varying the extraction rate in these areas various air movement control systems

could be obtained. The treatment room, with a constant air supply rate of 390 ft.^{3}/min. could be, with respect to the air lock: (*a*) positive—air extraction rate of zero; (*b*) balanced—air extraction rate of 390 ft.^{3}/min.; or (*c*) negative—air extraction rate of 780 ft.^{3}/min. This meant that for a positive system 390 ft.^{3}/min. flowed from treatment room to air lock via the pass-through damper and/or the door. For a negative system the direction of airflow was reversed and, under balanced conditions, there was no designed air flow through the doorway or pass-through damper. Similarly, the air lock could be positive or balanced with respect to the ward corridor.

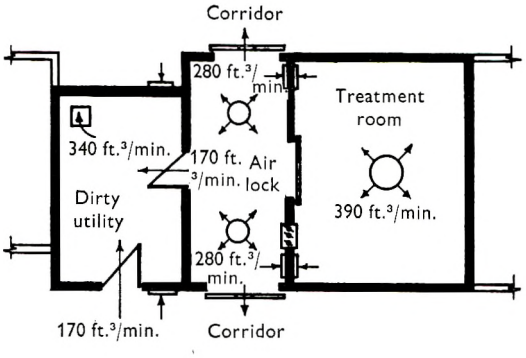

Fig. 1

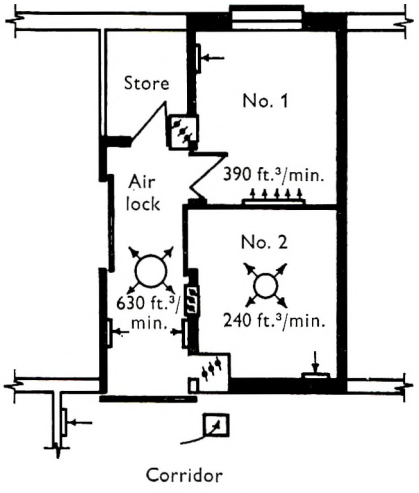

Fig. 2

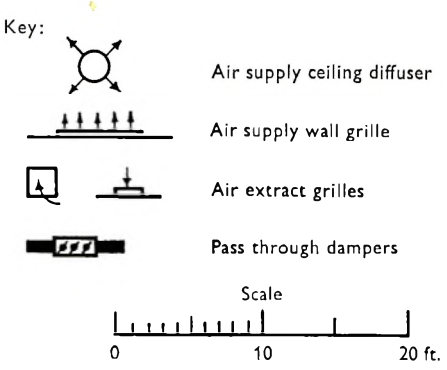

Fig. 1. The treatment area.
Fig. 2. The isolation rooms.

The system could also be operated at about two-thirds of its designed air-flow capacity when the supply rates to treatment room and air lock were 240 and 370 ft.^{3}/min. respectively. Tests were run under these conditions, the air extraction rates being reduced proportionately.

These air-supply rates of 390 and 240 ft.^{3}/min. were equivalent to a nominal 15 and 10 air changes/hr., respectively, in the treatment room under a balanced or

positive system. When a negative system was selected an additional 15 or 10 air changes/hr. were drawn in from the air lock to give a total of 30 or 20 air changes/hr.

The isolation rooms

The two isolation rooms, together with their associated air lock, are shown in Fig. 2. Isolation room no. 1 had an air supply of 390 ft.^{3}/min. and isolation room no. 2 had 240 ft.^{3}/min. These rates were the same as the alternative supply rates to the treatment room. The air lock had a constant supply of 630 ft.^{3}/min. Pass-through dampers were fitted between each of the isolation rooms and the air lock and between the air lock and the ward corridor.

By extracting air through grilles at low level in a similar way to that described above for the treatment area, each of the isolation rooms could be positive, balanced or negative with respect to the air lock and the air lock could be positive or balanced with respect to the corridor.

Method of assessing the efficiency of the air movement control systems

Nitrous oxide was used to simulate airborne bacteria throughout these tests. The gas was released at a constant rate over a period of 2 or 3 hr. in one room of the treatment or isolation areas. The atmosphere was sampled in both it and the adjacent rooms and the concentration of nitrous oxide determined by infra-red analysis. The tests were repeated under a variety of conditions. The efficiency of any system was given by

$$\text{system efficiency} = \left(1 - \frac{\text{gas concentration in the sampling room}}{\text{gas concentration in the source room}} \times 100\right)\%$$

this being an expression of the system's ability to prevent transfer of tracer gas to or from a room. This meant, for example, that if a gas concentration of 5 parts/million was measured in the sampling room, while the source room had 100 parts/million, the system efficiency would be 95%. Most of the tests were carried out with the rooms in normal use.

The use of bacteria as a tracer substance was considered less convenient in view of the large number of observations required to obtain reliable quantitative results. The use of nitrous oxide gas simulated the worst conditions possible as it did not allow for natural sedimentation or loss of viability. However, the distance the gas or bacteria would have to travel was only a few feet and the efficiencies quoted must be close to, although probably less than, those which would be achieved with naturally occurring bacteria.

RESULTS

The following variables were measured during each test: (*a*) the air supply and extract volumes; (*b*) the air flow through the doorway; (*c*) the length of time the door was open; (*d*) the number of times the door was opened and shut; (*e*) the temperature difference between the rooms.

It was thought that these variables might influence the systems' efficiencies. The results of all the treatment and isolation room tests were analysed by a

multiple regression technique on a K.D.F. 9 computer and the following over-all prediction equations obtained.

A variable was considered to influence the air flow through the door if it reached a 5 % level of significance.

Air supply rate = 390 ft.³/min.—treatment room and isolation room no. 1

Positive and negative schemes (air flow through door = 390 ft.³/min.):

$$\text{efficiency } (\%) = 98{\cdot}53 - 0{\cdot}027T \pm 1{\cdot}19C; \quad (1)$$

Balanced schemes:

$$\text{efficiency } (\%) = 93{\cdot}60 - 0{\cdot}105T \pm 3{\cdot}01C \quad (2)$$

Air supply Rate = 240 ft.³/min.—treatment room and isolation room no. 2

Positive and negative schemes (air flow through door = 240 ft.³/min.):

$$\text{efficiency } (\%) = 97{\cdot}87 - 0{\cdot}032T; \quad (3)$$

Balanced schemes:

$$\text{efficiency } (\%) = 89{\cdot}58 - 0{\cdot}158T, \quad (4)$$

where T = percentage time the door was open (this ranged from 0 to 100 % throughout the tests), and C = a comparison factor between isolation room no. 1 and the treatment room.

Temperature differences did not influence the results. This was not surprising as they had been kept to a minimum in this series of experiments. However, it would be wrong to discount this possible factor since room temperatures inevitably differ in practice. In a subsequent series of as yet incomplete tests conducted in the isolation rooms, it was found that a 5° F. temperature difference, in conjunction with a balanced system, would cause an air transfer of around 400 ft.³/min. in each direction through the fully opened doorway. Even when a flow of 390 ft.³/min. of air was passing through the doorway as would be the case with the positive or negative ventilation systems used here, a 5° F. temperature difference caused around 250 ft.³/min. of air flow in through the top of the doorway while 640 ft.³/min., i.e. 250 + 390 ft.³/min. of air passed out through the bottom.

Contrary to what one might expect, especially in the case of swing doors, it was found that the number of times the doors opened and shut had no significant influence on the efficiencies and gave no hint of doing so. Comparison of the swing and sliding doors showed that there was no difference between them so far as isolation efficiency was concerned.

The method of air distribution was found to influence the efficiencies of the systems. At the higher air supply rates and where ceiling diffusers were being used, air could be directed out through the open doorway. The use of high-level wall grilles (as in isolation room 1), which did not blow the supply air out of the door, gave improvements in efficiency of the order of 2 % for positive and negative systems and 6 % for balanced systems. This is indicated by the comparison factor C in equations (1) and (2). In the case of equation (1), the factor 1·19 should be added to the constant factor 98·53 to give the efficiency equation for isolation room no. 1. To obtain the corresponding equation for the treatment room, 1·19

should be subtracted from the constant factor. The same applies to equation (2), describing the balanced systems with an air supply rate of 390 ft.^{3}/min., the difference between the two being just over 6 % in this case, as stated above.

In addition to those tests previously described, where the release room and sampling room were next to one another, further tests were conducted where the tracer gas was released in the corridors. The purpose of those tests was to assess the effectiveness of the air lock.

The barrier afforded by the air lock was found to be fairly effective. As measured by the quantity of tracer gas detected in the air lock, its efficiency was about 90 %, falling to between 60 % and 70 % for the treatment area with two doors and to 84 % for the isolation area with one door, if the doors were left open. Samples taken in the treatment and isolation rooms simultaneously with those taken in the air lock indicated that the provision of an air lock did little to improve the over-all efficiency of the system. Under average-use conditions, over-all efficiency figures in the order of 88–95 % were obtained for gas released in the corridor. The corresponding figures for gas released in the air lock were in the range 83–99 %, depending on the system in operation. The over-all figures include systems with the air lock both positive and balanced with respect to the corridor, and with the treatment or isolation rooms both positive and balanced with respect to the air lock. The figures for systems with the room negative and the air lock balanced were not included since these systems are intended to prevent air escaping from the room. However, even in these cases, over-all efficiencies of the air lock in preventing air entering from the corridor into the isolation or treatment rooms were of the order of 75 %.

In another set of tests, tracer gas was released in the dirty utility room and sampled in the treatment room. It was found, under all the ventilation schemes, that the concentration in the treatment room rarely exceeded 1 % of that in the dirty utility room.

DISCUSSION AND CONCLUSIONS

A large demand exists for positively ventilated treatment and isolation rooms in order that patients susceptible to infection are not contaminated with pathogenic organisms from the ward environment. This demand has been satisfied in a number of new hospitals by supplying air to the room and allowing the air to be pushed out from the room through pressure-relief-flap dampers in the door. From our prediction equations it can be seen that this type of system will afford a very high degree of protection to the patient from external contamination. Provided the method of air distribution is satisfactory, and temperature differences are kept to a minimum, when the door is shut, efficiencies of over 99 % will be obtainable with an air supply rate of 390 ft.^{3}/min. (fifteen air changes), and over 97 % with an air supply rate of 240 ft.^{3}/min. (ten air changes). Even if the room door is left open all the time these efficiencies would not fall by more than about 3 %.

In new hospitals, provision is usually made for patients with infective areas to be nursed in isolation rooms. The patients' dressings may then be changed away from the ward environment. A report presented by the Newcastle Regional

Hospital Board (Report 1965) stated that in a general surgical unit 22 % of patients would require some form of isolation; 10 % because they were potential sources of infection. At present, few wards would have sufficient isolation rooms to meet these requirements. Where patients with infective areas must be dressed or inspected in a central treatment room we envisage three possible solutions.

The first of these is to provide a separate 'dirty' treatment room with a negative air-movement control system. In our experimental system negative ventilation was achieved by keeping the same air supply as under positive ventilation and extracting more air than was supplied. From our results an airflow of between 250 and 400 ft.^{3}/min. through the doorway would appear to give a high degree of protection to areas outside the room. With isolation rooms, an extract system would be sufficient to maintain isolation requirements but some air supply would be necessary to give comfortable conditions in the room. With treatment rooms an additional air supply would be necessary in order that the pathogenic bacteria disseminated from dirty areas could be quickly reduced to a reasonable level. In our case the total amount of air extracted under a negative ventilation system was 780 ft.^{3}/min., which was around 30 air changes/hr., 15 of them being filtered air. The use of a separate room for clean and infected wounds is uneconomical unless treatment rooms are centralized so that they serve a number of wards.

The second solution would be to equip a single treatment room with a ventilation system capable of providing a positive or a negative air-movement control scheme as the need arose. This would place the perhaps undesirable onus on the nursing staff of ensuring the correct system was in use.

The third solution would be to provide a balanced ventilation system to the treatment room. Although balanced systems are not quite so efficient, for the same rate of air supply, as positive and negative systems, they do have the advantage of simplicity, with fewer problems of design and maintenance. In addition, when the door is shut, the air within the room is fairly effectively sealed in, and the provision of an air lock to dilute contamination approaching or leaving it is not so necessary as with positive or negative systems. However, care must be taken to ensure that the system is correctly balanced, i.e. the same amount of air is extracted as supplied.

The test results indicated that, for efficiencies in the range 90–95 % one would require about double the air supply rate to achieve the same efficiency as the positive and negative systems. However, for higher efficiencies the difference in air supply rate would not be so great. A slight extrapolation of the test results indicates, for example, that a balanced system with an air supply rate of 520 ft.^{3}/min. would give about the same efficiency as positive and negative schemes with about 460 ft.^{3}/min. The reason for this is that the efficiency of isolation is mainly influenced by the air supply rate, and the higher this rate the less the effect of the duration of door opening.

An air lock was shown to be of little advantage in preventing the transfer of air in an already efficient system but would be of advantage in diluting the contaminants coming out of the room when under positive pressure or going into the room under a negative system. One of the main functions of an air lock may well be in

acting as an 'environmental lock', preventing adverse conditions of temperature and pressure affecting the rooms in question. Differences in temperature are expected to have a large effect and this 'environmental lock' would be necessary if rooms were installed in a naturally ventilated ward especially if a balanced system was contemplated.

We have so far discussed the requirements to ensure that there is no adverse movement of contamination in or out of rooms. What has not been considered is the quantity of air required to provide a 'safe' level of bacteria in the rooms. As a treatment room is only used for an hour or two each day and the ventilation system can be shut off at other times, or reduced in volume, it would probably be economical to supply air volumes of around 500–1000 ft.^{3}/min. to control the higher levels of bacterial contamination and the residual contamination carried over from patient to patient in these rooms; we found that the bacterial concentration was around 3–4 times higher in the treatment room than in the ward area and the time between patients' dressings very short. Using these large quantities of air a balanced system, although the least efficient of the three methods, would ensure almost absolute protection against the movement of bacteria across the door, provided the temperature differences were small. With such a balanced system it should not be necessary to provide an air lock in a mechanically ventilated ward. It is felt that it would still be advisable to dress the clean wounds first.

In isolation rooms, where the air will be supplied all the time, the choice of a ventilation scheme suitable for most purposes is complicated by the fact that between 250 and 500 ft.^{3}/min. of air would probably be regarded as an economical amount of air. With an air supply of less than 400 ft.^{3}/min. a balanced system would not give as full protection as a negative or positive system. The situation is further complicated by the variable use to which isolation rooms may be put. The decision as to what type of ventilation scheme should be used must ultimately depend on the type of patient to be nursed and the type of facility which may be economically provided. The lower the resistance of a patient to infection the greater the degree of protection which must be given. For the extremely critical situation a laminar flow system could be used (Michaelson, Vesley & Halbert, 1967).

Whatever ventilation scheme is chosen, the degree of elaboration built into the experimental systems we investigated would not be necessary, or indeed desirable, in the normal hospital. Their general complexity meant that in the treatment room the nurse had the option of five different ventilation schemes. A simple system which can be easily understood and seen to work would be much better.

SUMMARY

The degree of protection provided by various air movement control systems, as applied to isolation and treatment rooms, has been assessed. The main factors influencing the efficiency of isolation were the air supply rate to the room and the time the door was left open. The effect of some other possible factors, such as temperature differences and method of air distribution, are discussed.

The type of facilities provided must depend on the type of patient and economics,

but prediction equations, which allow the assessment of positive, negative and balanced ventilation systems, are presented. The ventilation requirements for isolation rooms should be assessed individually, but for a treatment room a balanced system of ventilation with between 500 and 1000 ft.^{3}/min. should be very effective. Precautions should be taken to ensure the air quantities are balanced and temperature differences are minimized. An air lock should not be necessary for balanced systems provided these rooms are in a mechanically ventilated ward.

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Requests for reprints, or a fuller description of the ward unit and test results, should be addressed to George Baird, Building Services Research Unit, 3 Lilybank Gardens, Glasgow, W.2.

REFERENCES

BLOWERS, R., MASON, G. A., WALLACE, K. R. & WALTON, M. (1955). Control of wound infection in a thoracic surgery unit. *Lancet* ii, 786.

LOWBURY, E. J. L. (1954). Air conditioning with filtered air for dressing burns. *Lancet* i, 292.

MICHAELSON, G. S., VESLEY, D. & HALBERT, M. M. (1967). Laminar flow studied as aid in care of low resistance patients. *Hospitals* **41**, 91.

REPORT (1965). Isolation requirements for surgical units. *Lancet* ii, 895.

REPORT (1968). *The Hairmyres Project—Experiments in Hospital Ward Ventilation*. Glasgow: Building Services Research Unit.

SHOOTER, R. A., TAYLOR, G. W., ELLIS, G. & ROSS, J. P. (1956). Postoperative wound infection. *Surgery Gynec. Obstet.* **103**, 257.

WOODRUFF, M. F. A., NOLAN, B., BOWIE, J. H. & GOULD, J. C. (1968). The Nuffield Transplantation Surgery Unit. *Lancet* i, 905.

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The effect of the tapeworm *Hymenolepis nana* on immunity to tuberculosis in mice

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Some years ago we began a study of BCG-induced immunity to tuberculosis in mice. At first we had some unexpected results, apparently because the animals were naturally infected with the tapeworm *Hymenolepis nana*. Since then a number of papers have appeared which may support the inferences made at that time. These were that the tapeworm may exert a significant effect on the course of experimental tuberculosis by decreasing the survival times of male mice, and thus causing an unequal response between the sexes.

MATERIALS AND METHODS

Tubercle bacilli

The BCG strain was obtained from Dr Mary Ann LeVan, Henry Phipps Institute, Philadelphia. The Vallée strain was obtained from Mr J. Albericci, Porton, Wilts. Both were grown for 7 days in Dubos Tween-albumin medium and were diluted in 0·1 % bovine albumin for intravenous injection.

Mice

Two strains of mice were used. The BALB/c strain was bred from stock supplied by the Laboratory Animals Centre, Carshalton. The FF strain had been obtained many years earlier from Glaxo Laboratories. Mice were given pelleted diet and water *ad lib.*

Tapeworm egg counts

From each mouse faeces were collected and weighed. Five hundred mg. of pellets were placed in 1 ml. of distilled water and roughly broken up by incubating at 37° C. with frequent agitation. Formalin was added to 10 % and incubation was continued to hasten sterilization, in case the faeces were contaminated by tubercle bacilli. The faeces were then thoroughly emulsified on an MSE homogenizer; 3·5 ml. of saturated brine was added and the mixture was thoroughly shaken. Three samples of each mixture were collected, each immediately after shaking, and placed into specially constructed counting chambers. The eggs floated to the surface of the liquid, and were counted over a measured area of the slide. The eggs counted were of course derived from a measured sample of the faecal mixture, and the results could have been converted to eggs per gramme of faeces by multiplying by 83·3. The method gave reasonably reproducible results.

RESULTS

Challenge dose

We wished to find a dose of the Vallée strain which would kill unvaccinated mice in the 20- to 30-day survival range. Since a preliminary test suggested that about 0·2 ml. of week-old culture might be suitable, doses of this order were tested in larger groups of mice.

Three groups of ten mice each (half male, half female; all 8-week-old FF strain) were challenged with different doses (0·4, 0·2 and 0·1 ml.) of 7-day culture of the Vallée strain. The survival time of each mouse after challenge was recorded in days.

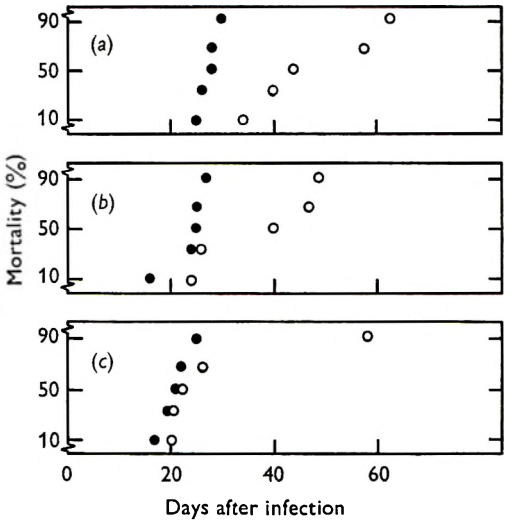

Fig. 1. Survival times of male and female FF strain mice, after intravenous challenge with three different doses of Vallée strain. (*a*) 0·1 ml.; (*b*) 0·2 ml.; (*c*) 0·4 ml. ●, male; ○, female.

Survival times are presented in Fig. 1. With all doses the mean survival time of the females was greater than that of the males—significantly so ($0{\cdot}05 > P > 0{\cdot}02$) at the 0·1 ml. dose.

Now it appears that there is no recorded instance of a mouse strain in which the females are noticeably more resistant to tuberculosis than the males. Hoyt, Moore, Knowles & Smith (1957) and Youmans & Youmans (1959) reviewed the literature and concluded that for most mouse strains the susceptibilities of the sexes was the same, although in some strains the males outlived the females after intravenous challenge. It therefore seemed possible that either we had an unusual strain of mice, or the apparent superiority of the females in this experiment was due to some non-specific factor. This was investigated later.

Apart from this unexpected finding it seemed that 0·2 ml. of Vallée culture should be tried for future experiments on FF strain mice of similar age.

Response to BCG vaccination

Having found a suitable challenge dose of the Vallée strain for the FF mice, we now wished to find whether BCG vaccination significantly prolonged the survival times of mice challenged with this dose. If so, the FF strain would be suitable for further studies on acquired immunity to tuberculosis.

A group of 20 mice (half male, half female, all 6-week-old FF strain) were vaccinated with $0{\cdot}2 \times 10^{-2}$ ml. of BCG. Twenty unvaccinated controls received diluent only. All animals were challenged twenty eight days after vaccination with 0·2 ml. of Vallée strain. The survival time of each mouse after challenge was recorded in days (Fig. 2).

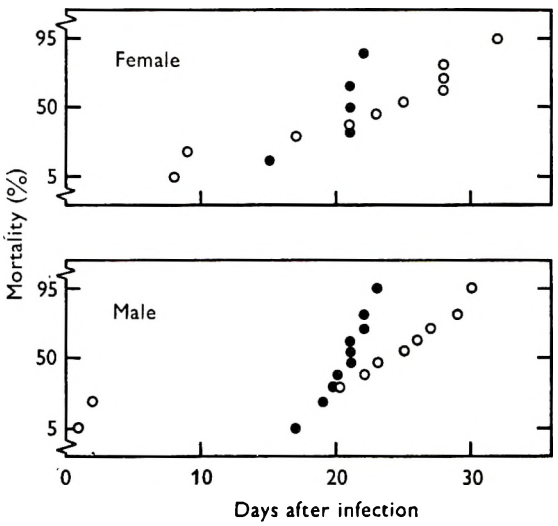

Fig. 2. Survival times of vaccinated and unvaccinated mice of each sex, after intravenous challenge with Vallée strain. ●, Unvaccinated; ○, vaccinated.

These mice were housed in temporary quarters and five control females in one cage died of overheating; their survival times are not recorded. Moreover, four of the vaccinated mice survived only 9 days or less. It was found that these mice, and some others, were suffering from heavy infestations with the tapeworm *Hymenolepis nana*. In view of these accidental interferences, we did not attempt to evaluate the statistical significance of the result. We noted, however, that the average survival time of the remainder of the vaccinated animals was somewhat greater than that of the control.

Tapeworm burdens of mice

In the vaccination experiment above, the deaths of two mice within 2 days of challenge were attributed to heavy infestations with *H. nana*. However, as the animals had previously appeared quite healthy, it seemed that the added stress of challenge was partly responsible for their deaths.

The routine practice in this Department was to segregate the sexes at weaning into different compartments in a battery of cages. The cleaning of the cages in use at

that time may have been inefficient, and, in that case, the cages may have been unequally contaminated with helminth eggs. This suggested a means whereby males and females could be unequally subjected to a non-specific factor. If *H. nana* infestations affected the survival time of tuberculous mice, here was a possible explanation for the sex difference in survival time demonstrated in the first experiment. Therefore an attempt was made to assess the tapeworm burden of each of the remaining mice in the vaccination experiment. This was done by counting the eggs in samples of faeces on day 3 of that experiment.

The mean egg counts for the four groups of mice are illustrated in Fig. 3. The counts were higher in the males than in the females. Hunninen (1935a) noted that female mice tend to be more resistant than males to *H. nana* infestation.

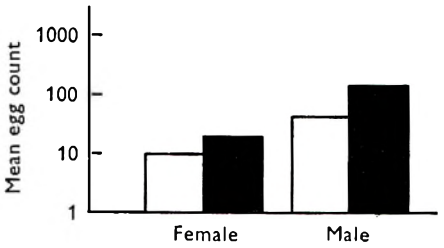

Fig. 3. Mean counts of *Hymenolepis nana* eggs in the faeces of groups of vaccinated and unvaccinated mice of each sex. The values in the figure, when multiplied by 83·3, give the mean numbers of eggs per gramme of faeces for the group. □, Unvaccinated; ■, vaccinated.

It is of further interest that for each sex the vaccinated mice had higher egg counts than did the non-vaccinated. Hunninen (1936) found that *H. nana* infestations become heavier with concurrent paratyphoid infections. It seems that BCG infections similarly may provide sufficient stress to allow *H. nana* populations to increase.

The greatest difference (between vaccinated males and unvaccinated females) illustrated in Fig. 3 is of probable significance ($P = 0{\cdot}05$). Thus it appears that *H. nana* infestation might provide a non-specific stress that would operate unequally in vaccinated and control mice and in the two sexes. Moreover, it appeared that mice with heavy worm burdens died sooner than others. In some cases intussusception caused by massive worm infestation was the obvious cause of death. We appeared to be faced with a non-specific condition which could seriously interfere with tuberculosis immunity studies.

Experimental Hymenolepis nana *infection*

At this stage attempts to test the suitability of the FF strain for acquired immunity studies in tuberculosis had been thwarted by two unexpected findings: an apparent difference in the resistance of the sexes to tuberculosis, and non-specific infestation with *H. nana*. It now seemed probable that the tapeworm infestation was the cause of the apparent sex difference. Possibly our husbandry practices would cause unequal exposure of the sexes to tapeworm infestation after weaning. Alternatively the observed superiority of the female mouse in resistance

to *H. nana* infestation (Hunninen, 1935*a*) could be responsible. This latter possibility was interesting and it was investigated experimentally.

A group of BALB/c strain mice (half male, half female) were reared tapeworm-free by Hunninen's method (Hunninen, 1935*a*). When 8 weeks old, ten of each sex were given about 2000 eggs of *H. nana* by mouth (Hunninen, 1935*b*). The eggs were recovered from the faeces of infested mice and washed by repeated centrifugation in water. Control mice, ten of each sex, were given supernatant from the same suspension. Subsequent examination of faecal samples showed that *H. nana* infestation had been successfully induced in the test groups. The controls remained free. Twenty-one days after infestation five mice of each sex-treatment group were

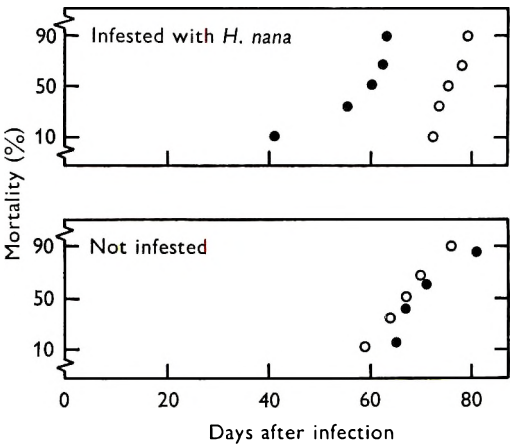

Fig. 4. Survival times of mice infested with *Hymenolepis nana* and of uninfested controls, after intravenous challenge with Vallée strain. ●, Male; ○, female.

challenged with 0·2 ml. of 50-fold dilution of Vallée culture. The rest were kept as unchallenged controls. The survival times were recorded in days.

All mice not challenged with tubercle bacilli, whether infested with *H. nana* or not, survived indefinitely and were killed in good condition at 150 days. The survival times of the rest—those challenged with tubercle bacilli—are shown in Fig. 4.

Whereas, in the absence of *H. nana* infestation the resistance of the sexes to tuberculosis was similar, in the tapeworm-infested group the males died significantly earlier than the females ($0{\cdot}01 > P > 0{\cdot}001$). This and previous findings suggested that the influence of *H. nana* should be avoided in tuberculosis immunity experiments. Accordingly, for further studies it was decided to use mice from another source, to change the diet, and to use only one sex. When this was done no further trouble was experienced with *H. nana*.

DISCUSSION

We have presented evidence that *H. nana* may influence the course of experimental tuberculosis. First, intussusception and acute enteritis killed some tuberculous, but not non-tuberculous, mice which harboured the tapeworm. Second, the experimental infestation showed that the tapeworm caused male mice to die earlier

than females following challenge with tubercle bacilli. Conversely, BCG infection seemed to alter resistance to *H. nana* as judged by faecal egg counts.

Bailenger, Roger & Pautrizel (1964) showed that female mice were more resistant than males to *H. nana*, as judged by the number of worms they harboured, and that this difference was accentuated by injecting males with testosterone proprionate or females with oestradiol benzoate. Beck (1951*a*) found that the production of eggs by individual *Hymenolepis diminuta* was greater in male rats than in females, even when the worms were approximately the same size. Later (1951*b*) Beck showed that egg production was lowered by castration of male hosts; it could be restored to the previous level by daily injections of testosterone. Addis (1946) demonstrated that worms of *H. diminuta* were smaller in immature or castrated male rats, and that this effect was overcome by daily injections of testosterone. On the other hand, immaturity or castration of females did not affect the size of the *H. diminuta* they harboured. If one assumes that carriage of large worms, infestation by a large number of worms, and high output of worm eggs are all indices of lowered resistance of the host, then these reports suggest that the resistance of the male rodent may be lessened by these tapeworms.

Similarly, our experimental infestation lowered the resistance of male mice to tuberculosis. This effect might be explained by a direct effect of *H. nana* on the non-specific resistance mechanisms of the host, similar to those reported by the other authors just mentioned.

We have not attempted to investigate further the precise mechanisms involved. However, it seems likely that the interaction is mediated by cellular rather than humoral mechanisms. There is now general agreement that immunity to mycobacteria is cell-mediated, and evidence is accumulating that cellular factors are concerned in resistance to helminths (Larsh, 1967). Indeed transfer of immunity to *H. nana* by spleen cells has been demonstrated by Friedberg, Neas, Faulkner & Friedberg (1967). However, since the cells concerned would include both macrophages and lymphocytes, their work did not demonstrate that the transferred immunity was not mediated by humoral antibody.

SUMMARY

Mice which carried the tapeworm *Hymenolepis nana* gave anomalous results when they were infected with tubercle bacilli. Males died earlier than females. The cause of death in some was intussusception or acute enteritis. More tapeworm eggs were excreted in the faeces of male mice, and, for each sex, egg counts were higher in BCG-vaccinated animals.

This work forms part of the studies on immunity to tuberculosis for a Ph.D. thesis, and was carried out while I was in receipt of a Harrison-Watson Studentship of Clare College and a Wellcome Fellowship of the Animal Health Trust.

REFERENCES

ADDIS, C. J. (1946). Experiments on the relation between sex hormones and the growth of tapeworms (*Hymenolepis diminuta*) in rats. *J. Parasit.* **32**, 574.

BAILENGER, J., ROGER, G., & PAUTRIZEL, R. (1964). Etude de l'immunité des rongeurs a l'égard d'*Hymenolepis nana*. *Annls Parasit. hum. comp.* **39**, 33.

BECK, J. W. (1951*a*). Effect of diet upon singly established *Hymenolepis diminuta* in rats. *Expl Parasit.* **1**, 46.

BECK, J. W. (1951*b*). Effect of gonadectomy and gonadal hormones on singly established *Hymenolepis diminuta* in rats. *Expl Parasit.* **1**, 109.

FRIEDBERG, W., NEAS, B. R., FAULKNER, D. N. & FRIEDBERG, M. H. (1967). Immunity to *Hymenolepis nana*: transfer by spleen cells. *J. Parasit.* **53**, 895.

HOYT, A., MOORE, F. J., KNOWLES, R. G. & SMITH, C. R. (1957). Sex differences of normal and immunised mice in resistance to experimental tuberculosis. *Am. Rev. Tuberc. pulm. Dis.* **75**, 618.

HUNNINEN, A. V. (1935*a*). Studies on the life history and host–parasite relations of *Hymenolepis fraterna* (*H. nana* var. *fraterna*, Stiles) in white mice. *Am. J. Hyg.* **22**, 414.

HUNNINEN, A. V. (1935*b*). A method of demonstrating cysticercoids of *Hymenolepis fraterna* (*H. nana* var. *fraterna*, Stiles) in the intestinal villi of mice. *J. Parasit.* **21**, 124.

HUNNINEN, A. V. (1936). An experimental study of internal autoinfection with *Hymenolepis fraterna* in white mice. *J. Parasit.* **22**, 84.

LARSH, J. E. (1967). Delayed (cellular) hypersensitivity in parasitic infections. *Am. J. trop. Med. Hyg.* **16**, 735.

YOUMANS, G. P. & YOUMANS, A. S. (1959). The relationship of sex to the susceptibility of normal and immunised mice to tuberculosis. *Am. Rev. resp. Dis.* **80**, 750.

J. Hyg., Camb. (1969), **67**, 241
With 1 *plate*
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Detoxification of an immunogenic fraction from a culture filtrate of *Pseudomonas aeruginosa*

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(*Received* 12 *September* 1968)

INTRODUCTION

In a recent communication (Jones, 1968) it was shown that burned mice infected with virulent strains of *Ps. aeruginosa* were protected from septicaemia and death by active immunization with fractions separated from culture filtrates of *Ps. aeruginosa*. The mice were immunized during a 3-week period before burning and infection. The protective fractions contained molecules of high molecular weight and were separated from 5-day aerated batch cultures of *Ps. aeruginosa* by gel filtration through Sephadex G-200 (Carney & Jones, 1968).

Immunization with the Sephadex G-200 fraction protected mice against the strain from which the immunizing fraction had been made and also against strains of different serotype. It was found that a fraction from an avirulent serotype of *Ps. aeruginosa* protected burned mice against virulent strains of *Ps. aeruginosa* of different serotype (Jones, 1968).

The Sephadex-separated fractions killed mice at dosages 400 times those of the protective immunizing dosages (Carney & Jones, 1968). Their usefulness as potential human immunogens would be greatly enhanced if their lethal and toxic effects could be reduced without loss of protective properties. Further refinement of the Sephadex G-200 fractions by gel filtration through Sepharose-4B was shown by Jones (1968) to produce immunizing fractions that were less lethal.

In this study an attempt was made to detoxify an immunogenic fraction from a culture filtrate of a virulent strain of *Ps. aeruginosa* (strain B4), by treating the fraction with alcohol or formalin solutions.

A Sepharose-4B fraction was further refined by gel filtration through Sepharose-2B; this produced a fraction (*b*) that gave a single precipitation band on immunodiffusion (Carney & Jones, 1968). The lethal and toxic effects of treated and untreated Sepharose-2B fractions were determined by intraperitoneal inoculation of mice, and the protective properties of the fractions were assessed in burned mice using techniques described by Jones (1968).

MATERIALS AND METHODS

Isolation of the immunogenic fraction

The strain B4 from which the immunogen was separated was isolated from a patient with burns and had a phage-typing pattern of 21/68/Col.21/+ and a serotype of 2/5 (B.T. Thom, personal communication).

Ps. aeruginosa (B 4) was grown for 5 days in an aerated batch culture containing 20 l. of a synthetic dialysable medium (Carney & Jones, 1968). After growth the bacteria were removed by centrifugation and positive pressure filtration (GS Millipore membrane); the volume of the culture was reduced to 1 l. by removing water at 20° C. in a circulatory cyclone evaporator; the unused medium constituents were removed by dialysis; the volume was further reduced to 100 ml. by ultrafiltration through cellophane and the contents of the sac were freeze-dried. The freeze-dried material was separated into fractions of different molecular size by gel filtration through G-200 Sephadex, Sepharose-4B and Sepharose-2B (Pharmacia Fine Chemicals, Uppsala). A fraction that was partly retained by Sepharose-2B, with a Kav value between 0·28 and 0·79, was used in this study.

Detoxification of pseudomonas immunogens

Alcohol precipitation. Thirty ml. of absolute alcohol containing a few drops of a saturated solution of sodium acetate were added to a solution of 10 mg of the Sepharose-2B fraction in 10 ml. of distilled water. After shaking the solution was left at 4° C. for 18 hr. and the precipitate was deposited by centrifugation. The precipitate was washed twice in absolute alcohol, dried at room temperature and redissolved in saline.

Formolization. Two methods were used.

(*a*) Ten mg. of the Sepharose-2B fraction from strain B 4 was dissolved in 10 ml. of isotonic saline containing 0·1 % formalin solution and stored for 7 days at 4° C. before use.

(*b*) Ten mg. of the Sepharose-2B fraction from strain B 4 was dissolved in 10 ml. of isotonic saline containing 0·5 % formalin solution. After 7 days storage at 4° C. the formolized fraction was dialysed overnight against saline.

Toxicity tests

The methods described by Carney & Jones (1968) were used.

Protection tests

Groups of mice, weighing 10–12 g. at the beginning of the experiment, were inoculated intraperitoneally with 0·1 mg./kg. of either formolized, alcoholized or untreated B 4 Sepharose fraction, once a week for 3 consecutive weeks. Ten days after the last inoculation, mice were depilated and burned in a standard way (Jones & Lawrence, 1964), then challenged with 0·1 ml. of a saline suspension of *Ps. aeruginosa* containing 700×10^6 organisms, spread over the surface of the burn as described by Jones, Jackson & Lowbury (1966). Half of each group of immunized mice was challenged with the same strain of *Ps. aeruginosa* from which the fraction had been prepared (B 4), the other half with a different virulent strain of *Ps. aeruginosa* (P 14) of serotype 6*c* and phage typing pattern 16/13/24/F 8/119X/352/1214/4 obtained from Dr M. T. Parker.

RESULTS

Toxicity of alcoholized, formolized and untreated Sepharose fractions from Pseudomonas aeruginosa *strain B4*

The number of mice which died after receiving a single intraperitoneal inoculation of various concentrations (80, 40, 20 10, 5 and 1·0 mg./kg.) of alcoholized, formolized (0·1 % and 0·5 %) and untreated Sepharose-2B fraction from strain B4, is shown in Table 1, together with LD50 of the four preparations, which were calculated from dose–response curves constructed from the percentage mortalities and dosages shown in Table 1.

Table 1. *Toxicity for mice of pseudomonas immunogens treated with alcohol and formalin solutions*

Treatment of immunogen	Dose of immunogen (mg./kg.)*						LD 50 of immunogen
	80	40	20	10	5	1	
Alcohol	3/3†	3/4	3/5	0/5	0/5	0/5	18·0
Formalin 0·1%	3/3	0/5	1/5	0/5	0/5	0/5	59·0
Formalin 0·5%	1/3	0/5	0/5	0/5	0/5	0/5	> 80·0
Untreated	3/3	2/4	1/5	0/5	0/5	0/5	40·0

* Given as a single intraperitoneal inoculation in 1·0 ml of saline.
† Number of mice dying/number inoculated.

Treatment of the Sepharose-2B fraction with 0·1 % and 0·5 % formalin solution was found to reduce its lethal effect for mice; the LD50 for the 0·1 % formolized fraction was 59·0 mg/kg, the LD50 for the 0·5 % formolized fraction was > 80 mg./kg. compared with an LD50 of 40 mg./kg. of the untreated fraction. Treatment of the Sepharose-2B fraction with alcohol lowered the LD50 of the fraction from 40 mg./kg. to 18 mg./kg., thereby increasing the lethal effect of the fraction more than twofold.

Even though few mice died in the groups of mice challenged with the 0·1 % formolized fraction, these mice, together with those challenged with the alcoholized and untreated fraction, showed external signs of illness—immobility, refusal of food and water, ruffled appearance and exudate in eyes (Plate 1)—at dosages of 10 mg./kg. and above. The external symptoms became worse during the 24 hr. period after challenge, reaching a maximum between 24 and 30 hr. after challenge. The mice which survived for 2 days returned to a normal rate of growth, reflected by a steady increase in body weight (Fig. 1), by the 3rd–5th day after a 40 mg./kg. challenge and by the 2nd day after a 1 mg./kg. challenge.

The groups of mice challenged with 40 mg./kg. of 0·5 % formolized fraction showed only slight external symptoms of illness 24 hr. after challenge and no signs of illness 48 hr. after challenge. Mice challenged with 1·0 mg./kg. of 0·5 % formolized fraction showed no external signs of illness. However, the body weights of mice challenged with both 40 mg./kg. and 1·0 mg./kg. of 0·5 % formolized fraction fluctuated in a similar way to the weights of mice receiving comparable dosages of

other preparations of Sepharose-2B fractions (Fig. 1), showing that even though the external symptoms resulting from the challenge had been reduced by treatment with 0·5 % formalin, the fraction still affected the mice systemically.

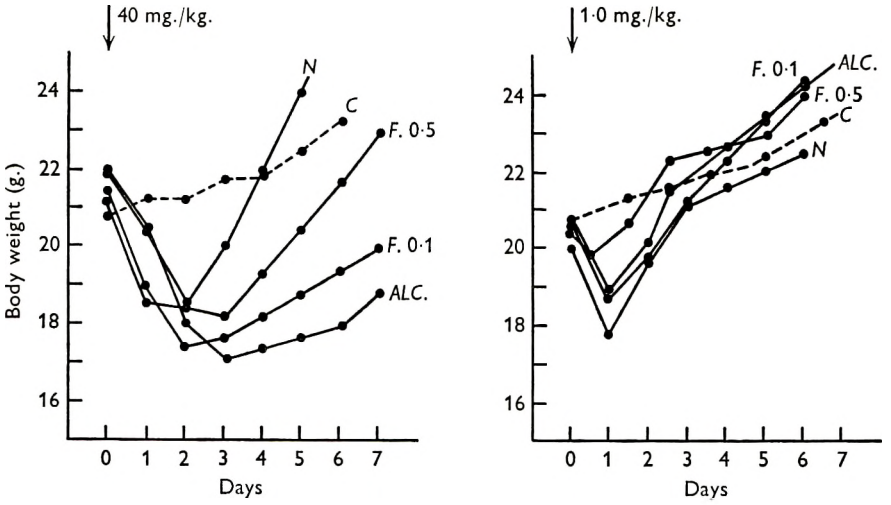

Fig. 1. Comparison of mean weights of groups of mice after intraperitoneal inoculation with fractions from a pseudomonas culture filtrate that have been treated with alcohol or formaldehyde. *C*, Uninoculated mice; *N*, mice inoculated with untreated fraction; *F* 0·5, mice inoculated with fraction treated with 0·5 % formalin solution; *F* 0·1, mice inoculated with fraction treated with 0·1 % formalin solution; *ALC*, mice inoculated with fraction precipitated with alcohol.

Protection against Pseudomonas aeruginosa *infection with pseudomonas immunogens*

Table 2 shows how effective four immunizing fractions, prepared from the Sepharose-2B fraction from strain B4, were in protecting burned mice from pseudomonas septicamia. Different groups of mice were immunized weekly for 3 weeks with formalin-treated (0·1 % or 0·5 %), alcohol-treated or untreated Sepharose-2B fraction. After immunization the mice were given whole-skin-thickness burns of approximately 5 % of body surface and challenged by infecting the surface of the burn with virulent strains of *Ps. aeruginosa*, B4 or P14. All mice given untreated or formalin-treated (0·1 % or 0·5 %) immunogen survived. The same challenge was found to kill 80 % of unimmunized burned control mice (Table 2). In the same experiment it was found that the alcohol-precipitated fraction was a less effective immunogen than the untreated or formolized fractions, as 20 % of the mice immunized with the alcohol-precipitated fraction then burned and challenged with strains P14 and B4 died. The mean survival times (Table 2) of the mice which were immunized with the alcohol-precipitated fraction indicates that the mice which died received some protection against pseudomonas invasion, since they survived 2–3 days longer than unimmunized mice which were burned and infected in a similar way.

Table 3 summarizes an experiment in which an attempt was made to find out

Table 2. *Protective efficacy of pseudomonas immunogens treated with alcohol and formalin*

Treatment of immunogen	Immunizing dose* (mg./kg.)	Infecting strain	Mouse mortality	Mean survival time days	No. of mice showing *Pseudomonas* in heart blood
Alcohol	0·1	B 4	2/9†	5·0	2
		P 14	2/10	6·0	2
Formalin 0·1 %	0·1	B 4	0/10	—	—
		P 14	0/10	—	—
Formalin 0·5 %	0·1	B 4	0/9	—	—
		P 14	0/10	—	—
Untreated fraction	0·1	B 4	0/9	—	—
		P 14	0/10	—	—
Unimmunized controls	None	B 4	8/10	3·7	8
		P 14	8/10	2·7	8

* Given once a week for three consecutive weeks before burning and infection.
† Number of mice dying/number treated.

Table 3. *Determination of the minimum protective dose of pseudomonas immunogen treated with* 0·5 % *formalin solution*

(The mice were all infected, after burning, with *Pseudomonas aeruginosa*, strain B 4)

Treatment of immunogen	Immunizing dose (mg./kg.)	Mouse mortality	Mean survival time days	No. of mice showing *Pseudomonas* in heart blood
None	0·1	1/10*	(5·0)	1
	0·01	1/10	(6·0)	1
	0·001	4/9	4·3	4
Formalin 0·5 %	0·1	0/10	—	—
	0·01	3/10	7·0	3
	0·001	1/10	(9·0)	1
Control	None	7/10	4·2	7

* Number of mice dying/number treated.

whether treatment of the protective Sepharose-2B fraction with 0·5 % formalin had altered its immunizing properties. Groups of mice were immunized with different dilutions (0·1, 0·01 and 0·001 mg./kg.) of 0·5 % formolized fraction and the protective properties of these fractions were compared with the protective properties of similar dilutions of untreated fractions, by challenging the immunized mice, after burning, with a suspension of *Ps. aeruginosa* (B4) that killed 70 % of the unimmunized burned control mice.

The dose of untreated fraction that effectively protected 50 % (EPD50) of the mice against pseudomonas septicaemia was approximately 0·001 mg./kg. mouse; the EPD50 of the 0·5 % formolized fraction was less than 0·001 mg./kg. mouse.

DISCUSSION

These experiments have shown that treatment with 0·5% formalin of a protective fraction from a culture filtrate of *Ps. aeruginosa* reduced both its lethal and systemic toxic effects and seemed to improve its protective properties for burned mice infected with virulent strains of *Ps. aeruginosa*. Other treatments—0·1% formalin solution and alcohol precipitation—were less effective than 0·5% formalin solution in reducing the lethal effects in mice.

Alcohol precipitation increased the lethal effects of the fraction for mice (LD 50 18 mg./kg.) and treatment with 0·1% formalin solution had only a small detoxifying effect on the fraction, raising the LD 50 from 40 mg./kg. to 59 mg./kg. After treating the fraction with 0·5% formalin solution only one mouse out of three was killed by the largest dose (80 mg./kg.) used in these experiments, and even though only small numbers of mice were used, the toxicity tests show that treatment with 0·5% formalin solution reduces the lethality by at least 50% compared with the untreated fraction.

Apart from reducing the lethal effects of the fraction, treatment with 0·5% formalin solution also reduced the duration of some of the systemic toxic side-effects of the fraction (exudates in eyes, ruffled appearances and lethargy).

In mouse protection experiments the lethal and toxic effects of the fractions were of little practical importance as the smallest protective doses used were well below (over 80,000 times) the lethal or systemic toxic doses. Mice immunized with 0·1 mg./kg. of untreated 0·1% and 0·5% formolized fractions given weekly for 3 weeks, received better protection against death from pseudomonas septicaemia than mice immunized with a similar dosage of alcoholized fraction. At lower immunizing doses (0·001 mg./kg.) the fraction treated with 0·5% formalin solution gave better protection against the strain from which it was prepared than the untreated fraction, and protected 9/10 mice from pseudomonas septicaemia compared with 5/9 mice that were immunized with the untreated fraction.

These preliminary experiments are encouraging since they suggest that simple chemical procedures may reduce the toxicity hazard of immunogens from culture filtrates of *Ps. aeruginosa* without impairing their immunizing properties, and this should considerably improve their usefulness as potential human immunogens.

SUMMARY

Mice immunized with a fraction from a culture filtrate of *Ps. aeruginosa*, then burned and infected with virulent serotypes of *Ps. aeroginosa*, were protected from septicaemia and death.

The immunogenic fraction killed mice at dosages 400 times those of the minimum protective dosages.

Treatment of the immunogenic fraction with 0·5% formalin reduced its lethal effect for mice and slightly improved its immunizing potency so that the difference between the smallest immunizing dose used and the smallest amount that killed mice was more than 80,000 doses.

REFERENCES

CARNEY, S. A. & JONES, R. J. (1968). Biological and immunochemical properties of culture filtrates of virulent and avirulent strains of *Pseudomonas aeruginosa*. *Br. J. exp. Path.* **49**, 395.

JONES, R. J. (1968). Protection against *Pseudomonas aeruginosa* infection by immunization with fractions of culture filtrates of *Ps. aeruginosa*. *Br. J. exp. Path.* **49**, 411.

JONES, R. J., JACKSON, D. M. & LOWBURY, E. J. L. (1966). Antiserum and antibiotic in the prophylaxis of burns against *Pseudomonas aeruginosa*. *Br. J. plast. Surg.* **19**, 43.

JONES, R. J. & LAWRENCE, J. C. (1964). Studies on extracts of heated and normal skin. *Br. J. exp. Path.* **45**, 198.

EXPLANATION OF PLATE

Appearance of mouse 2 days after intraperitoneal inoculation of 40 mg./kg. of a fraction separated by Sepharose-2B from a culture filtrate of *Pseudomonas aeruginosa* B4.

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Cross-contamination by cooked-meat slicing machines and cleaning cloths

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In recent years two large food-borne outbreaks of salmonella infection have stressed the importance of cooked-meat slicing machines, among other things, in the spread of salmonellas from contaminated meat products to other meats. Since many different cold meats may be sliced during the day on one machine, a contaminated product can constantly recontaminate the machine, thereby increasing the spread of the organism to other meats. Such an accident was likely in the Aberdeen typhoid outbreak (Report, 1964), in which a history of eating various cold meats from the infected shop was given by 373 patients who remembered the association out of the 507 recorded cases (Walker, 1965). The second outbreak occurred in Washington, U.S.A., in May–June 1965, in which there were 356 known cases of salmonellosis due to *Salmonella meleagridis*, all related to one combined caterer–delicatessen–restaurant (Kaufmann, Hayman, Heath & Grant, 1968). The same serotype was isolated from many of the patients, from 64 of 115 employees, from 17 of 29 different types of ready-to-eat foods examined including several cold meats (e.g. corned beef, roast beef, tongue and salami), and from the environment; as might be expected, the slicing machines concerned were found to be contaminated with *S. meleagridis*—one by repeat sampling after what was thought to be an adequate cleaning and disinfection programme. The purpose of this paper is to provide laboratory evidence of the obvious—that a contaminated slicing machine will easily cross-contaminate other products passed through it.

MATERIALS AND METHODS

Sealed cans of corned beef (2·7 kg.), chopped pork (1·8 kg.) and brisket of beef (1·8 kg.) were opened aseptically when required. To facilitate slicing, these canned products were stored for 3 days at 4° C. before opening. The salami (0·9 kg.) used was in the form of a sealed sausage.

Cross-contamination experiments

A central core of meat (6 cm. long, 2 cm. diam.) was removed from one end of half the contents of a can of chopped pork using a sterile cork-borer. The core was inoculated in several parts with a total of 6×10^9 coagulase-positive staphylococci using a syringe with a wide-bore needle, replaced in the hole on the chopped pork and pressed gently down. The chopped pork was then sliced on a cooked-meat

slicing machine (Asco G.2 gravity-feed slicer), cutting the inoculated piece of chopped pork first. The same machine was then used for cutting 20 slices of chopped pork from the uninoculated half of the can, 10 slices of corned beef, 10 slices of brisket of beef and 10 slices of salami. After homogenizing some of the slices of each meat in separate Atomix beakers, approximately 2–3 g. samples of each were inoculated into five bottles of cooked-meat medium containing 10 % (w/v) sodium chloride for enrichment of staphylococci. Total viable and staphylococcal counts were made by spreading 0·5 ml. samples of dilutions of the homogenized meat in quarter-strength Ringer's solution on the surface of duplicate plates of blood agar and phenolphthalein diphosphate agar with polymyxin (Hobbs, Kendall & Gilbert, 1968); plates were incubated for 48 hr. at 35° C.

The slicing machine was then cleaned using the method given by Gilbert & Maurer (1968). To avoid the risk of spoiling further experiments, the cleaning procedure was repeated twice.

The experiments were repeated using half the contents of a can of corned beef inoculated with 3×10^9 *Salmonella oranienburg* in a central core. After slicing the corned beef, the same machine was then used for cutting 20 slices of corned beef from the uninoculated half of the can and a freshly opened can, 10 slices of chopped pork and 10 slices of brisket of beef. After homogenizing some of the slices, 25 g. samples were incubated in 100 ml. volumes of selenite broth at 35° C. Subcultures on Wilson and Blair bismuth sulphite agar and deoxycholate citrate sucrose agar were made after 24 and 72 hr. and the plates incubated for 48 hr. Total viable counts were made on blood agar as previously described.

Experiments were then made with the cotton cloths normally used by shops and restaurants for wiping down surfaces and equipment and for washing-up. One piece of sterile cloth (10 × 10 cm) was dampened with warm water, wiped over the gravity feed and knife centre disk of the slicing machine used previously for cutting meat infected with *S. oranienburg*, and then placed in 100 ml. of selenite broth. One larger piece of sterile cloth (30 × 10 cm) was dampened with warm water, wiped over the upper and lower surfaces of the cutting blade and then cut into three pieces (approximately 10 × 10 cm). On piece was placed directly in selenite broth, one piece was quickly rinsed (10 sec.) in warm water at 50° C. before addition to selenite broth and one piece was soaked in warm water containing 0·75 % (w/v) of detergent/disinfectant for 10 min. and rinsed in water before addition to selenite broth. Subcultures on Wilson and Blair agar and deoxycholate citrate sucrose agar were made after 24 and 72 hr. and the plates incubated for 48 hr.

RESULTS

Samples of the three freshly opened canned products gave no growth from enrichment cultures and they were assumed to be sterile. The viable counts on salami were $3{\cdot}4–4{\cdot}3 \times 10^6$/g.

Table 1 shows the effect of cutting cold meats on a slicing machine contaminated with coagulase-positive staphylococci from a previously sliced product. Coagulase-positive staphylococci were found up to the 41st slice of meat cut on the machine.

Total viable counts and counts of coagulase-positive staphylococci from the sliced chopped pork, corned beef and brisket of beef were similar: coagulase tests showed that all colonies tested were coagulase-positive staphylococci. Only the first slice of the salami gave any positive results on enrichment culture for coagulase-positive

Table 1. *Effect of cutting cold meats on a slicing machine contaminated with coagulase-positive staphylococci*

Product sliced	Slice no.	Total viable count/g.	Count of coagulase-positive staphylococci/g.	Enrichment cultures (5) positive for coagulase-positive staphylococci
Chopped pork	1	970	1100	5
	2	520	580	5
	3	370	390	5
	4	260	290	5
	5	170	350	5
	10	190	210	5
	15	60	110	5
	20	110	80	5
Corned beef	21	120	110	5
	22	40	40	5
	25	60	60	5
	30	< 20	< 20	5
Brisket of beef	31	50	60	5
	32	< 20	20	5
	35	< 20	< 20	1
	40	< 20	< 20	4
Salami	41	$6{\cdot}9 \times 10^6$	< 20	1
	42	$1{\cdot}4 \times 10^7$	< 20	0
	45	$4{\cdot}0 \times 10^6$	< 20	0
	50	$3{\cdot}6 \times 10^6$	< 20	0

Table 2. *Effect of cutting cold meats on a slicing machine contaminated with* Salmonella oranienburg

Product sliced	Slice no.	Total viable count/g.	Enrichment culture for *Salmonella oranienburg*
Corned beef	1	210	+
	2	80	+
	3	120	+
	4	50	+
	5	50	+
	10	20	+
	15	30	+
	20	< 20	+
Chopped pork	21	< 20	+
	22	50	+
	25	< 20	+
	30	20	+
Brisket of beef	31	< 20	+
	32	< 20	−
	35	< 20	−
	40	< 20	−

staphylococci. This may have been due to the small number of staphylococci left on the slicing machine or to overgrowth on enrichment of any staphylococci by the background flora of the salami.

Table 2 shows the effect of cutting cold meats on a slicing machine contaminated with *S. oranienburg*. This serotype was isolated up to the 31st slice of meat cut on the machine. Numerous colonies from the total viable counts were serotyped and all were shown to be *S. oranienburg*.

Table 3 shows the results of experiments on the cloth used for cleaning the slicing machine contaminated with *S. oranienburg*. This serotype was isolated from a piece of cloth wiped over the gravity feed and knife centre disk and from the pieces of cloth wiped over the upper and lower surfaces of the cutting blade both before and after rinsing the cloth in warm water for 10 sec. However, a piece of cloth wiped over the contaminated blade and then soaked in warm water containing detergent/disinfectant for 10 min., followed by rinsing, was negative for salmonellas on enrichment culture.

Table 3. *Experiments on cloths used for cleaning slicing machines*

Area wiped with cloth	Treatment of cloth after wiping	Enrichment culture for *Salmonella oranienburg*
Gravity feed and knife centre disk	None	+
Upper and lower surfaces of cutting blade	None	+
	Rinsed in warm water (10 sec.)	+
	Soaked in warm water with detergent/disinfectant (10 min.) and rinsed	−

DISCUSSION

The results confirm the part played by slicing machines in the cross-contamination of various sliced meats. The results show also that cleaning cloths contaminated with pathogens could readily contaminate other equipment and utensils. In at least three recent outbreaks of food poisoning due to *Salmonella reading* and *S. tennessee* (Cruickshank, 1965; Burnett & Davies, 1967) and *S. meleagridis* (Kaufmann *et al.* 1968), the organisms have been isolated from slicing machines.

The dangers associated with dirty dish-cloths and tea-towels have been stressed recently by Davis, Blake & Woodall (1968). Similar dangers are associated with cotton meat cloths used for wrapping raw meats which may be contaminated with salmonellas from animal hosts. Such cloths are widely used in kitchens, shops and restaurants and every effort should be made to educate the public in the hygienic value of disposable paper. In the meantime it is necessary to emphasize that all dish and cleaning cloths should be washed and disinfected daily, either by boiling, or by soaking overnight in freshly made up detergent/disinfectant solution, or washed out in detergent and soaked in disinfectant solution overnight.

Although the 32nd, 35th and 40th slices of meat cut on the machine were negative on enrichment for *S. oranienburg* (Table 2), and also presumably the intervening slices which were not examined, experiments with the cloths (Table 3) showed that the slicing machine used for cutting this meat was still contaminated. Positive cultures from an area of low contamination are much more likely to be obtained by wiping a large surface area with a piece of cloth or a large swab than a small area with a normal swab.

Recommendations concerning the slicing and storage of cold meats and the cleaning of slicing machines and associated equipment, namely carving knives and can-openers, have been given recently by Gilbert & Maurer (1968), who recommend that such equipment be cleaned at least twice daily. Where possible it is also desirable that a whole can of meat be sliced at one time and the equipment cleaned again before re-use. It is only necessary here to re-emphasize the importance of efficient and regular cleaning by responsible persons using hot water containing detergent/disinfectant or detergent followed by disinfectant applied with clean cloths or preferably disposable paper.

SUMMARY

In view of recent food-borne outbreaks of salmonella infection in which cooked-meat slicing machines have been implicated in the spread of organisms from contaminated meat products to other meats, experiments have been made to provide laboratory evidence that a contaminated slicing machine will easily cross-contaminate other products passing through it. Chopped pork inoculated with coagulase-positive staphylococci was cut on a slicing machine; staphylococci were isolated up to the 41st slice of various cooked meats cut on the same machine. The experiments were repeated with *Salmonella oranienburg*; this serotype was isolated up to the 31st slice of various cooked meats cut on the same machine, and from pieces of damp cloth wiped over the gravity feed, knife centre disk and cutting blade.

The importance of efficient and regular cleaning of slicing machines with hot water containing detergent/disinfectant, or detergent followed by disinfectant, applied with clean cloths or preferably disposable paper, is stressed.

I thank Dr Betty C. Hobbs, Director of the Food Hygiene Laboratory, and Dr J. C. Kelsey, Administrative Director of the Central Public Health Laboratory, for their help throughout the work and the compilation of the paper.

REFERENCES

Burnett, R. C. S. & Davies, B. I. (1967). Salmonella food poisoning associated with imported canned meat. *J. Hyg., Camb.* **65**, 1.

Cruickshank, R. (1965). An account of two outbreaks of food poisoning in Scotland associated with imported canned meats. *C.D.C. Salmonella Surveil.* Report no. 40, p. 9.

Davis, J. G., Blake, J. R. & Woodall, C. M. (1968). A survey of the hygienic condition of domestic dish-cloths and tea-towels. *Med. Offr.* **120**, 29.

Gilbert, R. J. & Maurer, I. M. (1968). The hygiene of slicing machines, carving knives and can-openers. *J. Hyg., Camb.* **66**, 439.

HOBBS, B. C., KENDALL, M. & GILBERT, R. J. (1968). Use of phenolphthalein diphosphate agar with polymyxin as a selective medium for the isolation and enumeration of coagulase-positive staphylococci from foods. *Appl. Microbiol.* **16**, 535.

KAUFMANN, A. F., HAYMAN, C. R., HEATH, F. C. & GRANT, M. (1968). Salmonellosis epidemic related to a caterer–delicatessen–restaurant. *Am. J. publ. Hlth* **58**, 764.

REPORT (1964). *The Aberdeen Typhoid Outbreak* 1964. Edinburgh: H.M.S.O.

WALKER, W. (1965). The Aberdeen typhoid outbreak of 1964. *Scott. med. J.* **10**, 466.

J. Hyg., Camb. (1969), **67**, 255
Printed in Great Britain

Patterns of infections with adenovirus types 4, 7 and 21 in military recruits during a 9-year survey

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Numerous studies have shown the importance of adenoviruses as a cause of acute respiratory disease in military recruits. However, there are few reports on surveys of respiratory infections in military populations conducted for a period of years in order to obtain reliable and detailed data on the epidemiological pattern of adenovirus infection (Bloom *et al.* 1964; Rosenbaum *et al.* 1965). Such data are required to provide a well-founded base for the control of adenovirus illness. The present study was established for this purpose.

This paper summarizes the results of a survey of respiratory infection in military recruits over a 9-year period. It was designed primarily to acquire information on the behaviour of adenovirus infections. From 1963 to 1967 additional studies were made to assess the part played by other respiratory pathogens. Results of studies undertaken during brief periods before 1963 and designed to appraise the incidence of infection with adenovirus type 21, *Mycoplasma pneumoniae* and Coxsackie A-21 virus were described previously (van der Veen & Dijkman, 1962; van der Veen & van Nunen, 1963; Oei & van der Veen, 1967).

MATERIALS AND METHODS

Study population

The investigation was conducted from February 1958 through January 1967 in the military training centre at Ossendrecht, The Netherlands. A recruit is defined as any newly enlisted soldier, without previous military service, receiving basic training. The recruits were predominantly between the ages of 19 and 21 and came from all areas of The Netherlands. At intervals of 2 months, groups of recruits entered the camp to receive an 8-week course of basic training. The size of the groups varied from about 750 to 1350 men between 1958 and 1961 and from about 1600 to 2600 men between 1961 and 1967. During the period of basic training no other men were introduced into the camp.

The recruits were formed into companies of slightly more than 200 men, all of which followed a similar training schedule. The companies were trained separately and were housed in separate barracks. Each barrack was partitioned into rooms, $23 \times 26 \times 9{\cdot}5$ ft. housing up to 20 men, who slept in bunks of the double-decker type. Recruits from the same company ate together. Opportunities for contact

between men from different companies were limited by these circumstances to occasional associations at the common mess hall and to attendance at the theatre during off-duty hours. Following $2\frac{1}{2}$ weeks service, recruits received a $1\frac{1}{2}$-day leave every week. There was an interval of 4–5 days between two successive courses of basic training. During this interval the barracks were not occupied.

After completing the course of basic training, a portion of the recruits varying from one-third between 1958 and 1961 to three-quarters between 1961 and 1967 were shipped to duty at other posts. The remaining recruits stayed for another 8 weeks for further training. These advanced recruits were formed into separate companies. After completing the course of continued training they were transferred elsewhere. Opportunities existed for contact between fresh and advanced recruits at the mess hall and theatre. The permanent personnel who operated the centre and instructed the recruits varied during the study period between 200 and 750 men. The total strength of the camp varied between 1500 and 3500 men. The investigation was limited to recruits in basic training.

Collection of specimens

Specimens were collected from patients who were admitted to the sick quarters with symptoms of acute respiratory disease associated with rectal temperature greater than 38·0° C. (100·4° F.). Acute phase blood specimens and throat swabs for virus isolation were taken on the first or second day after admission. In addition throat swabs for isolation of streptococci were obtained shortly after admission over a 3-year period from 1962 to 1965. Convalescent blood specimens were collected 10–14 days after the first sample.

Furthermore, paired sera were collected from randomly selected recruits from each company of each group. The recruits were bled within 2 days after arrival in the camp and again at the end of the eighth week of basic training. Of the recruits of two groups who received training in February and March 1958 and in April and May 1959, 6 % and 4 % respectively were bled. Of the recruits of the remaining groups the percentage of men bled varied between 8 and 14. In all, paired sera were obtained from 8765 randomly selected men.

Sera from blood samples were frozen and stored at − 20° C. until tested. During the first two years of the study throat swabs were mailed to the laboratory. The swabs were then transferred to a tube containing 2 ml. of Hanks's balanced solution, and were squeezed against the glass. Subsequently, fluids from the swabs were frozen and preserved at − 20° C. After 1960, throat swabs for virus isolation were placed in tubes containing 2 ml. of GLY medium (0·5 % gelatin, 0·5 % lactalbumin hydrolysate, and 0·1 % yeast extract in Hanks's balanced salt solution). Fluids from the swabs were kept at 2–4° C. until arrival in the laboratory. They were then stored at − 50° C. Before inoculation, fluids from the swabs were thawed and centrifuged at 3000 rev/min. for 10 min.; the supernatants were used.

Isolation tests

From February 1958 to October 1962 throat swabs from patients who were serologically positive for adenovirus were tested routinely for virus. The specimens

were inoculated into cultures of HeLa cells. After October 1962 throat swabs were inoculated routinely into cultures of HeLa cells, rhesus monkey kidney cells and human diploid cells, irrespective of the results of serological tests. Specimens from patients serologically positive for adenovirus were also inoculated into cultures of human thyroid cells. In addition to virus isolation tests, throat swabs collected between October 1962 and October 1965 were tested for the presence of haemolytic streptococci. Cultures of human diploid cells consisted of WI-38, 'Gabi' or N-3 cells. The WI-38 cell strain was purchased from Flow Laboratories Inc., Rockville, Md. The 'Gabi' cell strain was kindly supplied by Dr R. Gispen, Utrecht. The N-3 cell strain, derived from human embryonic lung, was established in our laboratory. HeLa cells were maintained in medium consisting of 5 % horse serum and 0·5 % lactalbumin hydrolysate in Hanks solution. For maintenance of the other cell cultures, medium 199 (Difco) supplied with 2·5 % chicken serum was used. An inoculum of 0·2 ml. of the virus isolation specimen was added to each of duplicate tubes of each kind of cell culture. During the first years of the study inoculated cultures were subjected to stationary incubation. After October 1962 a drum rotating 12 times/hr. was used for incubating inoculated cultures. Passages were made by inoculation of 0·2 ml. of fluid into fresh tubes of cell cultures. The total incubation period was 21 days. Following one or two passages, all inoculated cell cultures were tested for haemadsorption with guinea-pig erythrocytes at 4° and 37° C. Viruses isolated were typed by neutralization or haemadsorption-inhibition tests with rabbit antisera against prototype strains.

Throat swabs for the isolation of streptococci were transferred to tubes containing Pike's enrichment medium. After incubation at 37° C. for 4–18 hr., subcultures on blood agar plates were made. The plates were incubated aerobically and anaerobically at 37° C. for 24 hr. and subsequently were examined for colonies of haemolytic streptococci. Representative colonies were tested for sensitivity to bacitracin. Sensitive strains were considered to be of group A.

Serological tests

Tests with sera from recruits for neutralizing antibody titre against adenoviruses were carried out according to procedures described previously (van der Veen, Abarbanel & Oei, 1968). The viruses used were the prototype strains of adenovirus types 4, 7 and 21.

The following strains of viruses were used as antigens for complement-fixation (CF) tests: prototype strain of adenovirus type 4; influenza A-1/Netherlands/49 and influenza A-2/Netherlands/60; influenza B (Lee); influenza C (Taylor); parainfluenza 1 (61–1264); parainfluenza 2 (Greer); parainfluenza 3 (24,249); Sendai; RS (Long); Coxsackie A-21 (58–5178); and *Mycoplasma pneumoniae* (Mac). The Coxsackie A-21 virus and the strains of parainfluenza 1 and 3 viruses were isolated in this laboratory. CF antigens were prepared from chorioallantoic membranes of infected chick embryos for influenza A and B viruses, from allantoic fluid of infected chick embryos for influenza C and Sendai viruses, and from infected cell cultures for the other viruses. CF antigen of *M. pneumoniae* was prepared according to the technique described by Chanock *et al.* (1962). With the exception of influenza

viruses, virus suspensions to be used as CF antigens were inactivated at 56° C. for 30 min.

Between 1958 and 1962, CF tests were performed in transparent plastic sheets with 0·125 ml. unit volumes and overnight fixation at 4° C. according to a minor modification of the Kolmer technique (van der Veen, 1955). After October 1962, CF tests were done in microtitre plates according to the technique described by Sever (1962). The lowest initial serum dilution tested was 1 in 5. Titres were expressed as the highest initial serum dilution causing complete or nearly complete (more than 3+) fixation of complement. Paired sera were always titrated simultaneously. A fourfold or greater rise in antibody between paired sera was considered significant.

RESULTS

Incidence of adenovirus illness and infection

During the 9-year study period, 89,200 recruits entering service at bi-monthly intervals received an 8-week course of basic training (section 1 of Fig. 1). The morbidity of patients admitted with febrile respiratory illness is shown in section 2 of Fig. 1. The expected influence of season is clearly seen, rates for the winter and spring being, in general, much higher than those for the other seasons.

Paired sera were obtained from 2776 of 4408 patients admitted to the sick quarters and were tested for increase in CF antibody titre against adenovirus. In all, 938 (34 %) of the 2776 patients tested showed serological evidence of infection with adenovirus. The incidence of adenovirus illness was estimated by applying the rate of serologically diagnosed cases to the total respiratory illness admission rate for each group of recruits (section 3 of Fig. 1). Outbreaks of adenovirus illness occurred in the winter and spring, and in some years in the late fall, whereas the incidence was very low or nil during the summer and early fall.

In addition to the admission rate for adenovirus illness, an estimate of the total incidence of adenovirus infection was made. Paired sera spanning the 8-week training interval were collected from roughly 10 % of all recruits. In all, 8765 recruits were randomly selected. The sera were tested for CF antibody titre against adenovirus. It is assumed that a rise in CF antibody against adenovirus may be detected by the eighth day after infection. Thus, the 8-week interval allowed for the detection of antibody which might have developed if infection occurred during the seventh week of basic training or earlier. The percentages of positive serum pairs are shown in section 4 of Fig. 1. These percentages give an estimate of the total incidence of adenovirus infection in the various groups. The seasonal distribution of adenovirus infection was similar to that of adenovirus illness. The incidence was high during the cold season. This contrasted with the summer, when infections were virtually absent. During epidemic periods about 20–60 % of the recruits were infected with adenovirus at some time during training.

Prevalence of different adenovirus types

The recovery of adenovirus from throat swab specimens from patients with acute respiratory disease is summarized in section 5 of Fig. 1. In all, adenoviruses

were isolated from 633 patients. A remarkable finding was the successive appearance of different serotypes. Type 7 was prevalent from 1958 to 1960 and was replaced in 1960 by type 21. Three years later type 21 in turn was replaced by type 4. Besides strains of these types, one strain of type 1, five strains of type 2 and eight strains of type 3 were recovered during the study period.

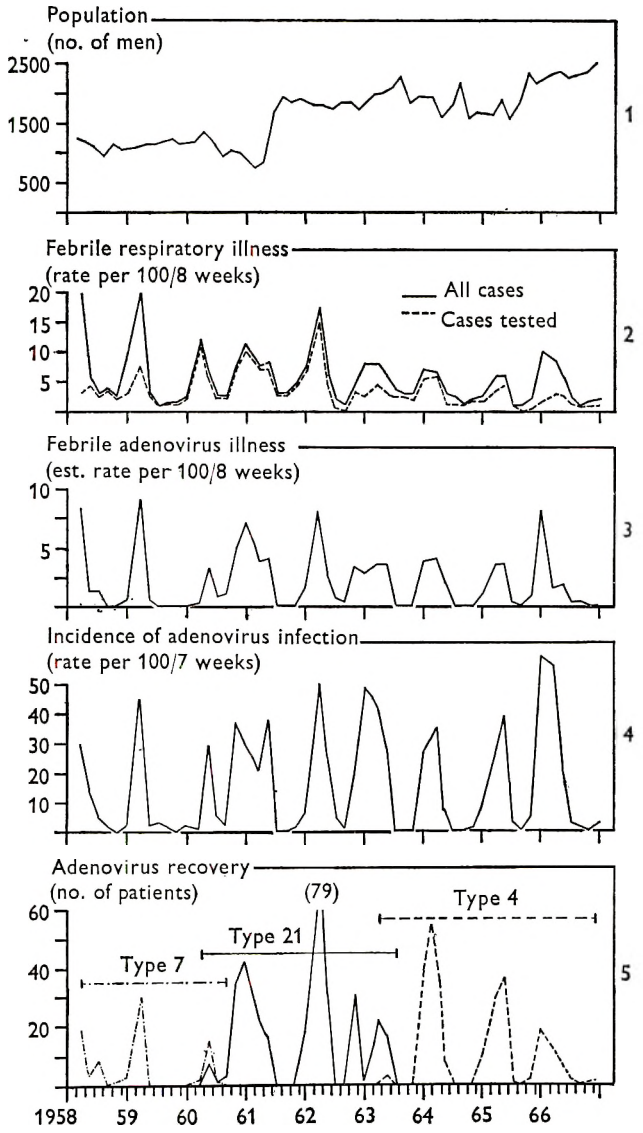

Fig. 1. Adenovirus infection among military recruits at Ossendrecht, 1958–67.

Serological studies were carried out to measure the population's prior experience with adenoviruses and to investigate whether changes in immunity of the general population occurred as estimated by frequency of neutralizing antibody. Sera collected from randomly selected recruits at the time of entry into service were

tested for neutralizing antibody against adenovirus types 4, 7 and 21. The sera were tested at a dilution of 1 in 4 and 1 in 16. Sera from nine groups of recruits who entered service in different years between 1958 and 1967 were used. The percentages of sera containing antibody to the various adenovirus types are shown in Table 1. The values give an estimate of the frequency of antibody among young adults of the general population.

No substantial changes in frequency of antibody at a serum dilution of 1 in 4 were found for any of the three types. This applied also to a comparison of the frequency of antibody at a dilution of 1 in 16. The data suggest that the immunity of incoming recruits remained fairly constant during the 9-year study period.

Table 1. *Prevalence of neutralizing antibody against adenovirus types in sera of incoming recruits in different years*

Time of collection of sera	No. of men studied	Percentage with antibody*		
		Type 4	Type 7	Type 21
June 1958	108	50 (24)	58 (37)	54 (34)
June 1959	116	63 (22)	57 (32)	54 (33)
June 1960	98	60 (23)	51 (29)	53 (34)
June 1961	117	66 (28)	62 (30)	44 (28)
June 1962	107	64 (26)	56 (31)	50 (31)
June 1963	107	65 (32)	51 (29)	49 (32)
June 1964	114	64 (26)	52 (31)	50 (33)
June 1965	116	68 (31)	52 (29)	56 (40)
June 1966	101	51 (31)	56 (29)	49 (34)

* Titres of 1/4 or more; in parenthesis titres of 1/16 or more.

Proportion of infections associated with illness

An attempt was made to estimate the proportion of adenovirus infections which was associated with clinical illness. Estimates were calculated for infections with type 7, type 21 and type 4. To allow comparison with the total incidence of infection, only cases occurring during the first 7 weeks of basic training were included and increase in CF antibody was used as the sole criterion for infection. The study was further confined to outbreaks in which a reasonably representative proportion (60 % or more) of the patients with acute respiratory illness was studied.

As seen in Table 2, the estimated ratio of the total amount of infection to the amount of clinical infection showed only minor fluctuations among different outbreaks. Thus, it would appear that the serotype had little influence upon the severity of infection. Likewise, there was no evidence that the infections occurring in the winter were more severe than those occurring in the fall or spring.

Adenovirus illness by week of training

For each group of recruits record was made of the number of patients admitted with adenovirus illness by week of training. Because of the small number of admissions in a group, the distribution of cases by week shows considerable chance fluctuation. To overcome this difficulty, the numbers of admissions in different

groups were combined to give the composite distribution of cases by week. The composite distribution was calculated for each of the three successive periods in which, respectively, adenoviruses type 7, type 21 and type 4 were prevalent. In Fig. 2 the cumulative percentages of cases by week are compared for the three serotypes.

Table. 2 *Ratio of total amount of adenovirus infection to amount of adenovirus illness*

Prevalent adenovirus type	Period	Est. rate/100/7 weeks		Est. ratio of total : clinical
		All adenovirus infections	Adenovirus illness	
Type 7	Apr.–May 1958	14	1·0	14:1
Type 21	Oct.–Nov. 1960	37	3·3	11:1
	Dec.–Jan. 1961	30	3·7	8:1
	Feb.–Mar. 1961	21	1·7	12:1
	Apr.–May 1961	38	3·3	12:1
	Feb.–Mar. 1962	50	7·1	7:1
	Apr.–May 1962	25	2·0	12:1
Type 4	Dec.–Jan. 1964	26	2·4	11:1
	Feb.–Mar. 1964	35	3·1	11:1

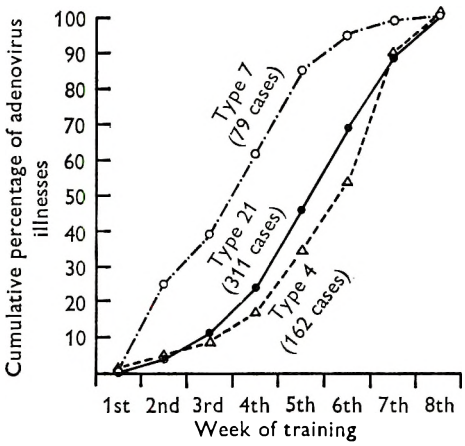

Fig. 2. Adenovirus illness by week of training.

Cases of illness due to type 7 occurred at a relatively early time during the training period. Over 50 % of these illnesses were observed by the fourth week of training. There was a delay of about 2 weeks in the cumulative percentages of cases due to type 21 and type 4 when compared to corresponding percentages of type 7 illnesses. Less than one fourth of the cases due to type 21 and type 4 fell within the first month of training and 50 % of the cases were observed by the sixth week.

Comparison of CF test and virus isolation

A comparison was made of the sensitivity of the CF technique and virus isolation tests in detecting adenovirus infection. Data relative to patients with acute respiratory illness from whom throat swabs were tested for the presence of virus,

irrespective of the results of the CF test, were employed. The comparative study was confined to patients admitted during outbreaks of adenovirus infection. In all, 1061 patients were included. The patients were divided into three groups according to the prevalent serotype (type 7, type 21 or type 4). Table 3 shows that in each group virus isolation tests were less sensitive in detecting infection than was the CF technique. In all, adenovirus was isolated from 72 % of the cases diagnosed by CF test and from only 8 % of the serologically negative cases.

Table 3. *Comparison of CF test and virus isolation test for detecting adenovirus infection*

Prevalent adenovirus type	CF antibody rise to adenovirus	No. of patients studied	Adenovirus isolated	
			No.	Percentage
Type 7	Positive	67	42	63
	Negative	18	2	11
Type 21	Positive	390	285	81
	Negative	98	18	18
Type 4	Positive	287	210	73
	Negative	201	5	2
Totals	Positive	744	537	72
	Negative	317	25	8

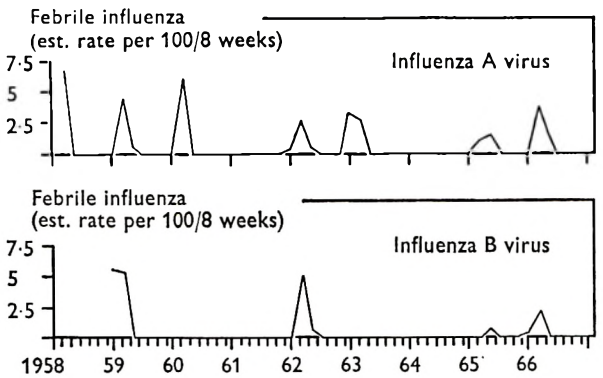

Fig. 3. Influenza among military recruits at Ossendrecht, 1958–67.

Other respiratory agents

Paired sera from the patients and randomly selected recruits who were studied for evidence of adenovirus infection were also tested for increase in CF antibody against influenza A and B viruses. As seen in Fig. 3, outbreaks of influenza A occurred at 1- or 2-year intervals, those of influenza B at 3- or 4-year intervals. Subclinical infections with influenza virus as evidenced by antibody rises in randomly selected recruits were found exclusively during outbreaks. The total incidence of infection with influenza A virus varied between 9 % and 44 % among different outbreaks of influenza A. The total incidence of infections with influenza B virus during outbreaks of influenza B ranged from 7 % to 36 %.

Between October 1962 and October 1966, paired sera from 570 patients

serologically negative for adenovirus and influenza A and B viruses were tested for CF antibody titre against various other respiratory agents. In addition, virus isolation tests were done routinely in these cases. Adenovirus was isolated from 23 of the 570 patients. Table 4 shows that infection with other respiratory pathogens as evidenced by virus isolation or antibody rise or both was detected in 87 of the remaining 547 patients.

Table 4. *Evidence of infection with various agents in patients with acute respiratory disease negative for adenovirus and influenza virus*

	Year of study				
	1962–3	1963–4	1964–5	1965–6	Totals
No. studied	189	176	87	95	547
No. positive					
Influenza C	15	2	3	1	21
Parainfluenza	3	2	5	4	14
RS	1	0	0	1	2
Coxsackie A-21	6	7	0	3	16
Echo-28	0	3	0	0	3
Picorna unidentified	4	13	2	3	22
M. pneumoniae	3	4	2	0	9

Influenza C virus was relatively more active in the spring and summer of 1963; during this period 15 of the 21 patients positive for influenza C were admitted. All but three of the CF antibody rises for Coxsackie A-21 virus occurred throughout a 12-month period between April 1963 and April 1964. It is dubious whether these rises were due to infection with Coxsackie A-21 virus, since no increase in neutralizing antibody to the virus was found and no virus was recovered in these cases. In a previous study we reported that Coxsackie A-21 virus was active in the military population at Ossendrecht in the fall of 1961 (Oei & van der Veen, 1967). CF antibody rises for parainfluenza virus and *M. pneumoniae* were observed throughout the 4-year period. Cases positive for *M. pneumoniae* were also tested for increase in amount of fluorescent stainable antibody against *M. pneumoniae;* antibody rises were found in all instances. As in the findings of the present study, *M. pneumoniae* was found to be a minor cause of acute respiratory disease in recruits at Ossendrecht during a separate study conducted in 1961 and 1962 (van der Veen & van Nunen, 1963).

Haemolytic streptococci of group A were recovered from 10 (3 %) of 286 patients with serological evidence of adenovirus infection and from 40 (10 %) of 397 patients who were serologically negative for adenovirus. The data do not allow a reliable appraisal of the role of haemolytic streptococci in producing acute respiratory disease, since the mere presence of *Streptococcus pyogenes* in the throat cannot be regarded as diagnostic of streptococcal illness (Williams, 1958). The difference in incidence of recoveries of streptococci between patients with adenovirus illness and those with illness not due to adenovirus was 7 %. If it is assumed that the excess of recoveries in the latter group or part of it was aetiologically associated with illness, and if, subsequently, the excess rate is applied to the total

number of patients studied, then it may be estimated that the proportion of total respiratory illness due to group A haemolytic streptococci was 4 % or less.

The percentage distribution of aetiological diagnoses in recruits with acute respiratory illness is graphically presented in Fig. 4. Adenovirus represented the major cause of illness for which a cause was found. The next most common diagnostic category was influenza. Other agents were of minor importance. A number of patients showed serological evidence of simultaneous infection with adenovirus

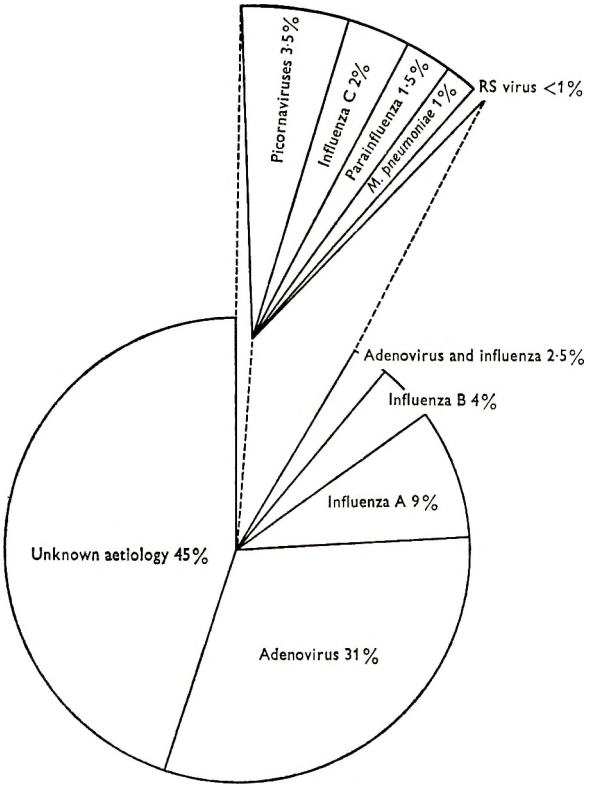

Fig. 4. Distribution of aetiological diagnoses in recruits with acute respiratory illness.

and influenza virus. Probably, the proportion of double infections was underrated, since no serological tests for parainfluenza, RS and Coxsackie A-21 viruses and *M. pneumoniae* were done in patients with adenovirus infection and influenza. In as many as 45 % of the cases, no aetiological diagnosis could be made. Although insensitivity of tests for detecting infection or loss of infectivity of agents during collection and processing of specimens may be a cause of failure in establishing diagnoses, it seems likely that at least a part of these illnesses were produced by presently unrecognized agents or families of agents. In a previous study we reported that an attempt to uncover agents in acute respiratory disease of unknown aetiology by application of interference tests was unsuccessful (Custers & van der Veen, 1967).

DISCUSSION

Perhaps the most interesting finding in this survey was the successive appearance of different serotypes. In a previous study we reported the prevalence of adenovirus type 14 in the winter of 1955 (van der Veen & Kok, 1957). The present data show that type 7 was prevalent from 1958 to 1960, type 21 from 1960 to 1963 and type 4 from 1963 up to the end of the study period in 1967. Studies conducted among Finnish military recruits between 1964 and 1966 suggested also that different adenovirus types may be prevalent in successive seasons (Mäntyjärvi, 1966). This pattern of adenovirus infection is substantially different from that reported for military populations in the United States (cf. review by Huebner, Rowe & Chanock, 1958). Types 4 and 7 were found to be the most prominent causes of adenovirus illness in American recruits, whereas type 3 played a less important role. No periodic variation in prevalence of these types was observed.

We thought that the successive appearance of different serotypes might be related to variation in the proportion of immunologically susceptible persons among the incoming recruit population. However, the data did not support this concept. The immunity of the population against types 4, 7 and 21 remained fairly constant during the 9-year study period. Possibly, the prevalence of type 7 during the first years of the study and the subsequent appearance of type 21 originated from the behaviour of adenovirus in the general population. Previous studies on children in hospital with acute respiratory disease in the Netherlands showed that type 7 was the predominant cause of adenovirus illness from 1958 to 1960 whereas type 21 was predominant in 1961 (Kapsenberg, 1962; van der Veen & van Zaane, 1963). Because of the relatively high frequency of contact between recruits and their families, there was ample occasion for the introduction of viruses into the training centre from the civilian population.

The important role of adenovirus in causing respiratory illness in recruits at Ossendrecht is evident. About 20–60 of every 100 recruits who received training in the winter or spring were infected at some time during the period of training, and about 7–14 of every 100 infections were severe enough to require admission to the sick quarters. Adenovirus accounted for about 30–70 % of all cases of acute respiratory illness occurring during the colder months of the year. Since reasons for reporting sick and criteria for admission of patients to sick quarters or to the hospital may vary in different training centres, in different years for the same centre and in different companies of the same population, the results of surveys of adenovirus illness in different military populations are difficult to compare. Studies designed to permit an estimate of the total incidence of adenovirus infection, irrespective of the presence and severity of illness, are better suited for comparison of incidence rates. Surveys of the total incidence of adenovirus infections in military recruits are scarce and are confined to relatively small numbers of recruits and brief periods of time. Studies conducted on United States Army and Navy recruits during an 8-week training interval in winter showed that about 50–80 % of the men became infected with adenovirus as measured by CF tests (Hilleman *et al.* 1955; Grayston *et al.* 1959; McNamara, Pierce, Crawford & Miller, 1962; Forsyth, Bloom, Johnson

& Chanock, 1964). In a training centre in Finland, unreported adenovirus infection as evidenced by a rise in CF antibody was found in 24 % of 159 recruits studied between February and April 1964 (Mäntyjärvi, 1966).

The seasonal pattern of adenovirus infection among recruits at Ossendrecht was characterized by the occurrence of extensive epidemics in the late fall, winter and spring and a very low incidence or absence of illness in the summer and early fall. A similar pattern was observed in military recruits at Fort Dix, N. J. (Hilleman *et al.* 1957), and at training centres in Finland (Mäntyjärvi, 1966), and in advanced recruits at Camp Lejeune, N.C. (Bloom *et al.* 1964). In contrast, studies at the Great Lakes Naval Training Centre showed that in this locality adenovirus illness occurred throughout the year, with the highest incidence usually occurring in the winter and early spring (Woolridge *et al.* 1956; Rosenbaum *et al.* 1965). Furthermore, during a comparative study of two companies at this centre who received training in the summer months and the winter months respectively no difference in the total amount of serologically detectable adenovirus infection was found (McNamara *et al.* 1962).

Several hypothetical explanations—such as crowding, variation in virus dosage, influence of the indoor relative humidity upon virus spread and variation in host defence mechanisms—might be advanced for the seasonal occurrence of epidemics of adenovirus infection. The present data offer no clue as to the nature of this phenomenon. However, the data do show that, at least in this population, the as yet undetermined seasonal factor influenced the incidence rate of subclinical as well as clinical adenovirus infection. During the warmer months of the year the activity of adenovirus appeared to be negligible.

SUMMARY

Investigations to define the epidemiological pattern of adenovirus infection in military recruits were carried out in a training centre at Ossendrecht, The Netherlands, during a 9-year period. Extensive outbreaks of adenovirus illness occurred in the winter and spring, and in some years in the late fall. The seasonal variation in total incidence of adenovirus infection as estimated by frequency of antibody rises in randomly selected recruits correlated well with that of adenovirus illness. In the cold season 20–60 of every 100 men were infected, whereas no or very little activity of adenovirus was found during the summer.

Type 7 was prevalent during the first 2 years of the study period, type 21 during the next 3 years and type 4 during the last 4 years. The proportion of adenovirus infection associated with clinical illness, the distribution of adenovirus illness by week of training and the immunity of incoming recruits against adenovirus infection as estimated by frequency of neutralizing antibody were compared for type 7, type 21 and type 4.

Adenovirus was responsible for about one third of all cases of acute respiratory illness. The next most common diagnostic category was influenza. Other respiratory agents (parainfluenza, RS and Coxsackie A-21 viruses, *Mycoplasma pneumoniae* and haemolytic streptococci) were of less importance.

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REFERENCES

Bloom, H. H., Forsyth, B. R., Johnson, K. M., Mufson, M. A., Turner, H. C., Davidson, M. A. & Chanock, R. M. (1964). Patterns of adenovirus infections in marine corps personnel. I. A 42-month survey in recruit and nonrecruit populations. *Am. J. Hyg.* **80**, 328.

Chanock, R. M., James, W. D., Fox, H. H., Turner, H. C., Mufson, M. A., & Hayflick, L. (1962). Growth of Eaton PPLO in broth and preparation of complement fixing antigen. *Proc. Soc. exp. Biol. Med.* **110**, 884.

Custers, J. H. E. & van der Veen, J. (1967). Failure to detect interfering agents in febrile respiratory illnesses. *Antonie van Leeuwenhoek* **33**, 213.

Forsyth, B. R., Bloom, H. H., Johnson, K. M. & Chanock, R. M. (1964). Patterns of adenovirus infections in marine corps personnel. II. Longitudinal study of successive advanced recruit training companies. *Am. J. Hyg.* **80**, 343.

Grayston, J. Th., Woolridge, R. L., Loosli, C. G., Gundelfinger, B. F., Johnston, P. B. & Pierce, W. E. (1959). Adenovirus infections in naval recruits. *J. infect. Dis.* **104**, 61.

Hilleman, M. R., Werner, J. H., Dascomb, H. E., Butler, R. L. & Stewart, M. T. (1955). Epidemiology of RI (RI-67) group respiratory virus infections in recruit populations. *Am. J. Hyg.* **62**, 29.

Hilleman, M. R., Gauld, R. L., Butler, R. L., Stallones, R. A., Hedberg, C. L., Warfield, M. S. & Anderson, S. A. (1957). Appraisal of occurrence of adenovirus-caused respiratory illness in military populations. *Am. J. Hyg.* **66**, 29.

Huebner, R. J., Rowe, W. P. & Chanock, R. M. (1958). Newly recognized respiratory tract viruses. *A. Rev. Microbiol.* **12**, 49.

Kapsenberg, J. G. (1962). Een epidemie bij kinderen veroorzaakt door adenovirus type 7. II. Etiologisch onderzoek. *Ned. Tijdschr. Geneesk.* **106**, 108.

Mäntyjärvi, R. (1966). Adenovirus infections in servicemen in Finland. *Annls. Med. exp. Biol. Fenn.* **44**, Suppl. 4.

McNamara, M. J., Pierce, W. E., Crawford, Y. E. & Miller, L. F. (1962). Patterns of adenovirus infection in the respiratory diseases of naval recruits. A longitudinal study of two companies of naval recruits. *Am. Rev. resp. Dis.* **86**, 485.

Oei, Kiem Giok & van der Veen, J. (1967). Epidemiological study of Coxsackie A-21 virus infections in military recruits. *Am. J. Epidem.* **85**, 93.

Rosenbaum, M. J., Edwards, E. A., Frank, P. F., Pierce, W. E., Crawford, Y. E. & Miller, L. F. (1965). Epidemiology and prevention of acute respiratory disease in naval recruits. I. Ten years' experience with microbial agents isolated from naval recruits with acute respiratory disease. *Am. J. publ. Hlth.* **55**, 38.

Sever, J. L. (1962). Application of a microtechnique to viral serological investigations. *J. Immun.* **88**, 320.

van der Veen, J. (1955). A rapid and simple method for conducting large series of complement fixation tests and antistreptolysin 'O' titrations. *J. Lab. clin. Med.* **45**, 323.

VAN DER VEEN, J., ABARBANEL, M. F. W. & OEI, KIEM GIOK (1968). Vaccination with live type 4 adenovirus: evaluation of antibody response and protective efficacy. *J. Hyg., Camb.* **66**, 499.

VAN DER VEEN, J. & KOK, G. (1957). Isolation and typing of adenoviruses recovered from military recruits with acute respiratory disease in The Netherlands. *Am. J. Hyg.* **65**, 119.

VAN DER VEEN, J. & DIJKMAN, J. H. (1962). Association of type 21 adenovirus with acute respiratory illness in military recruits. *Am. J. Hyg.* **76**, 149.

VAN DER VEEN, J. & VAN NUNEN, M. C. J. (1963). Role of *Mycoplasma pneumoniae* in acute respiratory disease in a military population. *Am. J. Hyg.* **78**, 293.

VAN DER VEEN, J. & VAN ZAANE, D. J. (1963). Infectie met adenovirus type 21 bij kinderen met acute aandoeningen van de onderste luchtwegen en longen. *Ned. Tijdschr. Geneesk.* **107**, 808.

WILLIAMS, R. E. O. (1958). Laboratory diagnosis of streptococcal infections. *Bull. Wld Hlth Org.* **19**, 153.

WOOLRIDGE, R. L., GRAYSTON, J. Th., WHITESIDE, J. E., LOOSLI, C. G., FRIEDMAN, M. & PIERCE, W. E. (1956). Studies on acute respiratory illness in naval recruits, with emphasis on the adenoviruses (APC–RI). *J. infect. Dis.* **99**, 182.

J. Hyg., Camb. (1969), **67**, 269
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Dose-response relationships of lymphocytic choriomeningitis viruses in mice and L cell tube cultures

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INTRODUCTION

Knowledge concerning the relationship between the dose of a virus and the response of the inoculated host is not only of importance for choosing the appropriate method for the estimation of the number of infectious units (IU) in a preparation; it also may aid in the understanding of the relation between infectious agent and its host. As part of our study of the virus of lymphocytic choriomeningitis (LCM) the dose–response relationships between two virus strains, WE_3 and Armstrong, in two hosts, L cell tube cultures and mice, were determined and analysed. If infectivity was taken as the response, its connexion with the dose was found to be compatible with the assumption that one and only one IU was capable of initiating infection. In contrast, if death of a mouse was regarded as signifying the response, its relationship with the dose was not so simple.

MATERIALS AND METHODS

Cell cultures

L cells (Earle, 1943; Sanford, Earle & Likely, 1948–49), kindly supplied by Prof. W. Schäfer, Tübingen, were grown routinely in Roux bottles with a growth medium consisting of Eagle's minimal essential medium (Eagle, 1959), supplemented with non-essential amino acids (Lockart & Eagle, 1959) and 5% heated calf serum. Screw-capped culture tubes, 16×125 mm., were seeded with 1 ml. of growth medium containing 3×10^5 or $1{\cdot}5 \times 10^5$ cells. These were incubated in a stationary position at 37° C., the former for 1 day and the latter for 2 days before use.

Mice

Randomly bred white albino mice of both sexes from a local dealer were employed. The average weights of the animals in different experiments will be given together with the experimental details. Five or ten mice were housed in Macrolon cages (Spiegel & Gönnert, 1961) types I and II respectively, and were fed on commercial mouse cubes.

* Dedicated to Prof. W. Scheid, Köln, on the occasion of his 60th birthday in token of my sincere respect and affection.

Viruses

The Armstrong strain of LCM virus (Armstrong & Lillie, 1934), obtained from the American Type Culture Collection, had been passaged approximately 200 times in the mouse brain after its isolation. A pool was prepared in L cells, and was stored in ampoules at − 60° C. The WE_3 strain (Jochheim *et al.* 1957), a descendant of the WE strain (Scott & Rivers, 1936), was supplied by Prof. W. Scheid, Köln, after passages in guinea-pigs, mouse embryo cells *in vitro*, and mice. Virus was prepared as 2nd passage in L cells, and was stored in ampoules at − 60° C.

Principal design of the experiments

In the case of mice, serial dilutions of the virus were made with balanced salt solution (BSS) (Hanks & Wallace, 1949) containing antibiotics and 1 % heated CaS. Employing semi-automatic syringes, 0·03 ml. were inoculated intracerebrally (IC). Animals which were found dead between the 5th and 21st day after inoculation were recorded as having died from the virus inoculation. Surviving mice were challenged on day 21 with approximately 10^3 LD 50 of Armstrong virus and observed for 2 more weeks. The infectivity of a given dose was based on mice now surviving, together with the numbers which had succumbed to the original inoculation.

For the determination of the dose–response relationship in L cells, dilutions of virus were made in maintenance medium (MM) (Lehmann-Grube & Hesse, 1967) and tube cultures were inoculated with 0·1 ml. volumes. After an adsorption period of 15 min. at room temperature with frequent rocking of the culture racks, 1·9 ml. of MM were added to each culture, which were then incubated at 37° C. in a stationary position. Infection was determined by assaying the culture media individually for complement-fixing antigen (CF Ag) 6 and 7 days after inoculation of Armstrong and WE_3, respectively. Details of the procedure have been published (Lehmann-Grube & Hesse, 1967).

Statistical analysis

The statistical evaluation of the results was based on the assumption that the IU in the inocula were distributed at random, and that one unit sufficed for evoking a response. The most probable number for each dilution was estimated with the aid of a formula developed by Halvorson & Ziegler (1933). For the calculation of the goodness of fit between observed and expected values the χ^2 method was employed, in accordance with the principles laid down by Haldane (1939). The 50 % lethal doses (LD 50) and the 50 % infectious doses (ID 50) were estimated according to Fazekas de St Groth (1955).

RESULTS

Preliminary experiments

For obvious technical reasons, it was not possible to keep single mice. Thus, the possibility had to be considered that infectious spread between cage mates might

influence the results. In order to rule out this possible source of error the following experiments were performed. A total of 120 mice were distributed into 20 cages. Two of each group of six mice, which were to be kept together, received IC approximately $10^{2.5}$ LD 50 of Armstrong or WE_3 virus. All inoculated mice died with typical symptoms; all non-infected animals remained healthy. On day 21 these mice were challenged with approximately 10^3 LD 50 of Armstrong virus. One mouse of 40 which had been housed together with Armstrong-infected animals survived. The 40 companions of WE_3-infected animals died. Because of its significance for our work, this experiment was repeated with some modifications. The total number of mice was increased to 200. Of five mice in each container, three were infected IC with either approximately 10 LD 50 of Armstrong or approximately 200 LD 50 of WE_3 virus. Of 60 mice thus infected with Armstrong, three survived and proved resistant to challenge. None of the 40 non-infected cage mates died or was immune to challenge infection on day 21. In the case of WE_3 the situation was comparable; of 60 infected mice, 58 died with typical symptoms. The two survivors were resistant to challenge. All 40 non-infected controls succumbed to the IC inoculation of 10^3 LD 50 of Armstrong virus. Thus of 160 mice, which, in these two experiments, had been kept together with infected animals, only one resisted later challenge with a deadly dose of Armstrong virus, presumably as a result of contact infection, and it can be concluded that infectious spread within cages can be neglected as a source of experimental error.

Table 1. *Relationship between dose of LCM virus, strain Armstrong, and response in mice (death)*

Relative virus dose (log_{10})	Response (death) Observed No.	Observed %	Expected (%)
0·0	106/109*	97·25	> 99·99
−1·0	108/108	100·00	> 99·99
−2·0	110/110	100·00	> 99·99
−3·0	110/110	100·00	> 99·99
−4·0	110/110	100·00	> 99·99
−5·0	110/110	100·00	> 99·99
−6·0	63/109	57·80	60·57
−7·0	14/108	12·96	8·89
−8·0	1/110	0·91	0·93

* Number of mice dead over number inoculated.

Armstrong virus

Dose–response in mice

Each dilution of a decimal series was inoculated into 110 mice, 55 of either sex. For control purposes a group of 110 mice was inoculated with diluent only. The average weights, determined at the time of inoculation, were 24·7 and 22·8 g. for 50 male and 50 female animals, respectively. Mortality was compared with expected values (Table 1). The apparently good agreement between observed and calculated figures could be substantiated by the computation of χ^2 which was found

to be 2·568, corresponding to $0{\cdot}3 > P > 0{\cdot}2$ (2 degrees of freedom). Unexpectedly, three mice, all inoculated with undiluted virus, survived. (They were not included in the estimation of χ^2.) What is more, they not only came from one group, but had been housed in one cage. Of 660 mice, inoculated with dilutions of virus ranging from 10° to 10^{-5}, these were the only survivors and the probability that this could have happened as a chance event must be considered exceedingly small. The possibility that they had not been inoculated has been ruled out; at the time of challenge (21 days after the original inoculation) two of these mice were killed, the brains were homogenized and titrated in mice. Each brain contained a minimum of 10^6 ID 50. The third mouse of this group was challenged and proved to be immune.

Table 2. *Relationship between dose of LCM virus, strain Armstrong, and response in mice (infection)*

Relative virus dose ($\log_{10}$)	Response (infection) Observed No.	Observed %	Expected %
0·0	109/109*	100·00	> 99·99
−1·0	108/108	100·00	> 99·99
−2·0	110/110	100·00	> 99·99
−3·0	110/110	100·00	> 99·99
−4·0	110/110	100·00	> 99·99
−5·0	110/110	100·00	> 99·99
−6·0	103/108	95·37	97·07
−7·0	37/106	34·91	29·74
−8·0	4/109	3·67	3·47

* Number of mice infected over number inoculated.

Of the 110 control mice, inoculated with diluent, one died on day 8 without apparent cause.

As regards infection, the close agreement between observed and expected proportions is again evident from the values (Table 2). χ^2 was estimated as 2·464, which, with 2 degrees of freedom, corresponds to $0{\cdot}3 > P > 0{\cdot}2$. Of the 109 control mice, none was resistant to IC challenge on day 21.

A repetition of this experiment with an Armstrong virus, prepared as 2nd passage in monkey kidney cells, led to essentially the same results.

WE_3 virus

LCM virus, strain WE_3, was diluted serially tenfold. Each dilution was inoculated into 110 mice, equal numbers of either sex. The mean weights, based on 50 male and 50 female animals, were found to be 23·4 and 22·3 g., respectively. The results, shown in Fig. 1, clearly show that the mortality at any given dose deviated significantly from expectation. With highest doses ($10^{6{\cdot}73}$ ID 50 per mouse) many animals survived. Upon successive reductions, increasing numbers died until a maximum (91·5 %) was reached at approximately 50 ID 50. Thereafter, deaths decreased again. By way of contrast, the infectivities were found to agree well with the expectation based on the Poisson distribution (Fig. 1); χ^2 was estimated

to be 3·246, which corresponds to $0{\cdot}2 > P > 0{\cdot}1$ (2 degrees of freedom). In order to obtain information on the question whether mice, which had survived the initial high WE_3 doses, did not respond to the challenge with Armstrong virus 21 days later because they had acquired active immunity or because they had become persistently infected carriers, a number of them were killed 80 days after the challenge inoculation, their brains were homogenized to 10 % suspensions and these were then inoculated into three mice each to test for infectivity. The results in Table 3 shows that most mice still had virus in their brains. However, from the proportions responding, the virus contents of the brains could be roughly estimated and were found to be very low, i.e. 10 ID 50 or less in most cases.

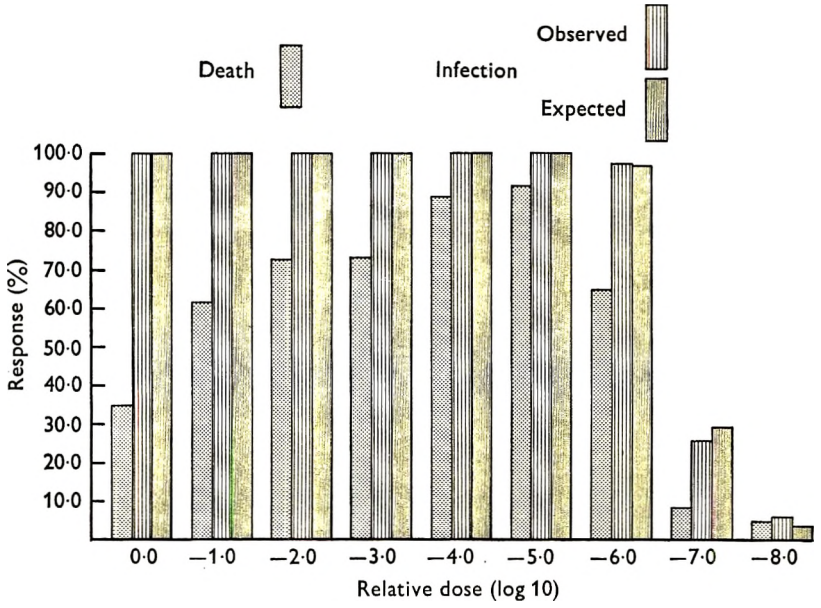

Fig. 1. Dose–response relationship between LCM virus, strain WE_3, and mice.

Table 3. *Detection of virus in brains of mice which had survived high doses of LCM virus, strain WE_3*

Original inoculum ($\log_{10}$ ID 50)	Number of mice*	
	Tested	Positive
6·73	15	13
2·73	11	8

* Challenged intracerebrally with approximately 10^3 LD 50 of Armstrong virus 80 days before sacrifice.

Armstrong virus

Dose–response in L cultures

In a preliminary experiment, L cell tube cultures were inoculated with serial tenfold dilutions of Armstrong virus and assayed individually for CF Ag following 6 days' incubation at 37° C. The results, given in Table 4, did not reveal zone phenomena at higher virus concentrations, as had been seen in WE_3-infected mice.

Table 4. *Dose–response relationship between LCM virus, strain Armstrong, and L cell tube cultures*

Relative virus dose (log_{10})	Observed response (infection)	
	No.	%
0·0	110/110*	100·00
−1·0	110/110	100·00
−2·0	110/110	100·00
−3·0	110/110	100·00
−4·0	110/110	100·00
−5·0	107/110	97·27
−6·0	31/110	28·18
−7·0	5/110	4·55

* Number of cultures infected over number inoculated.

Table 5. *Dose–response relationship between LCM virus, strain Armstrong, and L cell tube cultures*

Relative virus dose (log_{10})	Response (infection)		
	Observed		Expected
	No.	%	(%)
−4·0	109/109*	100·00	> 99·99
−4·5	109/109	100·00	> 99·99
−5·0	109/109	100·00	97·28
−5·5	77/109	70·64	68·01
−6·0	35/109	32·11	30·27
−6·5	6/110	5·45	10·77
−7·0	3/110	2·73	3·54
−7·5	1/109	0·92	1·13
−8·0	0/108	0·00	0·36

* Number of cultures infected over number inoculated.

Table 6. *Dose–response relationship between LCM virus, strain WE_3, and L cell tube cultures*

Relative virus dose (log_{10})	Observed response (infection)	
	No.	%
0·0	105/105*	100·00
−1·0	105/105	100·00
−2·0	105/105	100·00
−3·0	105/105	100·00
−4·0	105/105	100·00
−5·0	105/105	100·00
−6·0	105/105	100·00
−7·0	23/105	21·90
−8·0	2/105	1·90
−9·0	2/105	1·90

* Number of cultures infected over number inoculated.

The dose–response relationship was then more accurately determined by diluting the virus with the factor 3·162. Starting with $10^{-4\cdot0}$, 109 cultures were inoculated with each dilution and checked for infection 6 days later. As can be concluded from Table 5, observed and expected values agree well; χ^2 was found to be 4·013, which, with 4 degrees of freedom, corresponds to $0\cdot5 > P > 0\cdot3$.

WE_3 virus

With the WE_3 strain of LCM virus, again two large experiments were performed. As before, zone phenomena could not be detected (Table 6). In a further experiment (Table 7) the dilution factor was reduced to 3·162. Again, the observed values did not deviate significantly from expectation; χ^2 was estimated as 0·628, which corresponds to $0\cdot98 > P > 0\cdot95$ (4 degrees of freedom).

Table 7. *Dose–response relationship between LCM virus, strain WE_3, and L cell tube cultures*

Relative virus dose ($\log_{10}$)	Response (infection) Observed No.	Observed %	Expected (%)
−5·0	102/102*	100·00	> 99·99
−5·5	102/102	100·00	99·98
−6·0	95/102	93·14	93·11
−6·5	58/102	56·86	57·09
−7·0	26/102	25·49	23·48
−7·5	7/102	6·86	8·11
−8·0	2/102	1·96	2·64
−8·5	0/102	0·00	0·84
−9·0	0/102	0·00	0·26

* Number of cultures infected over number inoculated.

DISCUSSION

Dose–response curves in virology are, with few exceptions, adequately characterized by the zero term of the Poisson distribution, which is the same as saying that single infectious units act independently and hence one IU may elicit a response. The hosts have been found to contribute little to the effects in most instances (Meynell, 1957).

As regards infectivity our results fully agree with this general experience. In mice as well as in cell cultures the dose–response curves of both LCM prototype strains, Armstrong and WE_3, were fitted by single hit curves, based on the function e^{-xd}, where x is the number of IU per inoculated volume of the original material and d the dilution. The same type of response was found in the case of Armstrong virus with regard to the death of the mice. In contrast, the shape of the curve relating doses of WE_3 virus with deaths of mice was found to be quite different. Here, a maximum of lethality was found at approximately 50 ID 50; with higher, as well as with lower doses, the proportions of mice responding decreased.

Those who work with LCM viruses are well aware of the fact that high virus

concentrations frequently are less effective in killing mice than are lower ones. It is tempting to speculate with Hotchin & Benson (1963) that this sparing effect is caused by a mechanism which is akin to immunological paralysis in adult mice and is effected by the mass of antigen administered. There are, however, certain reservations to be made. In spite of the continuous multiplication of the antigen, this paralysis appears to be of short duration. A hundred and one days after the first IC inoculation and 80 days after a challenge with approximately 1000 LD 50, the brains of 5 of 26 mice which had survived high doses were found to be free of infectious virus, and in most of the 21 positive animals, little virus was demonstrated (10 ID 50 per brain or less). Furthermore, one is forced to ask why the two strains used here behave so differently, although their rates of multiplication in the brains of infected mice have been found to be indistinguishable (Lehmann-Grube, 1964*a*). However, as there can hardly be any doubt that a mechanism similar to immunologic tolerance protects newborn or unborn mice (Volkert & Hannover Larsen, 1965), it may well be that the administration of excessive amounts of antigen paralyses adults. Hannover Larsen (1968) has found adult mice protected when virus in high doses was injected frequently, which could be explained along similar lines. Certainly, our results do not confirm the opinion of others that LCM strains which exhibit such dose effects are of the 'docile' category, as defined by Hotchin, Benson & Seamer (1962). Both strains are 'aggressive' (Lehmann-Grube, 1964*b*), yet WE_3 spares mice at higher concentrations and Armstrong does not.

The survival of three mice from one cage which had been infected with high doses of Armstrong virus was contrary to expectation and needs some comment. We have on other occasions observed LCM-infected mice to live significantly longer, namely when suffering concurrently from an additional disease, such as a bacterial diarrhoea. Very probably this phenomenon is related to the sparing effect of X-rays (Rowe, 1956; Hotchin & Weigand, 1961) or antimetabolites (Haas & Stewart, 1956), and may indicate the animal's inability to mount an immune response to the virus resulting in an immunological conflict which is thought to be the mechanism of the LCM disease (Hotchin, 1962). It appears likely that these three mice survived longer because they were suffering from an unknown disease, infectious or not, which protected them from death due to LCM.

Much of the recent progress in animal virology can be ascribed to the introduction of accurate methods for the assay of infectivity, e.g. plaque titrations on monolayer cell cultures. Unfortunately, in the case of LCM, simple quantitative procedures are not generally available, and the titration of this virus still rests on the principle of quantal responses in dilution assays. The results presented here confirm our experience, reported previously (Lehmann-Grube & Hesse, 1967), that in cell cultures as well as in mice infection is a useful criterion for the assay of LCM viruses. Both the median effective dose (MED) as well as the most probable number (MPN) may be calculated from the data and provide an estimate of the concentration of infectious units in a given preparation. (It should be stressed that, while the MPN is a correct estimate and furthermore can quickly be looked up in tables, the MED is biased and requires calculations; its only advantage is its widespread use.)

In contrast to infectivities, deaths of mice cannot generally be used to indicate a response to LCM viruses. If a strain behaves like Armstrong, the MED or preferably the MPN may legitimately be calculated from the proportion of animals which succumb to the disease. A dose–response curve, however, as seen with WE_3 where death has been taken to indicate the effect, precludes the correct calculation of either value. Indeed, to my knowledge there is no method available to estimate the concentration of IU in a quantal assay if the underlying dose–response curve is as asymmetrical as the one just discussed.

SUMMARY

The dose–response relationships between two strains of lymphocytic choriomeningitis virus (WE_3 and Armstrong) and two hosts (mice and L cell tube cultures) were determined. The statistical analysis showed that, if infection was regarded as the response, and in the case of Armstrong virus in mice also death, the shapes of the empirical dose–response curves did not deviate from expectation which was based on the zero term of the Poisson distribution. Hence, the hypothesis that individual infectious units are capable of initiating infection and that co-operation is not required, was not contradicted. Furthermore, the units of assay were found to be equally susceptible under the experimental conditions applied.

By way of contrast, the relationship between WE_3 virus and mice dying after intracerebral inoculations was found to be more complex. In this case the empirical curve did not run the expected sigmoid course at all, but rather was bell-shaped with a maximum of mortality (92 %) at approximately 50 ID 50.

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REFERENCES

ARMSTRONG, C. & LILLIE, R. D. (1934). Experimental lymphocytic choriomeningitis of monkeys and mice produced by a virus encountered in studies of the 1933 St Louis encephalitis epidemic. *Publ. Hlth Rep., Wash.* **49**, 1019.

EAGLE, H. (1959). Amino acid metabolism in mammalian cell cultures. *Science, N.Y.* **130**, 432.

EARLE, W. R. (1943). Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. natl. Cancer Inst.* **4**, 165.

FAZEKAS DE ST GROTH, S. (1955). Production of non-infective particles among influenza viruses: Do changes in virulence accompany the von Magnus phenomenon? *J. Hyg., Camb.* **53**, 276.

HAAS, V. H. & STEWART, S. E. (1956). Sparing effect of a-methopterin and guanazolo in mice infected with virus of lymphocytic choriomeningitis. *Virology* **2**, 511.

HANKS, J. H. & WALLACE, R. E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. exp. Biol. Med.* **71**, 196.

HALDANE, J. B. S. (1939). Sampling errors in the determination of bacterial or virus density by the dilution method. *J. Hyg., Camb.* **39**, 289.

HALVORSON, H. O. & ZIEGLER, N. R. (1933). Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. *J. Bact.* **25**, 101.

HANNOVER LARSEN, J. (1968). Studies on immunological tolerance to LCM virus. 9. Induction of immunological tolerance to the virus in the adult mouse. *Acta path. microbiol. scand.* **73**, 106.

HOTCHIN, J. (1962). The biology of lymphocytic choriomeningitis infection: Virus-induced immune disease. In Basic Mechanisms in Animal Virus Biology. *Cold Spring Harb. Symp. Quant. Biol.* **27**.

HOTCHIN, J. & BENSON, L. (1963). The pathogenesis of lymphocytic choriomeningitis in mice: The effects of different inoculation routes and the footpad response. *J. Immun.* **91**, 460.

HOTCHIN, J., BENSON, L. M. & SEAMER, J. (1962). Factors affecting the induction of persistent tolerant infection of newborn mice with lymphocytic choriomeningitis. *Virology* **18**. 71.

HOTCHIN, J. & WEIGAND, H. (1961). The effects of pretreatment with X-rays on the pathogenesis of lymphocytic choriomeningitis in mice. I. Host survival, virus multiplication and leukocytosis. *J. Immun.* **87**, 675.

JOCHHEIM, K.-A., SCHEID, W., LIEDTKE, G., HANSEN, I. & STAUSBERG, G. (1957). Komplementbindende Antikörper gegen das Virus der lymphozytären Choriomeningitis im Serum von Versuchstieren und Beobachtungen zur Immunität. *Arch. ges. Virusforsch.* **7**, 143.

LEHMANN-GRUBE, F. (1964*a*). Lymphocytic choriomeningitis in the mouse. I. Growth in the brain. *Arch. ges. Virusforsch.* **14**, 344.

LEHMANN-GRUBE, F. (1964*b*). Lymphocytic choriomeningitis in the mouse. II. Establishment of carrier colonies. *Arch. ges. Virusforsch.* **14**, 351.

LEHMANN-GRUBE, F. & HESSE, R. (1967). A new method for the titration of lymphocytic choriomeningitis viruses. *Arch. ges. Virusforsch.* **20**, 256.

LOCKART, R. Z. & EAGLE, H. (1959). Requirements for growth of single human cells. *Science, N.Y.*, **129**, 252.

MEYNELL, G. G. (1957). Inherently low precision of infectivity titrations using a quantal response. *Biometrics* **13**, 149.

ROWE, W. P. (1956). Protective effect of pre-irradiation on lymphocytic choriomeningitis infection in mice. *Proc. Soc. exp. Biol. Med.* **92**, 194.

SANFORD, K. K., EARLE, W. R. & LIKELY, G. D. (1948–49). The growth *in vitro* of single isolated tissue cells. *J. natl. Cancer Inst.* **9**, 229.

SCOTT, T. F. M. & RIVERS, T. M. (1936). Meningitis in man caused by a filterable virus. I. Two cases and the method of obtaining a virus from their spinal fluids. *J. exp. Med.* **63**, 397.

SPIEGEL, A. & GÖNNERT, R. (1961). Neue Käfige für Mäuse und Ratten. *Z. Versuchstierk.* **1**, 38.

VOLKERT, M. & HANNOVER LARSEN, J. (1965). Immunological tolerance to viruses. *Prog. med. Virol.* **7**, 160.

J. Hyg., Camb. (1969), **67**, 279
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Sulphadiazine-resistant group A meningococci isolated during the 1968 meningitis epidemic in Greece

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INTRODUCTION

Since Millar *et al.* (1963) have reported the occurrence of sulphadiazine-resistance in a group B strain of *Neisseria meningitidis* at the U.S. Naval Training Center in San Diego, several authors have reported the increasing prevalence of sulphadiazine-resistant group B and group C meningococci in the military and civil population (Bories, Faucon, Audiffren & Bonzom, 1964; Bories, Faucon, Oddou & Audiffren, 1965; Leedom *et al.* 1965; Bristow, van Peenen & Volk, 1965; Eickhoff & Finland 1965; Feldman, 1966; Farrell & Dahl, 1966; Dowd *et al.* 1966; Sanders, 1967; and others). In fact, group B meningococci were reported to be less uniformly inhibited by sulphonamides than other meningococcal types by Branham, as early as 1940 and 1953 (cited by Leedom *et al.* 1965). Love & Finland (1954) also had detected, among 50 group B strains isolated before May 1954, 24 % highly resistant to sulphadiazine. However, the San Diego epidemic was the first large-scale outbreak due to highly resistant strains.

Such striking observations of sulpha-resistance were not reported in group A strains of *N. meningitidis*. Yet Bories *et al.* (1964), among 83 strains of group A meningococci tested, found one which was resistant *in vitro* to a concentration of 5 mg./100 ml. of sulphadiazine and also four others to 1 mg./100 ml. Moreover R. Faucon (personal communication), observed sulphadiazine-resistance in group A strains isolated in 1967 in Morocco and in 1968 in Tchad.

In Greece little was known about the prevailing serological groups and the sensitivity to sulphonamides of the meningococci responsible for the disease until the end of 1967. In this country, meningococcal meningitis occurs in endemic form, with an epidemic wave usually every 8–10 years. Some of the epidemiological features of the disease in the last years are shown in Tables 1 and 2 and in Fig. 1. (Data from the Archives of the Public Health Division, Ministry of Social Welfare, and from Publications of the Population Department of the National Statistical Service of Greece.)

It can be seen from Table 1 that the morbidity rate per 100,000 inhabitants fluctuates between 9·41 (1954) and 1·66 (1962). The decrease in morbidity in the last ten years may be partly attributed to the larger use of chemotherapeutic agents by the physicians.

The case mortality rate in the last 9 years shows a fluctuation between 3·46 in 1959 and 9 in 1961.

The seasonal distribution of the disease for the periods 1961–3, 1964–6 and for

the year 1967 is shown in Table 2 and in Fig. 1. It can be seen that, except for the period 1961–3, the disease follows the usual seasonal pattern; that is to say, a big rise of the curve during the spring with a peak in March. This seasonal form is more evident for the year 1967. A similar seasonal incidence of meningococcal meningitis is reported by Taylor & Knowelden (1957), Wilson & Miles (1964), Bevan-Jones & Miller (1967) and others. The influence of the season may probably be due to environmental conditions, which create specific microclimatic influences in some population groups (army barracks, schools, etc.).

Table 1. *Morbidity and case mortality rate of cerebrospinal fever during the years* 1950–67* (*case fatality rate for the years* 1959–67)

Years	No. of cases	Morbidity per 100,000	Years	No. of cases	Morbidity per 100,000	Case mortality rate (%)
1950	587	7·76	1959	433	5·24	3·46
1951	499	6·52	1960	240	2·88	5·83
1952	493	6·37	1961	200	2·38	9·00
1953	331	4·23	1962	140	1·66	7·86
1954	743	9·41	1963	161	1·90	3·73
1955	308	3·87	1964	166	1·95	6·67
1956	389	4·84	1965	215	2·51	5·55
1957	318	3·93	1966	198	2·30	3·53
1958	392	4·80	1967	374	4·33	5·08

* For 1968 see text.

Table 2. *Seasonal swing in morbidity of cerebrospinal fever in Greece during the years* 1961–3, 1964–6 *and* 1967

Months	1961–3	1964–6	1967
January	13·37	7·60	6·68
February	10·98	12·44	10·43
March	11·38	15·37	17·91
April	10·57	12·26	12·04
May	6·99	10·36	15·24
June	9·78	10·54	9·09
July	4·79	7·08	6·15
August	3·79	4·66	5·61
September	5·59	2·94	5·08
October	8·98	3·63	3·21
November	6·59	5·69	4·55
December	7·19	7·43	4·01
	100·00	100·00	100·00
Total cases	501	579	374

Following the endemo-epidemic rise of 1967, a severe and extensive epidemic of meningococcal meningitis developed during the winter and the spring of 1968. Thus from January to April 1968, 716 cases were notified (January 57 cases, February 266, March 231, April 162). This number is about twice the number of

cases which occurred throughout 1967.* The cases during the 1968 epidemic occurred in most regions of the country, especially in army and navy barracks and to a lesser extent in schools and in other civil population. Moreover, an increase in the case mortality rate was noted in 1967 and 1968. This rate was 5·08 % in 1967 and 4·33 % from January to April 1968. The high increase in the morbidity rate and the rise in the case mortality rate may be explained by the probable emergence, in the population, of more virulent strains. This assumption, without excluding an eventual change in the susceptibility of the host, is corroborated by the fact that in the last few years, in some countries of Europe, Africa and Oceania, an increasing incidence of the disease has been observed (*Epidemiological and Vital Statistics Reports*, WHO, 1960, 1962–1967).

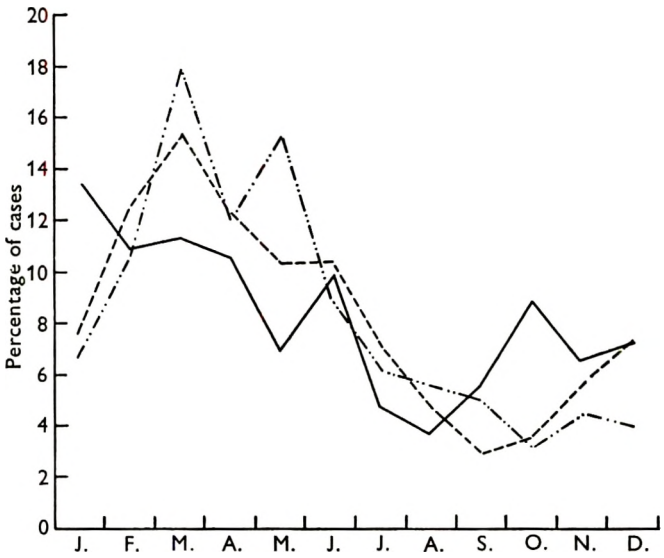

Fig. 1. Seasonal swing in morbidity of meningococcal meningitis in Greece during the years 1961–3 (——), 1964–6 (— —) and 1967 (—·· —··)

During the 1968 epidemic, we had the opportunity to isolate meningococci from patients and from healthy nasopharyngeal carriers and to determine their serological group and their susceptibility to sulphadiazine.

METHODS AND MATERIALS

Isolation media

For the detection of healthy carriers, nasopharyngeal swabs were taken and immediately streaked on Thayer–Martin selective medium (Thayer, Frank & Martin, 1965). This medium was prepared with Mueller–Hinton starch agar (Difco) to which supplement B (Difco), ristocetin (Abbott) and polymyxin B (Pfizer)

* Before this paper went to press the figures for the rest of 1968 became available. The total number of meningitis cases for the year was 1065, just over three times the annual average over the 18 years 1950–67, and nearly 50 % higher than the highest single annual total (743 in 1954.) Approximately two-thirds of these cases occurred between January and April, and one-third during the remaining eight months. The morbidity rate per 100,000 for the whole year was 12·09, and the case mortality rate was 4·03 %.

were added. The inoculated plates were incubated at 37° C. for 24–48 hr. under increased carbon dioxide tension (candle jar).

From clinical cases of meningitis the organisms were isolated by culturing the spinal fluid on chocolate agar.

Identification

The identification was based on conventional characters. Meningococci were Gram-negative, oxidase-positive, fermented dextrose and maltose but not sucrose, and failed to grow on nutrient agar (Difco) at 37° C. and on tryptose agar (Difco) at 22–25° C. after prolonged incubation. All strains were serologically identified by the slide agglutination method with group-specific meningococcal sera. Serial tube agglutination tests were done only on eight strains which gave uncertain results by the slide technique (probably of group B).

Tests for sulphadiazine susceptibility

The *in vitro* testing of the susceptibility to sulphadiazine was performed by the plate dilution method on Mueller–Hinton agar (Difco). The range of sodium sulphadiazine concentrations incorporated in the medium was 0·1, 1, 5, 10 and 20 mg./100 ml. Sodium sulphadiazine solution in ampoules for intravenous injection (Maizel Lab. Inc.) was employed for this purpose. Two inocula from each strain were used. They were prepared as follows: The strains were grown at least twice for 18–24 hr. on Mueller–Hinton agar. From the last growth on this agar a slightly opalescent suspension of organisms in Mueller–Hinton broth was prepared. From this suspension a 1/10 dilution, again in Mueller–Hinton broth, was also prepared.* Inocula from both suspensions from each strain (undiluted and diluted 1/10) were streaked with a 4 mm. loop over 1 or 2 $cm.^2$ of the surface of the sulphadiazine-containing plates. The plates were read after 24 and 48 hours. For this reading, use was made of the series of plates streaked with the inoculum which showed on the control plate, after 24–48 hr, an almost confluent layer of bacterial growth. These *in vitro* tests were repeated once more on strains of reduced sensitivity. In addition, we compared the method we used for the determination of sulphadiazine susceptibility with the technique used for the same purpose by Bories *et al.* (1964) in the International Reference Centre for meningococci. Their method differs from ours in that they use pure sulphadiazine which they dissolve in 0·1 N-NaOH, and in their inoculating the plates from a 4 hr. growth in Mueller–Hinton broth. The results of sulphadiazine susceptibility by both these methods on 9 group A and 1 group B meningococci were in general agreement.

Source of strains

A total of 105 strains of *N. meningitidis* were examined. They were isolated from the following sources:

(1) *From patients*

Twenty strains from the spinal fluid of patients were examined. Of these, ten

* Mueller–Hinton broth was prepared by soaking in water, Mueller–Hinton starch agar (Difco) for 1–2 hr. and by decanting the supernatant.

were isolated from cases which occurred in soldiers in army barracks situated in five different regions of the country and the remaining ten were from civilians, also from various areas of Greece. These isolations were done between January and April 1968, except for one in June 1968.

(2) *From nasopharyngeal carriers*

(a) *Army barracks.* Nasopharyngeal swabs were taken, on 21 February 1968, from 50 healthy soldiers of an army barracks in the region of Attica. In this barracks four cases of meningitis occurred between 29 December 1967, and mid-February 1968. The strains of three out of the four cases were examined and found to belong to serological group A.

One gramme of oral sulphadiazine, twice daily for 3 days, had been given to all the men of the barracks on three occasions. These were the dates on which each of the first three cases (i.e. 29 December 1967, 21 January and 8 February 1968) appeared. Prophylactic antibiotics had not been given.

After the last administration (8–10 February) of the sulphadiazine, 50–60 privates, who were on leave, returned to the barracks.

(b) *Naval Training Centre.* Swabs were taken on 14 March 1968 from 54 healthy recruits of a Naval Training Centre which is also in the Attica area but about 10 miles away from the army barracks already referred to. This centre consists of two communicating training centres, one of which had a population of 896 and the other one of 779 persons. Thus, the sample taken represented about 3% of the total population. Two-thirds of the total naval personnel were new recruits. The last recruits joined the centre on 7 January 1968. At the beginning of January a high proportion of cases of pharyngitis was noted.

In this centre, 11 cases of meningitis appeared between 23 January and 11 February 1968. All cases, except one, occurred among recruits. It is worth noting that in one of these training centres, which has five wards, four out of the five cases occurred in one ward.

On 23 January 1968 all the individuals of the centre were placed on prophylactic sulphonamides. They were given oral sulphadiazine 3 g. daily for about 6 days and thereafter 1 g. of sulphamethoxypyridazine daily, until 6 February 1968. After a 3–day interruption, sulphamethoxypyridazine was once again ordered for 3 more days.

No antibiotics had been used for prophylactic purposes in this centre.

(c) *Civilians.* Nasopharyngeal samples were taken from 51 healthy civilians on 6 March 1968. Out of these, 36 were students of the sanitarian branch of the Athens School of Hygiene and 15 were employees of the Athens Health Centre. No case of clinical meningitis had occurred among the contacts of these persons. Their homes were scattered in almost all parts of Athens. Sulphonamides or antibiotics had not been taken by any of them during the months preceding their examination.

RESULTS

Strains isolated

A total of 105 meningococcal strains were isolated from patients and from healthy nasopharyngeal carriers.

The number and the percentage of the strains of different serological groups isolated from various sources are analysed in Table 3.

In addition to the results shown in Table 3, the following data can be inferred from the reading of this table. Of the total number of 105 strains isolated, 66 belong to group A, 36 to group B and 3 to group C. Two of these meningococci, one of group A and one of group B, were harboured by the same healthy carrier. In the army barracks a total of 72 % healthy carriers of various groups of meningococci was found, while in the Naval Training Centre this rate was 59 %. Among civilians 31 % were carriers of only group B meningococci.

Table 3. *Numbers and groups of meningococci isolated from healthy carriers and patients*

(Figures in parentheses indicate percentages.)

Meningococcus groups isolated	Carriers*			Patients†	Total
	Soldiers	Sailors	Civilians		
Group A	26 (52)	21 (39)	0	18 (90)	65 (37)
Group B	6 (12)	11 (20)	16 (31)	2 (10)	35 (20)
Group C	3 (6)	0	0	0	3 (2)
Mixed A + B	1 (2)	0	0	0	1
Negative	14 (28)	22 (41)	35 (69)	0	71 (41)
Total examined	50	54	51	20	175

* From different areas of Attica. † From different regions of the country.

It is to be mentioned that all the ten cases of meningitis among soldiers were group A infections, while out of the ten civilian cases, eight were group A and two were group B infection.

Eight strains which gave equivocal results in the serological grouping were sent to the International Reference Centre at Marseille for examination. They were all found to belong to group B. In addition, for control purposes, eight group A and three group B meningococci, taken at random, were also sent to this Centre. Our typing on these 11 strains was confirmed.

Susceptibility to sulphadiazine

The susceptibility of 101 of the meningococci (four strains were lost) to sulphadiazine, tabulated according to their serological group and their origin, is shown in Table 4. The sulpha-susceptibility of eight group A and 11 group B meningococci, taken at random, was also examined by Dr R. Faucon, Director of the International Reference Centre for meningococci. Differences in susceptibility were found in

some strains. However, of the eight group A strains which were sent to this Centre, Dr Faucon found one resistant to 20 mg./100 ml. of sulphadiazine, another to 5 mg./100 ml. and four to 1 mg./100 ml.

It is of interest to note that Dr Faucon has also tested, by the disk technique, the susceptibility of these 19 strains to penicillin, streptomycin and chloramphenicol. They were all found to be sensitive to these antibiotics.

Table 4. *Susceptibility of strains of meningococci to sulphadiazine*

(Figures in parentheses indicate percentages.)

Origin of strains	Number of strains showing minimum inhibitory concentration of (mg./100 ml.): 0·1	1	5	10	20	>20	Total no. of strains
			Group A strains				
Soldiers*	1 (4)	0	16 (59)	10 (37)	0	0	27
Sailors*	0	0	2 (10)	10 (53)	6 (32)	1 (5)	19
Patients	0	1 (6)	9 (50)	4 (22)	2 (11)	2 (11)	18
Total group A	1 (1½)	1 (1½)	27 (42)	24 (37)	8 (12)	3 (5)	64
			Group B strains				
Soldiers*	5 (71)	1 (14)	0	1 (14)	0	0	7
Sailors*	0	1 (9)	2 (18)	5 (45)	3 (27)	0	11
Civilians*	12 (86)	0	2 (14)	0	0	0	14
Patients	0	1	0	0	0	1	2
Total group B	17 (50)	3 (9)	4 (12)	6 (18)	3 (9)	1 (3)	34
			Group C strains				
Soldiers*	2	1	0	0	0	0	3

* Carriers only

DISCUSSION

The high proportion of group A meningococci (90 %) isolated from patients during the 1968 epidemic of meningococcal meningitis points to the fact that organisms of this serological group were responsible for this epidemic.

An important aspect of our study is the isolation of group A meningococci resistant or of reduced sensitivity to sulphadiazine (see Table 4). These organisms came from different regions of the country. Three strains out of 64 were resistant to a concentration of 20 mg./100 ml.; two were isolated from patients of different regions and one from a healthy carrier. Two strains only were sensitive to concentrations of 0·1 mg./100ml. and 1 mg./100 ml., respectively. The minimal inhibitory concentrations of sulphadiazine for the remaining strains were as follows: 5 mg./100 ml., 27 strains; 10 mg./100 ml., 24 strains; 20 mg./100 ml., 8 strains.

Table 4 shows clearly that the group A meningococci isolated from carriers of the Naval Training Centre (naval recruits) were on the average more resistant to sulphadiazine than those isolated from carriers of the army barracks. This is probably due to the fact that persons in the Naval Centre had received larger and

more prolonged doses of sulphonamides and as a result more resistant mutants were selected in this centre.

The finding of *in vitro* resistance to sulphadiazine in group A meningococci isolated from individuals of the army barracks and of the Naval Training Centre is corroborated by the detection of high rates of group A healthy carriers in both military establishments, 52 % and 38·9 % respectively, despite the fact that all individuals in both establishments had been placed under chemoprophylaxis shortly before the samples were taken. In the army barracks, three standard prophylactic courses of sulphadiazine had been given (the last course was terminated 11 days before the samples were taken). In the Naval Training Centre large and prolonged doses of sulphadiazine and sulphamethoxypyridazine had been given $4\frac{1}{2}$ weeks before the nasopharyngeal swabs were taken.

Moreover, in both establishments several cases of meningitis appeared during, or shortly after, the chemoprophylaxis. The strains from three of these patients were examined and were found to belong to group A. They were also found of reduced sensitivity to sulphadiazine (minimal inhibitory concentration of 50 mg./100 ml.). In addition, sulpha-resistant group A meningococci with minimal inhibitory concentrations to sulphadiazine ranging from 50 mg./100 ml. to more than 200 mg./100 ml., were isolated from patients belonging to other army barracks, in which swabbing for healthy carriers was not done, but in which repeated courses of chemoprophylaxis had been administered before the appearance of the clinical cases. We may assume from these observations that group A meningococci, resistant to prophylactic doses of sulphonamides, were prevalent in these military establishments.

As a matter of fact, according to data accumulated by the U.S. army and quoted by Brown & Condit in 1965 (cited by Leedom *et al.* 1965), strains resistant to 0·1 mg./100 ml. of sulphadiazine or more cannot be eradicated from the nasopharynx of carriers with standard doses of this drug. Therefore, from the results of our *in vitro* tests which showed that all the group A meningococci, except one, were strains with minimal inhibitory concentrations of 1 mg./100 ml. or more, we may assume that they could be classified as sulpha-resistant on epidemiological grounds. On the other hand, in patients, on account of the concentration of sulpha-drugs attainable in the blood and spinal fluid without excessive toxicity, many of these strains could eventually be sensitive on clinical grounds. However, those with minimal inhibitory concentrations of more than 5 mg./100 ml. could be expected to be clinically resistant.

Concerning the civilians examined, it is worth noting that none of the 51 healthy individuals swabbed was a carrier of group A meningococci, whilst of the 10 cases of meningitis in civilians, eight were group A infections (80 %) and only two group B. Although the healthy group of civilians examined was a very small one and came only from the Athens area, the absence of group A carriers among them, in contrast to the frequency of group A infection in civilian patients, seems to suggest that the responsible strains in the case of meningitis in the civilian population originated from highly crowded military establishments, etc., in which a high carrier rate of group A meningococci was detected.

Group B meningococci were less prevalent than group A in all groups of individuals examined, with the exception of the healthy civilians who harboured only group B organisms.

The detection of a high proportion of sulpha-resistant group B meningococci in the Naval Training Centre is not surprising if we keep in mind that 'sulpha-prophylaxis' was undertaken repeatedly in this centre and that the occurrence of resistant group B strains has been well known since 1963 (see introduction).

In contrast to the Naval Centre, in the army barracks, in spite of previous chemoprophylaxis, six out of the seven group B and three group C meningococci recovered were strains with minimal inhibitory concentrations to sulphadiazine of 1 mg./100 ml. or less. It is likely that these sensitive strains were introduced in the army barracks during the period between the last administration of sulphadiazine and the sampling from the nasopharynx. In fact, 50–60 men returned to the barracks during this 11-day period. Another possible explanation is that not all the individuals in the barracks had taken sulphadiazine as prescribed by the physicians. However, the Medical Officers of the establishment consider this second possibility highly improbable.

SUMMARY

During the 1968 epidemic of meningococcal meningitis in Greece, 90 % of the meningococci recovered from the spinal fluid of patients belonged to serological group A. In one army barracks and in one Naval Training Centre, where cases occurred, a rate of 52 % and 38·9 % respectively of healthy nasopharyngeal carriers of group A meningococci was found, despite repeated courses of sulpha-prophylaxis given to all the population of these military establishments shortly before the swabs were taken.

The susceptibility to sulphadiazine of 64 group A meningococci isolated from patients in different regions of the country and from healthy carriers was tested *in vitro*. It was found that only one strain was sensitive to a concentration of 0·1 mg./100 ml. of sulphadiazine and another to 1 mg./100 ml. For the remaining strains, the minimal inhibitory concentrations of sulphadiazine ranged from 5 mg./100 ml. to more than 20 mg./100 ml.

Thirty-six strains of group B meningococci were isolated from 34 healthy carriers and from two patients. Some of these strains were resistant to sulphadiazine. Only three strains of group C meningococci were isolated from healthy nasopharyngeal carriers in one army barracks.

We wish to express our thanks to Dr R. Faucon, Director of the International Reference Centre for meningococci, for the valuable help he gave to us during our investigation, as well as for the supply of the group-specific agglutinating anti-meningococcal sera.

REFERENCES

BEVAN-JONES, H. & MILLER, D. L. (1967). Bacterial meningitis, 1962–63. *Mon. Bull. Minist. Hlth* **26**, 22–30.

BORIES, S., FAUCON, R., AUDIFFREN, P. & BONZOM, A. (1964). Etude de l'activité *in vitro* de la sulfanilamido-4-dimethoxy-5,6-Pyrimidine sur *Neisseria intracellularis*. *Méd. trop.* **24**, 9–17.

BORIES, S., FAUCON, R., ODDOU, A. & AUDIFFREN, P. (1965). Contribution à l'étude de la sensibilité *in vitro* aux sulfamides de *Neisseria intracellularis*. *Méd. trop.* **25**, 41–9.

BRISTOW, W. M., VAN PEENEN, P. F. D. & VOLK, R. (1965). Epidemic meningitis in naval recruits. *Am. J. publ. Hlth* **55**, 1039–45.

DOWD, J. M., BLINK, D., MILLER, C. H., FRANK, P. F. & PIERCE, W. E. (1966). Antibiotic prophylaxis of carriers of sulfadiazine-resistant meningococci. *J. infect. Dis.* **116**, 473–80.

EICKHOFF, T. C. & FINLAND, M. (1965). Changing susceptibility of meningococci to antimicrobial agents. *New Engl. J. Med.* **272**, 395–398.

Epidemiological and Vital Statistics Reports, W.H.O. (1960), **13**, nos. 2–3; (1962), ibid. **15**, no. 2; (1963). ibid. **16**, no. 9; (1964), ibid. 17, nos. 1, 2, 7; (1965), ibid. **18**, nos. 2, 8, 9; (1966) ibid. **19**, nos. 2, 7, 9; (1967), ibid. **20**, nos. 2, 8.

FARRELL, D. G. & DAHL, E. V. (1966). Nasopharyngeal carriers of *Neisseria meningitidis*. *J. Am. med. Ass.* **198**, 1189–92.

FELDMAN, H. A. (1966). Meningococcal disease, 1965. *J. Am. med. Ass.* **196**, 391–3.

LEEDOM, J. M., IVLER, D., MATHIES, A. W., THRUPP, L. D., PORTNOY, B. & WEHRLE, P. F. (1965). Importance of sulfadiazine resistance in meningococcal disease in civilians. *New Engl. J. Med.* **273**, 1395–401.

LOVE, B. D. & FINLAND, M. (1954). *In vitro* susceptibility of meningococci to 11 antibiotics and sulfadiazine. *Am. J. med. Sci.* **228**, 534–9.

MILLAR, J. W., SIESS, E. E., FELDMAN, H. A., SILVERMAN, C. & FRANK, P. (1963). *In vivo* and *in vitro* resistance to sulphadiazine in strains of Neisseria meningitidis. *J. Am. med. Ass* **186**, 139–41.

SANDERS, E. (1967). Use of sulfonamide carbonic anhydrase inhibitors in treatment of meningococcal carriers: rationale and report of a clinical trial of ethoxzolamide. *Am. J. med. Sci.* **254**, 709–16.

TAYLOR, I. & KNOWELDEN, J. (1957). *Principles of Epidemiology*, p. 245. London: J. A. Churchill Ltd.

THAYER, J. D., FRANK, P. F. & MARTIN, J. E. (1965). Thayer–Martin selective medium for the cultivation of *Neisseria meningitidis* from the nasopharynx. *Am. J. publ. Hlth.* **55**, 923–7.

WILSON, G. S. & MILES, A. A. (1964). *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th ed., Vol. II, p. 1738. London: Edward Arnold Ltd.

ADDENDUM

After this paper was sent for publication we read a paper (Alexander, Sanborn, Cherriere, Crocker, Ewald & Kay, 1968, *Science, N.Y.* **161**, 1019) in which the authors report that, during a meningitis epidemic in Meknes, Morocco, due to group A meningococci, most of the strains isolated from patients were resistant to sulphadiazine.

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The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses

I. Effect of reagents reacting with amino acids in the active centres

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The results of disintegration of influenza virus particles by ether treatment, and studies of the effects of proteolytic agents on the particles, show that the haemagglutinating, enzymic, and specific complement-fixing activities are closely related properties of the outer component of the virus particle, being associated with the surface projections. Some activity is probably also present in the interior of the particle. There are serological differences between the specific complement fixing antigens of different strains of virus, differences in the ability to agglutinate different types of red cell and in susceptibility to different mucoprotein inhibitors of haemagglutination, and differences in the enzymic activity against different substrates. By treatment of virus with chemical reagents acting on the reactive groups of protein molecules it was shown by Hoyle & Hana (1966) that there were differences in the chemical structure of the active centres of the haemagglutinins and neuraminidases of the DSP strain of virus A and the LEE strain of virus B. This work has now been extended to a range of strains of virus A.

Chemical reagents used in the work fall into two groups. The first consists of reagents which probably owe their activity to direct reaction with amino acids present in the active centres of the virus haemagglutinin or neuraminidase. The second group consists of reagents which do not act directly on the active centres but which alter the higher order structure of the protein molecule by reaction with hydrogen or other non-covalent bonds or with disulphide bridges. The results with the two groups of reagents were very different and they will be considered separately. This paper deals with reagents acting on the active centres, while the following paper (Hoyle, 1969) describes the results obtained with reagents of the second group.

METHODS

Virus preparations

Highly purified virus preparations were used in the work. Infected egg allantoic fluids were subjected to two cycles of adsorption–elution from guinea-pig red cells, concentrating $\times 15$, followed by a cycle of differential centrifugation. The final deposited virus was suspended in phosphate-buffered saline of pH 7·0 to a concentration of about 1 % by volume. This material is referred to as 'virus concentrate'.

For use in most of the tests the concentrate was diluted 1/10 with saline—'virus dilution'. In addition virus dilution preparations were disintegrated by shaking with ether at room temperature. After removal of ether and centrifugation to remove precipitated lipoprotein the supernatant 'ether-treated virus dilution' was used in the tests. The haemagglutinin titre of virus dilution preparations ranged from 8000 to 16,000 with predominantly spherical strains such as DSP or PR 8 to from 2000 to 8000 with filamentous strains. Ether-treated preparations were less variable, most giving titres of 16,000–32,000.

Eight strains of virus have been used in the work, the SWINE, PR 8, and DSP strains of virus A; two strains of A_1 virus, A_1 BURCH isolated in Northampton in 1947 and a Czechoslovak strain A_1 BRATISLAVA; two strains of A_2 virus, A_2/TAIWAN/64 and A_2/ENGLAND/67; and the LEE strain of virus B.

Conditions for chemical reaction

Virus dilution and ether-treated virus dilution preparations in volumes of 0·4 ml. were mixed with an equal volume of chemical reagent dissolved in buffered saline of suitable pH, 0·5% phosphate buffered saline being used for pH 5–8·0, and 0·5% borate saline for pH 9·0. Reagent-free controls were set up and exposed to the same pH and temperature for the same duration as the test samples.

After interaction with reagent the preparations were diluted to 2·0 ml. with buffered saline, pH 6·0, and immediately tested for haemagglutinin titre and neuraminidase activity.

Haemagglutinin titrations

Haemagglutinin was titrated by the Salk method with 0·5% guinea-pig red cells at pH 6·0, preparations being tested in dilutions from 1/32 to 1/65, 536.

Test for neuraminidase activity

Many of the chemical reagents used in the work interfere with the estimation of neuraminic acid by the Aminoff (1961) method and for this reason neuraminidase activity was tested by determining the ability of the treated virus to elute from red blood cells and to destroy the cell receptors.

After estimation of haemagglutinin titre the diluted virus-reagent mixtures were chilled to 4° C. and adsorbed with 0·05 ml. of a 1/3 dilution of packed guinea-pig red cells. The cells were then centrifuged out and resuspended in 1·0 ml. of buffered saline, pH 6·0, and incubated at 37° C., shaking at intervals of 10 minutes. At two intervals the tubes were centrifuged and the haemagglutinin titre of the supernatants measured. The first test was made as soon as the control preparation showed any sign of elution as indicated by a reduced rate of sedimentation of the red cells, and the second test was made after a four times longer period of elution. Thus with strains of virus which eluted rapidly tests were made at 20 and 80 min., with more slowly eluting strains at 30 and 120 min. while with very slowly eluting strains tests were made at 45 and 180 min.

The amount of virus used in the tests was much more than sufficient to saturate completely the red cells used in the elution test, so that neuraminidase activity

could be assessed provided the chemical reagent used did not reduce the haemagglutinin titre to less than 25 % of the original. But if the haemagglutinin was destroyed then none could be adsorbed or eluted. In such cases the elution test was carried out as if haemagglutinin was present and at the end of the elution period the red cells were centrifuged out and tested for agglutinability by fresh virus in a simple slide test. In all such cases it was found that the cells remained agglutinable, indicating that destruction of haemagglutinin also destroyed enzymic activity.

Design of experiments and assessment of results

Reaction of chemical reagents with the virus strains was assessed in two types of experiment. In the first a single strain of virus was tested simultaneously against a range of different reagents, and in the second several strains of virus were tested simultaneously against the same reagent. In each case both virus dilution and ether-treated virus dilution preparations were used. The activity of each reagent against each virus was thus tested in at least four different experiments. The results with intact and ether-disrupted virus were the same with reagents acting at pH 9·0 as at this pH the virus particles tend to be slowly disrupted, but at more acid reactions the two types of preparation showed slight differences in sensitivity, haemagglutinin of intact virus being more resistant than that of ether-treated virus, while neuraminidase activity was more resistant to attack in the ether-treated than in the intact virus preparations. These differences are explained in the following way. Intact virus particles carry several hundred surface projections and to prevent such a particle agglutinating red cells it might be necessary to destroy over 99 % of the haemagglutinin. The haemagglutinin particles in the ether-treated preparations carry a much smaller number of haemagglutinating units per particle and a smaller percentage reduction will be needed to prevent bridge formation between red cells. Neuraminidase activity of intact virus will be destroyed if the surface projections alone are inactivated, but on ether treatment internal neuraminidase is released and the enzyme is distributed over a much larger number of separate particles and may be more efficient. Ether-treated preparations frequently elute more rapidly in the elution test than intact virus.

In addition to these differences it is important to realize that chemical reaction with the active centre is more easily demonstrated in the case of the neuraminidase than with the haemagglutinin. A 50 % reduction in neuraminidase activity is detectable in the elution test, but a much greater destruction of haemagglutinin is necessary to prevent bridge formation between red cells.

From the mean results of four or more experiments the activity of each reagent against each virus was assessed and given a value from 0–4. In the case of haemagglutinin titrations the significance of the values is as follows:

0 = no reduction in haemagglutinin titre;
1 = reduction of titre to $\frac{1}{4}$ of the control;
2 = reduction of titre to $\frac{1}{16}$ of the control;
3 = reduction of titre to $\frac{1}{64}$ of the control;
4 = total destruction of haemagglutinin.

With the test for neuraminidase activity:

0 = haemagglutinin titres in the elution tests the same as in the control;
1 = reduced titre at the first elution test with complete elution at the second test;
2 = greatly reduced titre at the first test and some reduction at the second test;
3 = no elution at the first test and reduced titre at the second test;
4 = total failure to elute and complete sensitivity of the red cells used to agglutination by fresh virus.

Chemical reagents

Previous studies (Hoyle & Hana, 1966) did not suggest that reagents reacting with carboxyl or alcoholic hydroxyl groups could be usefully employed in the present work. Reagents reacting with —COOH groups precipitate the virus and destroy all virus properties. Reagents reacting with alcoholic —OH groups are almost all non-specific and results obtained with them were attributed to action on other groups. The following reagents have been used under the conditions described.

Sodium dichromate. Preparations were exposed to dichromate in a concentration of 1/10,000 at pH 6·0 for 4 hr. at 37° C. The reagent reacts specifically with the sulphydryl group —SH of cysteine.

Iodacetamide. Reacts specifically with the SH group when used in a concentration of 1/1000 at pH 6·0 for 4 hr. at 37° C.

Acrylonitrile. Used in a concentration of 1/200 at pH 7·0 for 2 hr. at 37° C. Reacts with —SH and amino groups (McKinney, Uhing, Setzkorn & Cowen, 1950).

Phenylisothiocyanate. Reacts primarily with amino groups. Virus preparations were treated at pH 9·0 for 18 hr. at 4° C. The reagent was added to virus–buffer mixtures in solution in 0·05 ml. of ether and shaken to give an emulsion with a final concentration of 1/1000. Control preparations were treated with reagent-free ether. This reagent could only be used in studies of the action on haemagglutinin as it rapidly lysed the red cells used in the elution test and this interfered with assessment of neuraminidase activity.

β propiolactone. Used in a concentration of 1/800 at pH 8·0 for 2 hr. at 37° C.; 2 % sodium acetate was added to give extra buffering. Reacts with amino groups and slightly with the —NH group of histidine.

Formaldehyde. Reacts rapidly with amino groups. Prolonged exposure to formaldehyde results in the formation of methylene bridges between amino and amide or guanidyl groups (Fraenkel-Conrat & Olcott, 1948). Preparations were tested under three different conditions. (*a*) Treatment with 1/1000 formaldehyde at pH 7·0 for 1 hr. at 37° C. Only amino groups react. (*b*) Treatment with 1/1000, and (*c*) with 1/100 formaldehyde at pH 7·0 for 24 hr. at 37° C. (under these conditions it was expected that amide and guanidyl groups would react in addition to amino groups).

Glyoxal. Used in a concentration of 1/200 at pH 8·5 for 4 hr. at 37° C. Reacts powerfully with amino groups and less actively with the guanidyl group of arginine (Nakaya, Norinishi & Shibata, 1967).

Xanthydrol. Reacts with amide and guanidyl groups at acid reactions. Reagent was added to virus–buffer mixtures in solution in 0·05 ml. of methanol and the mixture shaken to produce an emulsion with a final concentration of 1/1000. Controls were treated with reagent-free methanol. Preparations were treated at pH 5·5 for 4 hr. at 37° C. The reagent was more active at pH 5·0 but some of the virus strains were susceptible to acid at this reaction. Only ether-treated virus preparations gave satisfactory results; variable results were obtained with intact virus apparently because the reagent produced a slow disruption of the virus particle with release of internal haemagglutinin.

Iodine. Iodine at pH 6·0 reacts with cysteine, methionine, tryptophan, tyrosine, histidine, cystine and proline, but there are very great differences in the rate of reaction. In a concentration of 1/10,000 at 0° C. iodine reacts instantly with cysteine and methionine and in a few seconds with tryptophan. Tyrosine reacts more slowly with a half-life of some 20 min. Histidine reacts very slowly with a half-life of over 24 hr. Cystine and proline do not react at 0° C. At 37° C. cysteine, methionine, tryptophan and tyrosine react rapidly and histidine within 1 hr. Cystine and proline react much more slowly. Iodine was used at pH 6·0 in a concentration of 1/10,000 (dilutions prepared from a stock solution of 1/1000 iodine in 1 % KI) under three conditions:

(*a*) At 0° C. for 2 min.—Reaction with the SH group of cysteine, the CH_3S group of methionine and the indole ring of tryptophan.

(*b*) At 0° C. for 1 hr.—reaction also with the phenol ring of tyrosine.

(*c*) At 37° C. for 1 hr.—reacts also with the imidazole ring of histidine.

After interaction the iodine was neutralized with thiosulphate. The controls were treated with iodine previously neutralized with thiosulphate.

2,4-*dinitro*-1-*fluorobenzene* (*FDNB*). The activity of this reagent increases with increasing pH. Sulphydryl and amino groups react rapidly and the —NH group of histidine and the phenolic hydroxyl group of tyrosine more slowly. Because of the reaction with amino groups it was not possible to determine directly the rates of reaction with histidine and tyrosine but studies were made using iminazole and phenol and these indicated that at pH 8·0 the —NH group reacted more rapidly than the phenolic hydroxyl group. At pH 9·0 both groups reacted rapidly. In an attempt to differentiate between action on histidine and tyrosine the reagent was used at both pH 8·0 and 9·0. In each case the reagent was added to the virus–buffer mixture in solution in ether and the mixture was shaken to give an emulsion with a final concentration of 1/800. The mixtures were held at 20° C. for 30 min. followed by 18 hr. at 4° C. Controls were treated with reagent-free ether.

At pH 8·0 it was considered that effects produced would be due mainly to action on the —NH group of histidine (apart from reaction with —SH and amino groups). No significant hydrolysis of the reagent occurred at pH 8·0. At pH 9·0 the mixtures developed a yellow colour indicating hydrolysis of the reagent and it was considered that at pH 9·0 both the histidine NH group and the tyrosine OH group would react.

Diazotized sulphanilic acid. Reacts with histidine, tyrosine and tryptophan. It was used in a concentration of 1/5600 at pH 9·0 for 18 hr. at 0° C.

RESULTS

The haemagglutinin titre and enzymic activity of all the virus strains tested were completely unaffected by treatment with dichromate, iodacetamide or acrylonitrile. This shows that the SH group is not involved in the interaction of virus with red cells, and that the active centres of the haemagglutinins and neuraminidases do not contain cysteine.

The amino group also is apparently not involved. The haemagglutinin titre of all the virus preparations was unaffected by treatment with acrylonitrile, phenyl isothiocyanate, β-propiolactone, glyoxal, or short periods of treatment with

Table 1. *Action of reagents reacting with aromatic amino acids on the haemagglutinin of influenza virus strains*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees of reduction of haemagglutinin titre; 0 = no action. Trypt. = tryptophan; tyr. = tyrosine; hist. = histidine; int. = intact virus; E.T. = ether-treated virus.)

	Iodine 1/10,000, pH 6·0						Fluorodinitrobenzene, 1/800, 30 min. at 20° C + 18 hr. at 4° C.			Diazotized sulphanilic acid, 1/5600 pH 9·0, 18 hr. at 0° C.
	0° C.				37° C. 1 hr.					
Probable reacting groups ...	2 min. SH, CH_3S, trypt.		1 hr. SH, CH_3S, trypt., tyr.		SH, CH_3S, trypt., tyr., hist.		pH 8·0 SH, NH_2, NH (hist.)		pH 9·0 SH, NH_2, NH (hist.), OH (tyr.)	Hist., tyr., trypt.
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int. + E.T.
A SWINE	0	0	0	0	1	2	0	0	4	4
A PR 8	0	0	0	0	1	4	0	0	4	4
A DSP	0	0	0	0	2	4	0	0	4	4
A_1 BURCH	0	0	0	0	3	4	2	2	3	4
A_1 BRATISLAVA	0	0	0	0	2	4	2	3	4	4
A_2 TAIWAN	0	0	0	0	1	4	3	2	4	4
A_2 ENG/67	0	0	0	0	2	2	2	1	3	4
B LEE	0	0	0	0	0	2	2	2	4	4

formaldehyde. In studies of neuraminidase activity phenyl isothiocyanate could not be used because of its lytic effect on red cells, but the neuraminidase activity of all the virus strains was unaffected by acrylonitrile, glyoxal or by short periods of formaldehyde treatment. In some experiments a very slight reduction in neuraminidase activity was found on treatment with β-propiolactone, but these effects were probably due to the slight reaction of the reagent with histidine.

Action of reagents acting on the aromatic amino acids

Significant effects were produced by agents reacting with the imino group of histidine, the phenolic hydroxyl group of tyrosine, or with the aromatic rings. Effects of these reagents on the haemagglutinins of the various virus strains are shown in Table 1. None of the haemagglutinins were affected by iodine at 0° C., showing that the —SH group of cysteine, the —CH_3S group of methionine, and tryptophan play no part in haemagglutination. All the haemagglutinins were completely destroyed by diazotized sulphanilic acid, completely or nearly completely destroyed by FDNB at pH 9·0, and were reduced in titre by iodine at 37° C., ether-treated preparations being more sensitive than intact virus. All these reagents

react with both tyrosine and histidine. The results of treatment with FDNB at pH 8·0 show that the virus strains fall into two groups. The haemagglutinin titres of the SWINE, PR 8, and DSP strains of virus A were unaffected by FDNB at pH 8·0, while significant reductions in titre occurred with the A_1 and A_2 strains and with the LEE strain of virus B. The haemagglutinin titres of the A_1 and A_2 strains and the LEE virus were unaffected by 1 hr. exposure to iodine at 0° C., so that it appears that the haemagglutinating centres of these viruses contain histidine but not tyrosine. The haemagglutinin titres of the A strains were unaffected by 1 hr. exposure to iodine at 0° C., which reacts with tyrosine, and were also unaffected by FDNB at pH 8·0 which reacts with histidine, and with these viruses haemagglutinating activity can only be destroyed by agents acting on both tyrosine and histidine.

Table 2. *Action of reagents reacting with aromatic amino acids on the neuraminidase activity of influenza viruses*

(4 = complete destruction of neuraminidase; 3, 2, 1 = intermediate degrees of reduction in activity; 0 = no action. Trypt. = tryptophan; tyr. = tyrosine; hist. = histidine; Int. = intact virus; E.T. = ether-treated virus.)

	Iodine 1/10,000, ph 6·0						Fluorodinitrobenzene, 1/800, 30 min. at 20° C. + 18 hr. at 4° C.			Diazotized sulphanilic acid, 1/5600, pH 9·0, 18 hr. at 0° C.
	0° C.				37° C					
Probable reacting groups ...	2 min. SH, CH_3S, trypt.		1 hr. SH, CH_3S, trypt., tyr.		1 hr. SH, CH_3S, trypt., tyr.		pH 8·0 SH, NH_2, NH (hist.)		pH 9·0 SH, NH_2, NH (hist.), OH (tyr.)	Hist., tyr., trypt.
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int. + E.T.
A SWINE	0	0	1	0	4	4	2	2	4	4
A PR 8	0	0	3	1	4	4	2	3	4	4
A DSP	0	0	0	0	4	4	2	2	4	4
A_1 BURCH	0	0	0	0	4	4	4	4	4	4
A_1 BRATISLAVA	0	0	0	0	4	4	4	4	4	4
A_2 TAIWAN	0	0	0	0	4	4	4	3	4	4
A_2 ENG/67	0	0	0	0	4	3	2	1	4	4
B LEE	0	0	1	1	4	4	2	2	4	4

The results suggest the existence of two types of haemagglutinating centre, one containing histidine and one tyrosine. The SWINE, PR 8 and DSP strains appear to contain both types of centre, but only the histidine-containing centre can be detected in the A_1, A_2 and LEE strains.

The action of these reagents on the neuraminidase activity is shown in Table 2. No reduction in activity of any of the strains was produced by 2 min. exposure to iodine at 0° C., indicating that cysteine, methionine and tryptophan are not present in the active centres. All the neuraminidases were completely destroyed by agents reacting with both tyrosine and histidine. But the results obtained with 1 hr. exposure to iodine at 0° C. and with FDNB at pH 8·0 show that two types of neuraminidase can be detected. The neuraminidase activity of the DSP, A_1 and A_2 strains is reduced or destroyed by FDNB at pH 8·0 but is unaffected by iodine at 0° C., so that the active centres of these strains appear to contain histidine but not tyrosine. With the SWINE, PR 8 and LEE strains neuraminidase activity is reduced both by FDNB at pH 8·0 and by iodine at 0° C. but is only completely

destroyed by reagents acting on both tyrosine and histidine. These strains appear to contain two types of neuraminidase: one containing histidine and one tyrosine.

Action of reagents acting on amide and guanidyl groups

Results obtained with these reagents are shown in Tables 3 and 4. The haemagglutinating and neuraminidase activities of all the strains were unaffected by glyoxal or by 1 hr. exposure to 1/1000 formaldehyde, showing that amino groups

Table 3. *Action of reagents reacting with amino, amide and guanidyl groups on the haemagglutinin of influenza virus strains*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees of destruction of haemagglutinin; 0 = no action. Int. = intact virus; E.T. = ether-treated virus.)

Probable reacting groups ...	Formaldehyde, pH 7, 37° C.						Glyoxal, 1:100, pH 8·5, 4 hr., 37° C.	Xanthydrol, 1:1000, pH 5·5, 4 hr., 37° C.	
	1:1000, 1 hr. Amino		1:1000, 24 hr. Amino, amide guanidyl		1:100, 24 hr. Amino, amide, guanidyl		Amino, guanidyl	Amide guanidyl	
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int.	E.T.
A SWINE	0	0	0	1	4	4	0	1	3
A PR 8	0	0	0	0	4	4	0	0	1
A DSP	0	0	0	0	0	4	0	2	3
A_1 BURCH	0	0	0	0	4	4	0	1	3
A_1 BRATISLAVA	0	0	0	2	4	4	0	0	3
A_2 TAIWAN	0	0	0	0	4	4	0	0	3
A_2 ENG/67	0	0	0	1	4	4	0	1	2
B LEE	0	0	0	1	4	4	0	0	2

Table 4. *Action of reagents reacting with amino, amide and guanidyl groups on the neuraminidase activity of influenza virus strains*

(4 = complete destruction of neuraminidase; 3, 2, 1 = intermediate degrees of neuraminidase destruction 0 = no action. Int. = intact virus; E.T. = ether-treated virus.)

Probable reacting groups ...	Formaldehyde, pH 7, 37° C.						Glyoxal, 1:100, pH 8·5, 4 hr., 37° C.	Xanthydrol, 1:1000, pH 5·5, 4 hr., 37° C.	
	1:1000, 1 hr. Amino		1:1000, 24 hr. Amino, amide, guanidyl		1:100, 24 hr. Amino, amide, guanidyl		Amino, guanidyl	Amide, guanidyl	
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int.	E.T.
A SWINE	0	0	2	2	4	4	0	2	3
A PR 8	0	0	3	1	4	4	0	1	3
A DSP	0	0	3	2	3	4	0	3	3
A_1 BURCH	0	0	4	2	4	4	0	1	4
A_1 BRATISLAVA	0	0	4	2	4	4	0	1	4
A_2 TAIWAN	0	0	1	4	4	4	0	2	4
A_2 ENG/67	0	0	0	2	4	4	0	1	2
B LEE	0	0	1	2	4	4	0	2	2

are not involved in the activities. All the strains showed a reduction in both haemagglutinating power and neuraminidase activity as a result of treatment with xanthydrol. Prolonged exposure to 1/1000 formaldehyde reduced the enzymic activity of all the strains but had little effect on haemagglutinin titres, but with 1/100 formaldehyde both haemagglutinins and neuraminidases were destroyed,

except with intact DSP virus which showed some resistance even to 1/100 formaldehyde. These results suggest that an amide group is present in the active centres of both haemagglutinin and neuraminidase in all the virus strains.

It is, however, possible that the effects produced by these reagents are non-specific. The methylene bridges produced by prolonged treatment with formaldehyde may produce steric effects and distort the structure of the protein molecule with a resulting interference with the virus activities even if amide groups are not present in the active centres. Xanthydrol is specific for the amide group but is effective only at reactions close to the iso-electric point of the viruses and also produces a precipitate, so that loss of activity might be due to aggregation of virus or adsorption on the precipitate. However, the most probable explanation of the results is that an amide group is present in all the active centres of haemagglutinin and neuraminidase in all the virus strains. If this is so then the amino acid involved is probably not arginine, as the activities are unaffected by glyoxal, but may be either asparagine or glutamine.

DISCUSSION

The results described above indicate a very considerable similarity in the chemical constitution of the active centres of haemagglutinin and neuraminidase in all the strains of virus tested. The active centres do not appear to contain cysteine, methionine, tryptophan, lysine or arginine, while all the active centres are partially or completely inactivated by action on amide groups and are completely inactivated by agents acting on both tyrosine and histidine. The only differences observed were in the results of treatment with iodine for 1 hr. at 0° C. which reacts with tyrosine but not with histidine, and with FDNB at pH 8·0 which probably reacts mainly with histidine and only weakly with tyrosine.

It would, of course, be expected that the chemical structure of haemagglutinating or enzymically active centres acting on the same substrate molecule would be very similar, and the possibility has to be considered that the centres may in fact all be chemically identical and that the differences observed in experiments with iodine and FDNB were due to non-specific factors such as variations in morphology of different virus strains or differences in the degree of purification attained.

However, the great differences in the rate of elution of the strains from red cells suggests that the enzymes are not identical, as also does the well-known differences in susceptibility to different haemagglutinin inhibitors. Also, in experiments in which strains were tested simultaneously against the same reagent consistent differences in sensitivity were seen; thus in numerous experiments with FDNB the A_1 and A_2 haemagglutinins were always more sensitive than the haemagglutinin of the A strains.

If, therefore, we accept the observed results at their face value we can detect a number of different types of centre. The results with the A_1 and A_2 strains can be explained by supposing that these strains contain a single active centre containing histidine and an amide group but not containing tyrosine, the centre possessing both haemagglutinating and enzymic activity.

With the A and B strains the results indicate the presence of two types of active centre, one containing histidine and one tyrosine. With the SWINE and PR 8 strains both centres appear to possess both haemagglutinating and enzymic activity as haemagglutination can only be prevented by action on both histidine and tyrosine, while neuraminidase activity is reduced by action on either tyrosine or histidine but is only completely destroyed by action on both. With the DSP strain, however, the tyrosine-containing centre has no demonstrable neuraminidase activity. It is possible that a similar centre without neuraminidase activity may also be present in the SWINE and PR 8 strains as these strains elute very slowly from red cells, a finding which is difficult to explain if all the haemagglutinating centres present also had neuraminidase activity.

The LEE strain of virus B also has two centres: one containing histidine and one tyrosine. Both centres have neuraminidase activity and the strain elutes rapidly from red cells. The tyrosine-containing centre of the LEE strain does not appear to play any part in haemagglutination. As it is difficult to believe in the existence of enzymes which do not unite chemically with their substrates one would expect that all neuraminidases present in the intact virus would be potential haemagglutinins, and it may be that the tyrosine-containing centre of the LEE strain also contains histidine so that with this strain haemagglutination can be prevented by action on histidine alone. The neuraminidase activity of all the strains is reduced by FDNB at pH 8·0 and it is possible that histidine is an essential constituent of all neuraminidases, but that tyrosine can substitute for histidine in haemagglutination, so that there exist haemagglutinating centres without neuraminidase activity.

Finally, it must be recognized that the chemical procedures used represent a very crude method of investigating the active centres and that only active centres which are dominant in the virus particle can be demonstrated; minor components would probably be undetectable.

SUMMARY

Studies of the chemical reactions of the haemagglutinins and neuraminidases of eight strains of influenza viruses have been made by the use of chemical reagents reacting with chemically active groups in the protein molecule. The results indicate a close resemblance between the active centres of the haemagglutinins and neuraminidases in all the strains tested. In all cases the activities were unaffected by reagents reacting with the —SH group of cysteine, the —CH_3S group of methionine, the amino group of lysine, the guanidyl group of arginine, or the indole ring of tryptophan. In all cases both the haemagglutinating and enzymic activities were reduced or destroyed by agents reacting with amide groups or reacting with both tyrosine and histidine.

By the use of iodine under conditions in which tyrosine reacts but not histidine, and fluorodinitrobenzene under conditions in which histidine reacts more strongly than tyrosine, it was possible to detect a number of different active centres.

(1) An active centre containing histidine and an amide group but not containing tyrosine was present in all the virus strains and was the only centre detectable in

A_1 and A_2 strains. This type of centre appeared to possess both haemagglutinating and neuraminidase activity.

(2) Active centres containing tyrosine and an amide group were detected in strains of A and B viruses. There was some evidence suggesting that tyrosine-containing centres were of two types: one possessing both haemagglutinating and enzymic activity while the other was a haemagglutinin without neuraminidase activity.

The results could be explained by supposing that the presence of histidine in the active centre was essential for neuraminidase activity and that enzymically active tyrosine-containing centres also contained histidine, but that tyrosine could substitute for histidine in haemagglutinating centres.

REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384–92.

FRAENKEL-CONRAT, H. & OLCOTT, H. S. (1948). The reaction of formaldehyde with proteins. Cross linking between amino and primary amide or guanidyl groups. *J. Am. chem. Soc.* **70**, 2673.

HOYLE, L. (1969). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. II. Effects of reagents modifying the higher order structure of the protein molecule. *J. Hyg., Camb.* **67**, 301.

HOYLE, L. & HANA, L. (1966). The chemical reactions of influenza virus proteins. *J. Path. Bact.* **92**, 447–60.

MCKINNEY, L. L., UHING, E. H., SETZKORN, E. A. & COWAN, J. C. (1950). Cyanoethylation of α-amino acids. I. Monocyanoethyl derivatives. *J. Am. chem. Soc.* **72**, 2599–603.

NAKAYA, K., HORINISHI, H. & SHIBATA, K. (1967). States of amino acid residues in protein. XIV. Glyoxal as a reagent for discrimination of arginine residues. *J. Biochem., Tokyo* **61**, 345–51.

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The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses

II. Effects of reagents modifying the higher order structure of the protein molecule

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In previous work (Hoyle & Hana, 1966) it was found that the enzymic activity of the DSP and LEE strains of influenza virus was reduced by agents acting on disulphide bonds. The results, however, tended to be irregular, except in the case of mercuric chloride, these irregularities being attributed to reversibility of the reaction with many of the chemical reagents used. More recent work using dithiothreitol (Cleland, 1964) which reacts completely and irreversibly with the disulphide bond have shown that the disulphide bonds in the virus proteins are inaccessible to attack except under conditions permitting some disruption of hydrogen bonds. A study has therefore been made of the effects on the virus properties produced by reagents acting on non-covalent bonds alone and in combination with dithiothreitol.

METHODS

In this work purified 'virus concentrate' preparations have been used, prepared as described in the previous paper (Hoyle, 1969). Two mixtures were first made, one consisting of equal volumes of virus concentrate and buffer pH 8·0, and the second of virus concentrate and 1/100 dithiothreitol in buffer pH 8·0. The two mixtures in 0·05 ml. amounts were then mixed with 0·15 ml. of buffer pH 8·0 containing amounts of urea or guanidine sufficient to give final concentrations of 0, 4 M and 6 M urea or 2 M, 4 M and 6 M guanidine. Mixtures were held at 20° C. for 30 min. and were then diluted to 2 ml. with buffer pH 6·0. Haemagglutinin titres were then measured and neuraminidase activity assessed by the elution test as described in the previous paper (Hoyle, 1969). Each virus was therefore exposed to concentrations of urea of 0, 4 M and 6 M, and of 2 M, 4 M and 6 M guanidine, alone and in combination with 1/800 dithiothreitol.

In addition the action of mercuric chloride was tested by exposing virus dilution preparations to 1/10,000 $HgCl_2$ at pH 6·0 for 2 hr. at 37° C.

RESULTS

In contrast to the results obtained with reagents reacting directly with amino acids in the active centres of the virus haemagglutinin and neuraminidase, very

striking differences were observed between individual strains of virus and also between haemagglutinin and neuraminidase as a result of treatment with reagents acting on the higher-order structure of the protein molecule.

Exposure to high concentrations of urea disrupts hydrogen bonds. With concentrations of less than 4 M there was no visible disruption of the virus particle and no effects were produced on the virus properties, but with 4 M urea the virus preparations became less opalescent indicating some disruption of the particles and the opalescence completely cleared with 6 M urea. Guanidine is a more powerful reagent disrupting non-covalent bonds and significant effects were produced by concentrations as low as 2 M. The opalescence of concentrated virus preparations was completely cleared by 4 M guanidine.

Table 1. *Effect of reagents disrupting the higher-order structure of the virus protein on the virus haemagglutinin*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees of haemagglutinin destruction; 0 = no action.)

	A			A_1		A_2		B
Reagent	SWINE	PR 8	DSP	BURCH	BRATISLAVA	TAIWAN	ENG/67	LEE
4 M urea	0	0	0	0	0	0	0	0
6 M urea	2	4	0	0	0	0	2	0
4 M urea + 1/800 DTT	1	0	1	0	1	0	0	0
6 M urea + 1/800 DTT	4	4	4	4	4	0	1	4
2 M guanidine	4	4	0	0	0	4	4	0
4 M guanidine	4	4	0	0	0	4	4	0
6 M guanidine	4	4	0	0	0	4	4	0
2 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
4 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
6 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
Dithiothreitol (DTT) 1/800	0	0	0	0	0	0	0	0
Mercuric chloride 1/10,000	0	0	0	0	0	0	0	0

The results of treatment of eight strains of virus with these reagents is shown in Tables 1 and 2. The haemagglutinin titre and neuraminidase activity of all eight strains was completely unaffected by dithiothreitol alone, suggesting that the disulphide bonds are inaccessible to attack by this reagent. Haemagglutinin titres were not significantly reduced by mercuric choloride but with 6 of the 8 strains the neuraminidase activity was completely destroyed. The A_2 strains of virus were remarkable in the possession of a neuraminidase that was completely resistant to mercuric chloride.

With three strains of virus—the DSP strain of virus A and the two A_1 strains—there was a very considerable difference in sensitivity between haemagglutinin and neuraminidase. The neuraminidase activity of these strains was completely destroyed by mercuric chloride and by all the concentrations of urea and guanidine used. The haemagglutinins of these three strains were completely resistant to mercuric chloride and to urea and guanidine, but were destroyed by combinations

of guanidine and dithiothreitol and also by 6 M urea + dithiothreitol. It appears that with these strains neuraminidase activity is destroyed by any interference with the higher-order structure of the virus protein, but that the haemagglutinin is unaffected by reagents acting on non-covalent bonds and is also unaffected by mercuric chloride which introduces a mercury atom into the disulphide bridges without disrupting the bond. Haemagglutinin is only destroyed as a result of the complete disruption of the disulphide bonds by dithiothreitol in combination with urea or guanidine.

Table 2. *Effect of reagents disrupting the higher-order structure of the virus protein on neuraminidase activity*

(4 = complete destruction of neuraminidase activity; 3, 2, 1 = intermediate degrees of neuraminidase destruction; 0 = no action.)

	A			A_1		A_2		B
Reagent	SWINE	PR 8	DSP	BURCH	BRATISLAVA	TAIWAN	ENG/67	LEE
4 M urea	4	4	4	4	3	0	0	0
6 M urea	4	4	4	4	4	2	2	0
4 M urea + 1/800 DTT	4	4	4	4	4	1	0	2
6 M urea + 1/800 DTT	4	4	4	4	4	4	4	4
2 M guanidine	4	4	4	3	3	4	4	2*
4 M guanidine	4	4	4	4	4	4	4	2*
6 M guanidine	4	4	4	4	4	4	4	4
2 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
4 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
6 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
Dithiothreitol (DTT) 1/800	0	0	0	0	0	0	0	0
Mercuric chloride 1/10,000	4	4	4	4	4	0	0	4

* Two types of sub-unit present; one sensitive to guanidine and one resistant

The neuraminidases of the SWINE and PR 8 strains of virus A were also completely destroyed by all reagents acting on the higher-order structure of the virus protein. The haemagglutinin of these strains was highly sensitive to guanidine, but showed some resistance to urea, being unaffected by 4 M urea but reduced or destroyed by 6 M urea. Combinations of urea and dithiothreitol were only slightly more destructive than urea alone and mercuric chloride had no effect.

The remarkable sensitivity of the neuraminidase of the A and A_1 strains to urea and guanidine suggested the possibility that urea and guanidine did not actually destroy neuraminidase but split it off the virus particle in a form which was not adsorbed by red cells and therefore was not detectable in the elution test. Fortunately urea and guanidine do not significantly interfere with the estimation of neuraminic acid by the Aminoff (1961) technique so that it was possible to test for the presence of a non-haemagglutinating neuraminidase by the use of the Aminoff method.

Preparations of DSP virus and the A_1 BRATISLAVA virus in volumes of 0·05 ml. were exposed to 4 M urea and to 4 M guanidine for 30 min. at 20° C. They were then diluted to 1·0 ml. with buffer pH 6·0. Control preparations of untreated virus and

virus treated with diluted urea or guanidine were also set up. To all preparations 0·4 ml. of packed guinea-pig red cells was added as a substrate for neuraminidase action and the mixtures incubated at 37° C. for 30 min. The cells were then centrifuged out and the concentration of *N*-acetylneuraminic acid in the supernatant fluids measured. Results are shown in Table 3. With the DSP virus neuraminidase activity was unaffected by treatment with diluted urea but was greatly reduced by 4 M urea. The activity was completely destroyed by 4 M guanidine and was even slightly reduced by diluted guanidine. The neuraminidase activity of the A_1 BRATISLAVA strain was destroyed by 4 M urea or guanidine and was reduced by diluted guanidine and very slightly affected by diluted urea. These results show that urea and guanidine actually destroy neuraminidase and do not merely split it off the virus particle.

Table 3. *Action of urea and guanidine on the neuraminidase activity of A DSP and A_1 BRATISLAVA viruses*

(Effects tested by determining the amount of *N*-acetylneuraminic acid released from 0·4 ml. of guinea-pig red cells in 30 min. at 37° C. The figures given are absorptiometer readings at 549 μ.)

	Virus	
	A DSP	A_1 BRATISLAVA
Untreated virus control	0·205	0·240
Virus + diluted urea	0·200	0·200
Virus + 4 M urea diluted after 30 min. at 20° C.	0·035	0·000
Virus + diluted guanidine	0·160	0·135
Virus + 4 M guanidine diluted after 30 min. at 20° C.	0·000	0·005

With the A_2 strains there was much less difference in sensitivity between haemagglutinin and neuraminidase. Both activities were resistant to mercuric chloride and sensitive to guanidine. The neuraminidase of the A_2 TAIWAN strain was partially sensitive to urea while the haemagglutinin was resistant, and the neuraminidase of the A_2 ENG/67 strain was slightly more sensitive to urea + dithiothreitol than the haemagglutinin.

With the LEE strain of virus B also the reactions of haemagglutinin and neuraminidase were very similar. The haemagglutinin was resistant to urea and guanidine and to mercuric chloride but was highly sensitive to dithiothreitol in combination with urea or guanidine. The neuraminidase was also resistant to urea and highly sensitive to dithiothreitol in combination with urea or guanidine and was also sensitive to mercuric chloride. Treatment with guanidine alone led to an unusual type of result. With the LEE strain 2 M guanidine produced a considerable increase in haemagglutinin titre indicating disruption of the virus particle. When preparations treated with 2 M and 4 M guanidine were tested for neuraminidase activity in the elution test it was found that some of the adsorbed haemagglutinin eluted very rapidly, but the rest remained attached to the cells and at the end of the elution period, when the control preparation was completely eluted, the cells in

the guanidine-treated preparations still showed a fine agglutination and this agglutination was increased by addition of fresh virus. It appeared that there were two types of enzymic sub-unit present in the virus particle, one sensitive to guanidine and one resistant to it, and it seemed possible that these were reacting with different substrate molecules in the red cell since the guanidine-resistant enzyme was not able to release the guanidine-sensitive haemagglutinating particles from combination with red cells. This type of result has only been seen with the LEE virus.

DISCUSSION

The results described above suggest that the neuraminidase activity of A and A_1 strains of influenza virus is a function of a higher-order of structure of the protein molecule than is the haemagglutinating activity. There is much evidence that the protein of the outer component of the influenza virus particle can exist in a number of different structural states. Jagger & Pollard (1956), from studies of the resistance of virus properties to irradiation by deuterons, α-particles and electrons, calculated the haemagglutinin in the intact virus to have a molecular weight of 190,000. Mayron, Robert, Winzler & Rafaelson (1961) first showed that neuraminidase could be separated from the virus particle by trypsin treatment and Noll, Aoyagi & Orlando (1962) showed that the neuraminidase separable from LEE virus had a sedimentation constant of 9S corresponding to a molecular weight of 190,000. Laver (1963) separated a neuraminidase of sedimentation constant 9S from LEE virus by treatment with dodecyl sulphate, but similar treatment of A viruses destroyed the neuraminidase. This result agrees with the finding in the present paper that the neuraminidase of A viruses is destroyed by agents reacting on non-covalent bonds while the LEE virus possesses a neuraminidase resistant to these agents. Reginster (1966*a*, *b*) separated neuraminidase from PR 8 virus by treatment with caseinase C or pronase and found it to have a sedimentation constant of 6S, but in later work (Reginster, 1968) a value of 8S was found. Seto, Drzeniek & Rott, (1966) separated a neuraminidase of 8·8S from A_2 virus by pronase treatment, and Drzeniek, Seto & Rott (1966) obtained neuraminidases of S value 9–10 from A_2 and fowl-plague viruses. Reginster (1968) showed that treatment of PR 8 virus with caseinase C resulted in loss of the surface projections and the residual particles no longer agglutinated red cells and were not adsorbed by them.

The released neuraminidases do not agglutinate red cells and are either not adsorbed or adsorbed by them only with great difficulty. As it is difficult to believe in enzymes which do not unite with their substrate the probable explanation of the failure of the isolated neuraminidases to agglutinate red cells is that they carry only one active centre and are therefore unable to bridge red cells. Such a monovalent neuraminidase will be very difficult to adsorb on red cells. An intact virus particle carrying some 400–500 projections may be adsorbed by a single red cell receptor, but 400–500 receptors will be needed to adsorb the released separate projections.

Although the released neuraminidases do not agglutinate red cells they may act

as a blocking antigen in haemagglutination-inhibition tests (Reginster 1968) and antisera to them may inhibit haemagglutination (Rafaelson, Wilson, & Schneir, 1962; Reginster, 1968) though not apparently in all cases (Drzeniek *et al.* 1966).

It is very probable that the molecular weight of 190,000 represents the molecular weight of one of the surface projections on the virus particle and that the released neuraminidases of sedimentation constant 8–10S are single projections. The virus protein can, however, be disintegrated into smaller units.

Disruption of viruses by dodecyl sulphate results in the release of protein sub-units of sedimentation constant 3·4S which have no enzymic activity and do not agglutinate red cells (Laver, 1963). By treatment of WS virus with deoxycholate and ether Hobson (1966) produced a sub-unit which did not agglutinate red cells but which was active as a serum-blocking agent in haemagglutination-inhibition tests and also induced haemagglutinin-inhibiting antibody in guinea-pigs. Eckert (1966*a*, *b*, 1967) extracted the lipid from PR 8 virus and then treated the protein either with 8 M urea + dithiothreitol or with 67 % acetic acid. In each case the protein was disrupted to sub-units with a sedimentation constant of 2S which reassociated to a 4S unit. The 4S units produced by urea-DTT treatment had no enzymic activity and did not agglutinate red cells, but reacted as a blocking antigen in complement fixation tests. Treatment with dithiothreitol produces irreversible disruption of disulphide bonds and prevents restoration of the tertiary structure of the protein molecule. The sub-units produced by acetic acid treatment reassociated to give a product which agglutinated red cells in the cold and could be adsorbed by red cells if a sufficiently large amount of cells was used. It had no neuraminidase activity.

Relation between haemagglutinin and neuraminidase

Many workers have suggested that haemagglutinin and neuraminidase are separate protein molecules, and Laver & Kilbourne (1966) produced a recombinant strain X 7 from the NWS strain of virus A and the A_2 (R 1) 5 strain which apparently contained the haemagglutinin of the A parent and the neuraminidase of the A_2 strain.

There is, however, reason to believe that while haemagglutinin and neuraminidase are certainly not precisely identical they are more closely related than would be expected if they were entirely distinct proteins. Both are associated with the surface projections, both unite with the same substrate molecule and both appear to depend for their activity on the presence of active centres containing the same amino acids. All chemical reagents which destroy haemagglutinin also destroy neuraminidase. Neuraminidase activity is, however, more sensitive to destruction by heat and chemical reagents than haemagglutinin

The following hypothesis is advanced to explain the relation between haemagglutinin, neuraminidase, and specific antigen. The surface projections on the virus particle are protein polymers of sedimentation constant 8–10S and a molecular weight of about 190,000. Each polymer is made up of three or four monomers of sedimentation constant 4S and a molecular weight of 50,000–60,000. Each of the monomers carries an active centre capable of uniting with red cell receptors, but

neuraminidase activity is only developed when the active centres of the monomers become closely associated in the polymer. Neuraminidase activity is therefore a function of the quaternary structure of the projections while the haemagglutinating activity is a function of the tertiary structure of the monomers. Antigenic activity resides in the primary or secondary structure of the monomers which represent the various components of the V antigen complex. On this basis the results of treatment of virus strains with agents acting on the higher-order structure is interpreted as follows.

(1) With the A and A_1 strains the quaternary structure of the polymer is maintained by hydrogen bonds between the monomers, and neuraminidase activity is destroyed by 4 M urea and by guanidine. The haemagglutinin of the two A_1 strains and the DSP strain of virus A is resistant to both urea and guanidine and is only destroyed by combinations of these agents with dithiothreitol. The tertiary structure of the monomers in these strains is therefore maintained primarily by disulphide bonds.

The haemagglutinin of the SWINE and PR 8 strains is destroyed by urea + dithiothreitol but is also very sensitive to guanidine, so that in these strains both disulphide bonds and guanidine-sensitive urea-resistant bonds are needed to maintain the tertiary structure. This guanidine-sensitive bond may possibly be an ionic bond between amino acids with basic and acidic side-chains.

Neuraminidase activity is not only sensitive to disruption of the quaternary structure but is also highly sensitive to modifications of the tertiary structure of the monomers. Thus even the introduction of a mercury atom into the disulphide bonds produces enough distortion of the active centre to destroy neuraminidase activity.

(2) In the A_2 and LEE viruses the quaternary structure of the polymers is maintained by stronger bonds than with the A and A_1 strains. Neuraminidase activity is not destroyed by 4 M urea and Laver (1963) found that while treatment of A viruses with dodecyl sulphate produced sub-units of sedimentation constant 3·4S, with the LEE virus it only produced 9S sub-units.

The haemagglutinin of the A_2 strains is resistant to urea + dithiothreitol but is highly sensitive to guanidine, suggesting that the tertiary structure is maintained by guanidine-sensitive bonds. The neuraminidase of A_2 strains is guanidine sensitive and is resistant to 4 M urea + dithiothreitol, but is destroyed by 6 M urea + dithiothreitol and is slightly sensitive to 6 M urea alone. The quaternary structure may be maintained by a combination of hydrogen and disulphide bonds. The A_2 strains, however, are unique in the possession of a neuraminidase which is resistant to mercuric chloride. This would suggest that disulphide bonds are not involved in the tertiary structure and that introduction of a mercury atom into the disulphide bond does not disrupt the quaternary structure.

The haemagglutinin of the LEE virus is completely resistant to urea and guanidine but destroyed by combination of these agents with dithiothreitol, indicating that the tertiary structure is maintained by disulphide bonds. Neuraminidase activity is also sensitive to urea + dithiothreitol and to mercuric chloride but is resistant to urea. It is destroyed by high concentrations of guanidine and is

partially sensitive to low concentrations. The quaternary structure may be maintained by guanidine-sensitive bonds, but there is some evidence that there are two types of neuraminidase, one sensitive and one partially resistant to guanidine.

Table 4. *Classification of influenza viruses by the chemical reactions of their haemagglutinins*

(+ = haemagglutinin sensitive to reagent; − = heamagglutinin not sensitive to reagent.)

Group	Strain	Fluoro-dinitro-benzene, pH 8·0	Iodine, pH 6·0, 0° C.	Urea	Guanidine	Urea + dithio-threitol	Mercuric chloride
1	A SWINE	−	−	−	+	+	−
	A PR 8	−	−	−	+	+	−
2	A DSP	−	−	−	−	+	−
3	A_1 BURCH	+	−	−	−	+	−
	A_1 BRATISLAVA	+	−	−	−	+	−
4	A_2 TAIWAN/64	+	−	−	+	−	−
	A_2 ENG/67	+	−	−	+	−	−
	B LEE	+	−	−	−	+	−

Table 5. *Classification of influenza viruses by the chemical reactions of their neuraminidases*

(+ = neuraminidase sensitive to reagent; − = neuraminidase not sensitive to reagent.)

Group	Strain	Fluoro-dinitro-benzene, pH 8·0	Iodine pH 6·0, 0° C.	Urea	Guanidine	Urea + dithio-threitol	Mercuric chloride
1	A SWINE	+	+	+	+	+	+
	A PR 8	+	+	+	+	+	+
2	A DSP	+	−	+	+	+	+
	A_1 BURCH	+	−	+	+	+	+
	A_1 BRATISLAVA	+	−	+	+	+	+
3	A_2 TAIWAN/64	+	−	−	+	+	−
	A_2 ENG/67	+	−	−	+	+	−
	B LEE	+	+	−	±	+	+

Chemical classification of influenza virus strains

The virus strains used in the present work can be classified into groups by means of the chemical reactions shown by their haemagglutinins and neuraminidases (Tables 4, 5). The A virus haemagglutinins fall into four groups; SWINE + PR 8, A (DSP), A_1, and A_2, while the neuraminidases fall into three groups: SWINE + PR 8, A (DSP) + A_1, and A_2. Paniker (1968), studying the serological relationships of the haemagglutinins and neuraminidases of strains of influenza viruses, also found that the haemagglutinins fall into four groups: SWINE, A_0, A_1, and A_2, while the neuraminidases fall into three groups; SWINE, $A_0 + A_1$, and A_2. The results of chemical and serological classifications are therefore very similar, the only difference being that the PR 8 strain was chemically indistinguishable from SWINE.

SUMMARY

The results of treatment of influenza virus strains with chemical reagents acting on the higher-order structure of protein molecules shows that both the haemagglutinating and enzymic activities are susceptible to these agents but there are considerable differences between the different strains and the neuraminidase activity is more sensitive than the haemagglutinating activity.

The neuraminidase activity of A and A_1 strains is destroyed by urea, guanidine, urea + dithiothreitol and mercuric chloride. The haemagglutinin of the PR 8 and SWINE strains is resistant to urea and mercuric chloride but destroyed by guanidine and by urea + dithiothreitol. The haemagglutinin of the DSP strain of virus A and the A_1 strains is resistant to urea, guanidine and mercuric chloride but is destroyed by urea + dithiothreitol.

The neuraminidase activity of the A_2 strains is more resistant than that of the A and A_1 strains. It is resistant to mercuric chloride and partially resistant to urea but is destroyed by guanidine and by urea + dithiothreitol. The A_2 haemagglutinin is resistant to urea, urea + dithiothreitol, and mercuric chloride but is destroyed by guanidine.

The LEE virus neuraminidase is resistant to urea and partially resistant to guanidine but is destroyed by urea + dithiothreitol and mercuric chloride. The LEE haemagglutinin is resistant to urea, guanidine and mercuric chloride but is destroyed by urea + dithiothreitol.

It is suggested that the surface projections of the virus particle are protein polymers each made up of three or four monomers which are the components of the V antigen complex. Antigenic activity is a function of the primary or secondary structure of the monomers, haemagglutinin activity is a function of the tertiary structure of the monomers, while neuraminidase activity is a function of the quaternary structure of the polymer.

From studies of the chemical reactions of their haemagglutinins and neuraminidases strains of influenza virus A can be classified into groups. These groups are very similar to but not precisely identical with groupings made by serological methods.

REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384–92.

CLELAND, W. W. (1964). Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* **3**, 480–2.

DRZENIEK, R., SETO, J. T. & ROTT, R. (1966). Characterisation of neuraminidases from myxoviruses. *Biochim. biophys. Acta* **128**, 547–58.

ECKERT, E. A. (1966*a*). Envelope proteins derived from influenza virus. *J. Bact.* **91**, 1907–10.

ECKERT, E. A. (1966*b*). Characterization of a low molecular weight antigenic protein from the envelope of influenza virus. *J. Bact.* **92**, 1430–4.

ECKERT, E. A. (1967). Envelope protein of influenza virus. I. Haemagglutinating activity of reassociated sub-units. *J. Virol.* **1**, 920–7.

HOBSON, D. (1966). The strain-specific serological activity of a non-haemagglutinating fraction of influenza viruses. *Br. J. exp. Path.* **47**, 257–65.

HOYLE, L. (1969). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. I. *J. Hyg., Camb.* **67**, 289.

HOYLE, L. & HANA, L. (1966). The chemical reaction of influenza virus proteins. *J. Path. Bact.* **92**, 447–60.

JAGGER, J. & POLLARD, E. C. (1956). Inactivation with fast charged particles of infectivity and haemagglutination in influenza A virus. *Radiat. Res.* **4**, 1–19.

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

LAVER, W. G. & KILBOURNE, E. D. (1966). Identification in a recombinant influenza virus of structural proteins derived from both parents. *Virology* **30**, 493–501.

MAYRON, L. W., ROBERT, B., WINZLER, R. J. & RAFAELSON, M. E. (1961). Studies on the neuraminidases of influenza virus. 1. Separation and some properties of the enzyme from Asian and PR8 strains. *Archs Biochem. Biophys.* **92**, 475–83.

NOLL, H., AOYAGI, T. & ORLANDO, J. (1962). The structural relationship of sialidase to the influenza virus surface. *Virology* **18**, 154–7.

PANIKER, C. K. J. (1968). Serological relationships between the neuraminidases of influenza viruses. *J. gen. Virol.* **2**, 385–94.

RAFAELSON, M. E., WILSON, V. W. & SCHNEIR, M. (1962). The neuraminidases of influenza virus. *Med. Bull. Presb. St Luke's Hosp.* **1**, 34–9.

REGINSTER, M. (1966*a*). Release of influenza virus neuraminidase by caseinase C of *Streptomyces albus* G. *J. gen. Microbiol.* **42**, 323–31.

REGINSTER, M. (1966*b*). Effects of pronase on influenza virus. *Acta virol. Prague* **10**, 111–16.

REGINSTER, M. (1968). Recherches sur la structure du virus grippal. *Mém. Soc. r. Sci. Liège* **16**, fasc. 4.

SETO, J. T., DRZENIEK, R. & ROTT, R. (1966). Isolation of a low molecular weight sialidase (neuraminidase) from influenza virus. *Biochim. biophys. Acta* **113**, 402–4.

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Some rodenticidal properties of coumatetralyl

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INTRODUCTION

Coumatetralyl (3-(α-tetralyl)-4-hydroxycoumarin), an anti-coagulant rodenticide developed by Farbenfabriken Bayer A.G., has been used extensively against rats in Germany (Schultze, 1965). Comparatively little information is available, however, concerning its rodenticidal properties. The present laboratory study was therefore undertaken to investigate the toxicity of the anti-coagulant to warfarin-resistant and warfarin-susceptible rats (*Rattus norvegicus* Berk.) and to compare its palatability with that of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin).

METHODS

Wild rats, presumed not to be resistant to warfarin (NR rats) were caught in a Midlands refuse destructor and on a Surrey farm. Warfarin-resistant (WR) rats were trapped on farms in three different areas of Britain: Nottinghamshire (WR Notts), Gloucestershire (WR Glos) and the Salop–Montgomeryshire area (WR Welsh). Field investigations had shown that the rats on the Nottinghamshire and Gloucestershire farms could not be controlled with warfarin. All ten WR Glos rats used in tests with coumatetralyl had first been given, and had survived, unrestricted feeding for 6 days on 0·005 % warfarin in the laboratory and similarly all but six of the 23 WR Notts rats had first been given periods of from 3 to 6 days on warfarin. The 53 WR Welsh animals had survived either 6 days feeding on 0·005 % warfarin or a single subcutaneous injection of 200 mg./kg. of warfarin, or had shown a minimal increase in prothrombin time 24 hr. after subcutaneous injection of 1 mg./kg. of warfarin (Greaves & Ayres, 1967).

Animals allegedly resistant to coumatetralyl were obtained from two sources. The first (CR Welsh), a farm where a coumatetralyl treatment had failed, was in the Salop–Montgomeryshire area where warfarin-resistance is prevalent. Three of the rats from this source were first given 0·005 % warfarin in medium oatmeal for 6 days, their survival indicating them to be resistant to warfarin. The second source (CR Notts) was a Nottinghamshire farm where it had been found impossible to eradicate the rats with warfarin or subsequently with coumatetralyl. Allegedly coumatetralyl-resistant animals from both sources were thus most probably resistant also to warfarin.

Finally, 15 laboratory-bred WR rats were tested. These animals were also shown by preliminary laboratory tests to be resistant to warfarin. All animals to which warfarin was administered in preliminary tests were permitted a recovery period

of at least 3 weeks, except for five WR Welsh rats which were allowed only 12–19 days before being given coumatetralyl.

Rats were caged singly for all experiments. In toxicity tests approximately equal numbers of males and females were given unrestricted access to medium oatmeal containing coumatetralyl, without alternative food, for specified numbers of days or until death. Consumption of the toxic food was normally measured daily and mortality was recorded for at least 14 days after the start of each test. In palatability tests wild NR rats were given a choice for 2 days between plain medium oatmeal and the same food containing poison; after the first day fresh food containers were provided and the positions in the cage of the two foods were interchanged.

Pure compounds were used in the majority of experiments, but in others either of two proprietary pre-mixes containing 60 % or 0·75 % coumatetralyl and a balance of inert ingredients was employed. There was no indication that toxicity was affected by the method of formulation.

RESULTS

The results of toxicity tests are given in Table 1. The median lethal feeding periods (LFP_{50}) for groups 1–4 were calculated by the probit analysis method given by Finney (1952), employing a logarithmic transformation of feeding period. Analyses of relative potency for the same groups were carried out, taking an individual potency ratio to be statistically significant if its 95 % fiducial limits did not span the value of unity. Not unexpectedly, coumatetralyl was significantly less toxic at 0·005 % than at 0·05 % to NR rats. At 0·05 % it was significantly less toxic to WR Welsh than to NR rats and again at 0·05 %, the compound was significantly less toxic to CR Welsh than to WR Welsh rats.

A further analysis of relative potency involving groups 1–4 of Table 1 was performed in which coumatetralyl was compared with warfarin, an anticoagulant which, on account of its widespread use as a rodenticide, provides a useful reference standard. For this purpose data from Drummond & Wilson (1968) were used. These authors, who used methods essentially the same as ours, offered NR rats bait containing 0·005 % warfarin for 1, 2 and 3 days and obtained corresponding mortalities of 3/12, 19/29 and 30/30, leading to an LFP_{50} estimate of 1·2 days. Our results (groups 1–3) were not found to differ significantly from theirs. Coumatetralyl at 0·05 % was found, however, to be significantly less toxic to CR Welsh (group 4) rats than was 0·005 % warfarin to NR rats.

Though the results for groups 5–12 are not suitable for statistical analysis the feeding behaviour of the four WR rats (in groups 7, 8 and 12) that survived 6 days' feeding on 0·05 % coumatetralyl and also of the longest-surviving CR animal in each of groups 10 and 11 calls for comment. The daily consumption of the toxic food by these six rats fell almost to zero after 3–5 days and then rose again in three animals; doubtless the remaining rats would have shown a similar increase had the experiments continued. This oscillation in food consumption, which is attributable to a period of illness induced by the anti-coagulant alternating with a period of more or less complete recovery, occurred four times before the longest-surviving CR Welsh rat finally succumbed.

Table 1. *Mortality in rats after unrestricted feeding on medium oatmeal containing coumatetralyl*

Group	Type of rat	Conc. of coumatetralyl (%)	Feeding period (days)	Mean body weight (g.)	Mortality	Range of lethal doses (mg./kg.)	Highest dose survived (mg./kg.)	Range of days to death	Median lethal feeding period (days)
1	NR	0·005	1	250	3/10	3–5	5	4–7	
			2	262	6/10	6–8	8	4–7	1·6
			3	304	8/10	7–15	13	4–9	
2	NR	0·05	1	239	7/10	22–35	42	4–14	
			2	239	10/10	44–77	—	4–9	0·7
			3	190	9/10	25–118	101	3–6	
3	WR Wales	0·05	1	222	4/15	31–51	54	3–8	
			2	224	9/15	53–98	118	2–8	1·6
			3	167	13/16	58–160	160	4–7	
4	CR Wales	0·05	3	313	1/6	136	207	5	
			4	286	2/6	87–93	229	4	5·1
			6	190	4/6	112–187	260	5–7	
5	WR Wales	0·05	6	238	7/7	67–165	—	5–9	—
6	WR Notts	0·05	3	279	8/10	53–96	115	5–7	—
7	WR Notts*	0·05	6	281	14/15	49–176	128	5–8	—
8	WR Glos	0·05	6	245	9/10	72–205	200	4–9	—
9	CR Notts	0·05	3	231	0/2	—	115	—	—
10	CR Notts*	0·05	until death	252	2/2	120–123	—	6–13	—
11	CR Wales*	0·05	until death	188	17/17	54–401	—	4–26	—
12	WR Lab. bred	0·05	6	342	13/15	58–183	79	5–9	—

*Includes survivors of shorter feeding periods.

Table 2. *Palatability of coumatetralyl and warfarin to rats*

Poison and Concentration		Amounts eaten (g.) Plain food	Poisoned food	No. of rats preferring plain food and total no. in test	Significance of difference
Coumatetralyl	0·05 %	155·3	160·6	7/10	n.s.
Coumatetralyl	0·1 %	195·2	155·2	9/12	n.s.
Warfarin	0·005 %	170·2	221·7	4/12	n.s.
Warfarin	0·025 %	208·8	202·9	6/11	n.s.
Warfarin	0·05 %	301·8	104·3	9/10	0·01

The results of palatability tests of coumatetralyl, together with some comparative data for warfarin, are presented in Table 2. The significance of each mean difference between the amounts of the two foods eaten was assessed by means of Student's *t*. There was no evidence that pure coumatetralyl was unpalatable at concentrations of up to 0·1 %. Pure warfarin, in contrast, was markedly unpalatable at half this concentration.

DISCUSSION

It has been shown that coumatetralyl at 0·005 % or 0·05 % is about as toxic to NR rats as is warfarin at 0·005 %. Further, at concentrations of 0·05 %–0·1 % coumatetralyl is clearly more acceptable than warfarin to rats. Coumatetralyl is therefore to be regarded, concentration for concentration, as a good alternative to warfarin for use against *R. norvegicus* and will probably give better results than warfarin at concentrations of the order of 0·05 %.

Over a 6-day feeding period coumatetralyl at 0·05 % was lethal to all but two of the WR rats obtained from Nottinghamshire and Gloucestershire. The survival of one animal from each of these sources was evidently a result of the toxic effects of the poison preventing the ingestion of a lethal dose. In field conditions where many rats may often feed only partly on poison bait, animals of this type might well therefore survive treatments with coumatetralyl indefinitely—as perhaps happened with the CR rats, all of which, though eventually succumbing to coumatetralyl in the laboratory (groups 10 and 11, Table 1), had survived expertly conducted treatments with coumatetralyl in the field.

The finding that 0·05 % coumatetralyl is about as toxic to WR Welsh rats as 0·005 % warfarin is to NR rats suggests that coumatetralyl would be effective against WR Welsh rats in the field. The high toxicity of coumatetralyl to WR Welsh rats is also of considerable interest in the light of the report (Drummond & Wilson, 1968) that these animals can survive after feeding for 6 days or more on oatmeal containing concentrations of up to 1·0 % warfarin. An insight into this difference in toxicity between the two very similar compounds will probably depend upon elucidation of the mechanism of resistance to warfarin.

In assessing the possible value of coumatetralyl for destroying warfarin-resistant rats, the evidence that these animals were also somewhat resistant to coumatetralyl must be considered. Where resistance to warfarin occurs only sporadically, as it has in Nottinghamshire and Gloucestershire, such cross-resistance to coumatetralyl, if present, may be of relatively minor importance since by special efforts the few affected infestations can normally be eradicated by other means. In the Salop–Montgomeryshire area, however, where resistance to warfarin, apparently due to a major gene, occurs in rat populations extending over hundreds of square miles, cross-resistance is of greater potential significance. The discovery in the area of an infestation which could not be controlled by treatment with coumatetralyl taken together with the finding that coumatetralyl was, on average, less toxic to WR Welsh than to NR rats suggests that there may be reserves of variability in the warfarin-resistant population sufficient to allow an increase in resistance to evolve in response to the general use of coumatetralyl. Furthermore, to the extent that warfarin-resistant rats are more likely to survive treatments with coumatetralyl than are non-resistant animals, the use of coumatetralyl would be likely to increase the incidence of resistance to warfarin. Thus while coumatetralyl would be expected to give good results against warfarin-resistant rats initially, this use might eventually result in an increase in the incidence of resistance to both anti-coagulants.

SUMMARY

The toxicity and palatability of coumatetralyl (3-(α-tetralyl)-4-hydroxycoumarin) to rats (*Rattus norvegicus* Berk.) were investigated in the laboratory by means of feeding tests. Animals resistant to warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) and warfarin-resistant rats from infestations refractory to coumatetralyl, as well as non-resistant animals, were employed in the tests.

Medium oatmeal containing a concentration of 0·1 % coumatetralyl was not markedly less palatable than the same food unpoisoned. In comparison warfarin at 0·05 % but not at 0·025 % was significantly less readily eaten than the plain food. Coumatetralyl at 0·05 % and 0·005 % was about as toxic as 0·005 % warfarin is reported to be to non-resistant rats. Warfarin-resistant rats were significantly less susceptible to coumatetralyl than were non-resistant rats. Warfarin-resistant rats from an infestation refractory to coumatetralyl were significantly less susceptible to coumatetralyl than were animals from other sources.

It is considered that coumatetralyl at concentrations of the order of 0·05 % in bait would be a good alternative to warfarin against non-resistant rats. While it would be expected that, at this concentration, coumatetralyl would often give good results against warfarin-resistant infestations, this use might eventually produce an increase in the incidence of resistance to both anticoagulants.

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REFERENCES

Drummond, D. C. & Wilson, E. J. (1968). Laboratory investigations of resistance to warfarin of *Rattus norvegicus* Berk. in Montgomeryshire and Shropshire. *Ann. appl. Biol.*, **61**, 303–12.

Greaves, J. H. & Ayres, P. (1967). Heritable resistance to warfarin in rats. *Nature, Lond.* **215**, 877–8.

Schultze, G. (1965) Rat eradication and maintenance of the achieved state of clearance, from the aspect of the pest control operator. *Pflanzenschutz-Nachricht. Bayer* **18**, 173–82.

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Overlap and the errors of plaque counting

I. The overlap biases of observed counts and their correction

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INTRODUCTION

When viruses multiply in layers of host cells, the number of plaques counted will often underestimate the number of plaque-forming units, owing to overlapping. This 'overlap bias' is also present in other counting techniques and, because it may rival the random error in magnitude, is of practical importance.

In 1949 Irwin, Armitage & Davies developed an approximate mathematical model of overlapping which might be used to correct dust-particle or bacterial-colony counts. This model was improved by Armitage (1949) and shown to be effective in practice. Further results are given by Roach (1968).

However, the assumptions of particle or colony counting represented by this model differ from those of plaque counting in one important respect. Whereas in particle counting each clump is counted as one, in plaque counting the observer attempts to resolve and count all members of the large and irregular areas of lysis which are produced by plaque 'clumps'. A mathematical model representing such a counting technique must therefore make allowance for factors influencing resolution. This renders the problem both more complex and less tractable due to the subjective nature of overlap resolution.

The present paper describes an attempt to provide an acceptable solution.

THEORETICAL

(1) *The model*

In the simple model of Irwin *et al.* the concentration of circular particles of equal size, distributed at random on a plate, is measured by the quantity ψ, which is the ratio of the sum of areas of the particles to the area of the plate. Considering only circular plates and in the present notation,

$$\psi = \frac{Nd^2}{D^2}, \tag{1}$$

where N = number of particles, or true count, d = particle (or plaque) diameter, D = plate diameter. The formula relating the true count N and the observed count C is

$$C = \frac{N(1-e^{-4\psi})}{4\psi}. \tag{2}$$

This may be written more conveniently as

$$C = \frac{1}{K}(1 - e^{-KN}), \tag{3}$$

where

$$K = \frac{4d^2}{D^2}$$

and is called the assay constant.

Dulbecco & Vogt (1954) briefly considered the problem of overlapping in plaque counting and visualized this in terms of the critical distance (g) between plaque centres below which overlapping plaque pairs are not resolved. In effect the assay constant in their model is

$$K = \frac{4g^2}{D^2}. \tag{4}$$

A similar approach was adopted by Lidwell in appendix V of Bourdillon, Lidwell & Lovelock (1948). However, the practical value of the above model and that proposed by Cooper (1961), which is essentially the same, is limited, as the various factors which determine the critical distance have never been defined or separated.

One factor is certainly the average size of plaques, while others include plaque morphology, the variation of plaque size, etc., all of which influence the capacity of the observer to resolve overlapping plaques. The combined effect of these other factors may be represented by a single 'resolution factor', P, defined as the ratio, g/d, of the critical overlap distance to plaque diameter d. This leads to

$$K = \frac{4P^2d^2}{D^2}. \tag{5}$$

P will have values ranging from 0 for plaques which are always resolved no matter how close their centres, to 1 for plaques which become unresolvable as soon as they touch. The latter extreme corresponds to the 'clump' counting of particles considered by Irwin *et al.*

The replacement of g by its factors P and d allows evaluation of the effect which changes in plaque morphology will have upon an observer's capacity to resolve overlapping plaques. It also paves the way to practical application, for, once the value of P for a standardized assay system has been determined, the assay constant, K, can be obtained by measuring a number of plaques and performing a simple computation.

In practice equation (3) has been used in the form

$$N = -\frac{1}{K}\log_e(1 - KC). \tag{6}$$

The correction factor, the ratio N/C, is specified by KC. Hence a single table of correction factors for a range of values of KC will suffice for all assay systems. This has been prepared and will be presented elsewhere. (Howes & Fazekas de St Groth, in preparation).

(2) *The estimate of the resolution factor P*

The value of P may be estimated by measuring pairs of overlapping plaques, of approximately the same size, which have been barely resolved in the course of routine plaque counting. By definition, the smallest observed value for the ratio of the distance between their centres to their diameter should approximate the value of P.

An alternative method is to calculate the value of P from observed decreases in plaque counts which accompany a known increase in plaque size, i.e. are due to overlapping. As is shown in the appendix the relationship is

$$P^2 = \frac{D^2(C_1 - C_2)}{2(C_2^2 d_2^2 - C_1^2 d_1^2)}, \tag{7a}$$

where the subscripts 1 and 2 designate observed counts and mean plaque diameters for the first and second counting times respectively.

Estimation of the value of P^2 by means of equation (7*a*) requires the processing of considerable volumes of counting data, and although the mean counts observed on groups of replicate cultures may be used, this would often require that special experiments be carried out. This disadvantage is overcome if an estimate of P^2 is obtained from each culture, and all estimates are then weighted and pooled to give a combined estimate. Data provided by cultures drawn from routine assays may then be used.

The error of the initial count C_1 vanishes in this method of analysis, and the major component of the error of P^2 becomes the error in estimating the number of plaques missing from a particular plate at the second counting, $(C_1 - C_2)$.

To facilitate calculations, a further simplification of equation (7*a*) can be made,

$$P^2 = \frac{D^2(C_1 - C_2)}{2C_1^2(d_2^2 - d_1^2)}. \tag{7b}$$

Although a small systematic error results, which increases the value of P^2 slightly, this tends to compensate for the neglect of more complex forms of overlapping.

Regarding $C_1 - C_2$ as approximately a Poisson variate would lead to a weight, C_1^2, for each value of $C_1 - C_2/C_1^2$. When equation (7*b*) is used, as in these studies, the following convenient relationship is then obtained:

$$\text{estimator of } P^2 = \frac{D^2}{2(d_2^2 - d_1^2)} \left(\frac{\Sigma(C_1 - C_2)}{\Sigma C_1^2} \right). \tag{8}$$

(3) *The inadequacy of simple mathematical models*

Were a complete model of the effects of plaque overlap used, the estimates of P obtained by the two methods should be the same. However, it was found that the estimate of P derived from counting data in the present system was approximately 0·3, whereas that obtained by the inspection and measurement of barely resolved plaque pairs was 0·22 or less. Thus actually more plaques are obscured by overlap than can be explained by the simple model. Correction of this deficiency would

require either a more complex theoretical model or an empirical modification of the simple model. Of these alternatives the latter appeared to have more practical merit.

(4) *The empirical method*

The empirical adjustment of the simple model is achieved by accepting the higher value of P derived from counting data according to equations (7) or (8). Equation (6) then predicts overlap bias errors with acceptable accuracy over a substantial part of the possible counting range. Such values of P are, of course, not covered by its definition, and P^2 is replaced in the relevant equations by the empirical resolution factor 'R'. The estimator of K is then

$$\hat{K} = \frac{4Rd^2}{D^2}. \tag{9}$$

(5) *Allowance for variation in plaque size*

The plaques present on a culture at a given time always show some variation in size, and this will influence the magnitude of the overlap bias to some extent. Following Armitage (1949), d^2 was therefore replaced in equation (5) by

$$(d^2 + 0{\cdot}5s_d^2),$$

where s_d^2 is the variance of the distribution of plaque diameters. The contribution of the variance is, however, relatively small (usually less than 5 % of d^2). This refinement has therefore only a minor effect and may be safely omitted in less exacting routine assays.

MATERIALS AND METHODS

Cultures

The cynomolgus monkey kidney cultures used in these experiments were prepared as described by Thayer (1965).

Media

Phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) was used to wash cultures, and with the addition of 0·1 % calf serum, which had been heated to 68° C. for 1 hr. to destroy virus inhibitors, was used to prepare virus dilutions. The overlay medium, developed by Mr E. H. Ridge of this laboratory, had the following composition:

Two-fold nutrient solution	ml.
Tenfold concentrated Earle's salt solution	10
2·8 % $NaHCO_3$	5
2 M Tris, pH 7·3	1
2 M-$MgCl_2$	1·25
Calf serum, heated at 68° C. for 1 hr.	4
2 % yeast extract	5
Neutral red, 1/1000 solution in distilled water	2·5
Distilled water to	50

Penicillin and streptomycin were included in the above solution at 100 units and 100 μg./ml. respectively.

Prior to use, the nutrient solution was saturated with CO_2, raised to about 44° C., and mixed with an equal volume of 2% agar at the same temperature.

Virus strains

Two strains of poliovirus were used in four experiments: the attenuated type 1, LSc-2ab strain, and the virulent type 3, Saukett strain.

Inoculation and incubation

Cultures were rinsed twice with PBS, and a 0·5 ml. inoculum was then carefully distributed over each cell monolayer. One hour at room temperature, on a level surface, was allowed for adsorption. After adding 10 ml. of overlay, cultures were incubated at 35° or 37° C. in a humidified atmosphere of 5% CO_2–air mixture. The incubators had been extensively modified to give better control of temperature and gaseous environment than is usually possible with the bacteriological incubators commonly employed for this purpose.

Plaque counting

In each experiment, plaques were counted on three occasions during a period of from 2–3 days. The plaques present at the first counting time ('original' plaques) were marked with a felt-tip pen containing an acetone soluble dye. Immediately prior to recounting, at the second or third counting times, the relatively few new plaques were marked with indian ink, and the markings of the original plaques were selectively removed with acetone. The original plaques were then recounted again using the felt-tip pen.

Measurement of plaque diameters

In the four experiments the diameters of 194, 107, 107, and 94 plaques, respectively, were measured at each counting time. To do this, cultures inoculated with the highest dilutions of virus were used so that the number of plaques was usually less than ten. All plaques whose centres fell within a scribed concentric circle of 6·1 cm. diameter (internal diameter of the culture plate being 7·2 cm.) were measured on the three occasions.

Experimental design

Apart from the inherent random sampling errors, the random component of counting uncertainty, and the overlap bias, plaque counts may vary owing to non-uniformity of cell cultures, variation in the environment of cultures during incubation, and counting bias on the part of the operator. The following steps were taken to avoid or nullify these sources of error.

(1) All experimental cultures were numbered and then randomized with respect to the virus inoculum by means of sets of random numbers.

(2) Particular care was taken to ensure that all cultures received the same volume of inoculum, that this was distributed evenly, and that variation in the adsorption period was kept within narrow limits.

(3) After overlaying, cultures were placed on numbered trays in numerical order

and the trays were then placed in an incubator in a predetermined order, a procedure which randomized cultures with respect to their position in the incubator.

(4) Cultures were identified only by their assigned numbers and were counted without reference to the records of the inocula they had received.

(5) Plaque overlap also reduces the variance of plaque-count distributions, an effect which is considered in the accompanying paper (Howes & Fazekas de St Groth, 1969). Bias, which could arise if the observer knows or might successfully guess the inoculum which a culture has received, will mimic this effect of overlapping. To avoid this, dilution steps were closely spaced to make it difficult for the observer to guess the inoculum which a culture had received. In the first experiment a series of 1·6-fold dilutions was used but, although apparently successful, even better safeguards were introduced in the remaining three experiments. In these, two series of twofold dilutions of the same virus suspension were used as inocula for sets of replicate cultures, the lowest dilution of the second series being a 9/10 dilution of the lowest dilution of the first. Thus pairs of extensively overlapping distributions were provided at four more widely separated levels of virus concentration. As the observer can do no more than guess from which distribution a culture is derived, any unconscious tendency to bias will be as often as not in the wrong direction, and may be expected to increase rather than decrease the variance of either or both distributions.

ANALYSIS OF COUNTING DATA

Correction of counts

Mean observed counts were corrected by use of the table of correction factors mentioned above. The statistical analysis of ungrouped data was done by a computer, the programme being based on equation (6).

Identification of the limit to the counting range and the estimation of the resolution factor

The value of the empirical resolution factor R is estimated by means of equation (8), using only counting data which fall within the range of KC values for which the correction procedures are valid. However, the limit value of KC cannot be defined until the value of R is known, and both must therefore be estimated simultaneously using an iterative procedure.

Arbitrary values for both are adopted at the start of the first cycle of computations and the estimates provided by this cycle become the starting-point for the next cycle of computations. The recomputation of R during each cycle is simply achieved by adding or subtracting a few values of $(C_1 - C_2)$ and C_1^2 to or from their sums in equation (8). The limit value of KC is determined by testing correction procedures as described below.

The testing of the model and of correction procedures

Using mean counts for groups of replicate cultures

A widely used method analyses data obtained at the one counting time, the corrected counts at one inoculum level being compared with those at other levels. The criterion of success is the conversion of a curvilinear relationship between observed counts and relative virus concentration into a linear relationship for corrected counts. This approach has the disadvantage that it involves comparisons of independent samples, and conformity to or departures from linearity must be assessed against a background of substantial random sampling error.

The alternative method, designed to avoid this problem, is therefore more efficient and was used in this study. In this method, plaque counts observed on one group of replicate cultures, at one time, are compared with counts on the same cultures after further periods of incubation during which plaques increase in size.

Although successive observed counts of the plaques on a culture are expected to decrease as plaque size increases, each provides an estimate of the one true count after correction by the procedures under test. If these procedures are adequate, the ratios of the estimates to one another will fluctuate about the value of 1. In this form of analysis comparisons are made within groups, and the random error of the total true count for the group, so important in the first method, becomes irrelevant.

Using ungrouped cultures

The use of mean counts has implications which may be of little practical importance but must be considered in a detailed analysis of plaque counting. The mean counts are themselves subject to fluctuation due to departures of the observed frequencies from their theoretical distribution, and this scatter contributes to the variance of mean count ratios about the expected value of 1. However, by determining the individual ratios for each culture this effect is avoided. This procedure also permits other factors which cause fluctuation in the ratios of corrected counts to be assessed, and will be discussed with experimental results.

EXPERIMENTAL RESULTS

The effectiveness of correction procedures and the setting of counting limits

Data from groups of replicate cultures

Results of the four experiments are summarized in Fig. 1, and show that for the Saukett strain of virus ratios of corrected mean counts fluctuate about the expected value of 1, for all values of $K\bar{C} \leqslant 0{\cdot}2$. This value sets the limit to the acceptable counting range.

Ratios for the LSc-2ab strain of virus showed greater inherent variation, and although they fluctuated about the value of 1 for low values of $K\bar{C}$, the point at which corrections become inadequate could not be readily recognized.

Analysis of data from individual cultures

Using the same data, each of the three observed counts of each culture was first corrected by means of equation (6), and then the three ratios of corrected counts, N_2/N_1, N_3/N_2 and N_3/N_1 were calculated. The regression of these ratios on KC was computed, taking progressively widening ranges of the data. For each range of values of KC the following statistics were determined: the slope and intercept of the regression line, together with their errors; the mean ratio of corrected counts for each increment and its error; and the regression and error variances. All data provided by a culture were included in a given range if the value of K_3C_3 fell below the specified upper limit—all data provided by a culture were excluded if this condition was not met.

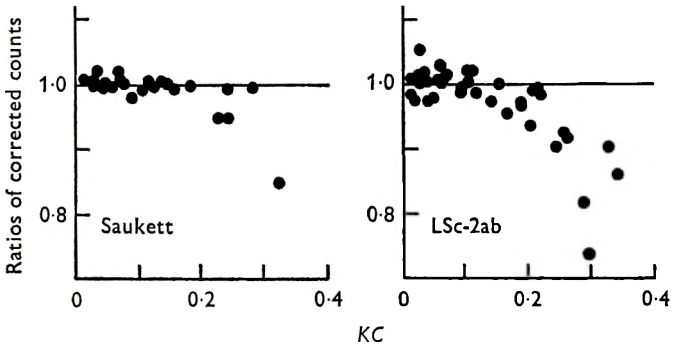

Fig. 1. Ratios of corrected mean plaque counts for the possible counting range. Plaques present at the first counting time on groups of from 13–30 replicate cultures were re-counted at two later times. Mean counts were corrected, and ratios of the second to first, and third to second corrected counts were determined. These fluctuate about the expected value of 1 in lower regions of the possible counting range.

The significance of departures of incremental mean ratios from the expected value of 1 were assessed in a t test. The significance of departures from linearity was assessed by Fisher's F-test.

The computer programme for these operations was written by Dr S. Fazekas de St Groth of the Division of Animal Genetics, C.S.I.R.O.

The results of these analyses are given for the Saukett strain of virus in Table 1, and for the LSc-2ab strain of virus in Table 2, and are discussed under several headings.

Limit to the acceptable counting range

The corrections are adequate as long as (1) the slope of the regression line does not significantly differ from zero, and (2) the regression variance does not show significant trend.

For the LSc-2ab strain of virus (Table 2) significant changes appeared in Expt. 3 as the upper limit to the range of values of KC was increased from 0·18 to 0·20, and in Expt. 2 during the increment from 0·20 to 0·22. However, in the latter experiment a significant change was barely avoided in the increment from 0·18 to 0·20. The limit to the acceptable counting range was therefore set as 0·18 for both experiments.

Table 1. *Statistical analysis of plaque-counting data: Saukett strain of poliovirus*

Range of values of KC	No. of observations in range		Statistics of regressions (ratios, N_2/N_1, etc. $= a+bKC$)						Incremental mean ratios of corrected counts (N_2/N_1, etc.)	
			Slope (b)		Error variance $\times 10^3$		Variance ratio† F $(1, n-2)$			
	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4
0–0·10	147	87	+0·135	+0·388	1·14	2·11	0·82	3·68	1·001	1·011
–0·12	183	99	+0·146	+0·401*	1·40	1·92	1·80	7·47*	1·007	1·038
–0·14	219	117	+0·094	+0·089	1·34	2·03	1·43	0·61	1·002	0·988
–0·16	255	144	+0·056	−0·032	1·46	2·08	0·78	0·15	1·004	0·987
–0·18	276	165	+0·093	+0·047	1·48	1·96	2·83	0·47	1·018	1·018
–0·20	300	186	+0·033	+0·022	1·64	2·03	0·42	0·14	0·991	1·006
–0·22	330	189	−0·004	+0·022	1·70	2·00	0·01	0·15	0·996	0·999
–0·24	363	189	−0·032	.	1·81	.	0·71	.	0·991	.
–0·26	405	195	−0·150*	+0·026	2·11	1·96	19·44*	0·25	0·958	1·008
–0·28	420	216	−0·147*	−0·046	2·06	2·15	21·53*	1·11	0·981	0·986
–0·30	441	240	−0·276*	+0·017	2·51	2·22	75·80*	0·21	0·871*	1·017
–0·32	474	258	−0·346*	+0·039	2·83	2·19	137·1*	1·39	0·904	1·018
–0·34	486	270	−0·360*	+0·037	2·83	2·13	162·9*	1·51	0·909	1·007
–0·36	498	273	−0·376*	+0·045	2·88	2·13	193·0*	2·39	0·893	1·057*
–0·38	507	.	−0·363*	.	2·93	.	190·8*	.	0·954	.

* Significant value ($P < 0·05$).

† Ratio $\frac{\text{regression variance}}{\text{error variance}}$.

Table 2. *Statistical analysis of plaque-counting data: LSc-2ab strain of poliovirus*

Range of values of KC	Number of observations		Statistics of regressions (ratio, N_2/N_1, etc. $= a+bKC$)						Incremental mean ratios of corrected counts	
			Slope (b)		Error variance $+10^3$		Variance ratio† $F\ (1, n-2)$			
	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
0–0·10	177	177	+0·142	−0·159	2·78	1·84	0·71	1·26	1·001	1·001
–0·12	204	138	+0·342*	−0·209	2·57	2·98	7·61*	1·99	1·033	0·978
–0·14	231	165	+0·075	−0·050	2·82	2·81	0·54	0·20	0·979	1·012
–0·16	249	180	+0·082	−0·109	2·81	2·75	0·89	1·27	1·013	0·990
–0·18	267	204	+0·011	−0·080	2·84	2·59	0·02	1·10	0·987	1·002
–0·20	285	210	−0·130	−0·188*	2·92	2·79	3·86	6·19*	0·953	0·914
–0·22	297	231	−0·137*	−0·190*	2·88	2·68	5·36*	9·44*	0·986	0·980
–0·24	306	258	−0·123*	−0·265*	2·90	3·49	5·00*	20·44*	0·998	0·961
–0·26	315	276	−0·154*	−0·231*	3·01	3·65	9·05*	18·73*	0·956	0·995
–0·28	342	288	−0·361*	−0·251*	3·87	4·01	59·38*	23·31*	0·894	0·956
–0·30	357	294	−0·462*	−0·238*	4·49	3·94	103·9*	23·37*	0·853	0·984
–0·32	390	300	−0·613*	−0·222*	5·19	4·14	228·5*	21·12*	0·826	0·957
–0·34	402	.	−0·613*	.	5·16	.	256·6*	.	0·876	.

* Significant value ($P < 0{\cdot}05$).

† Ratio $\dfrac{\text{regression variance}}{\text{error variance}}$.

For the Saukett strain of virus (Table 1) the limit was identified as $KC = 0{\cdot}24$ in Expt. 1, but exceeded a value of $KC = 0{\cdot}36$ in Expt. 4, probably because of the larger initial regression error.

Effectiveness of correction procedures within the acceptable counting range

The over-all effectiveness of corrections applied to plaque counts falling within the acceptable counting range is shown by Table 3, within which the essential elements of the experimental analysis are summarized. The slopes of regression lines and the mean ratios of corrected counts conform closely to the ideal values of 0 and 1 respectively.

Table 3. *Essential features of acceptable counting range*

	Poliovirus strain			
	Type 3, Saukett		Type 1, LSc-2ab	
Expt. no.	1	4	2	3
No. of observations	363	273	267	204
Limit value of KC	0·24	$\geqslant 0{\cdot}36$	0·18	0·18
Slope of regression line	−0·032	+0·045	+0·011	−0·080
Mean ratio of corrected counts	1·001	1·008	1·002	0·999
Error variance ($\times 10^3$)	1·81	2·13	2·84	2·59

Table 4. *Estimation of the value of the resolution factor R from counting data*

	Virus strain			
	Saukett		LSc-2ab	
Expt. no.	1	4	2	3
Range of values of KC	0–0·24	0–0·24	0–0·18	0–0·18
No. of obscured plaques considered	211	145	106	102
R				
For experiments	0·1074	0·1054	0·0835	0·0770
For virus strain	0·1066		0·0803	
$\sqrt{R}$	0·326		0·283	

The resolution factor, R

The above analysis shows that correction procedures provide an effective means of estimating true plaque counts. The same results also prove that variation in R is of no practical importance within the acceptable counting range.

It is also of interest that the value of R for a particular virus strain does not change substantially from experiment to experiment. Thus routine correction of observed counts may be based on predetermined values of R. This is shown by

the close agreement between two estimates of R obtained with each of the two virus strains in different experiments (Table 4). That the value of R must be determined for each virus strain, as might be expected, is shown by the significant differences between the strains considered here ($0{\cdot}02 < P < 0{\cdot}05$).

Counting uncertainty

Where corrections are applied to successive counts of the plaques on individual cultures, fluctuation of ratios of corrected counts about the value of 1 will be due to two main factors: (1) the random placement of plaques on the culture surface, which determines the potential number of plaques which may be obscured by overlapping, and (2) counting uncertainty on the part of the observer.

The first of these, together with the two minor sources of variation (the representation of the true count as a continuous instead of a discrete variate, and the residual bias attributable to the imperfect mathematical representation of plaque overlapping) was common to all the experiments described here. In addition the plaques were counted by the same observer under identical conditions. Systematic differences between viruses in the error variances for data within the acceptable counting ranges may therefore be attributed to differences in counting uncertainty.

The differences found (Table 3) were significant, ($P < 0{\cdot}05$), and indicate that the counting of plaques produced by the LSc-2ab strain of virus was associated with greater uncertainty than the counting of plaques produced by the Saukett strain.

Overlap biases of observed counts which exceed the limit to the acceptable counting range

It is well known that observed plaque counts must eventually become heavily biased by overlapping as plaque number is increased. It is therefore general practice to exclude cultures containing numbers of plaques judged to be too many, even though in fact it might be possible to count them.

Such attempts to limit the overlap bias are inefficient where the bias can neither be predicted nor accurately determined. Clearly, what is regarded as an acceptable number of plaques per culture will vary from one worker to another, and for each worker, from assay to assay and from virus to virus. Since the limit values of KC observed here are probably often exceeded, the relationship between plaque count and overlap bias beyond these limits is of practical importance. This relationship was therefore examined by comparing heavily biased counts observed at the second or third counting times with the estimated true counts for the same cultures, which were calculated by correcting the much smaller biases of the counts observed at the first counting time.

The results (Fig. 2) show (1) that the acceptable counting range is the lower third to half of the possible counting range (which terminates as cultures approach semiconfluency); (2) that the overlap bias increases more rapidly than predicted by the empirically modified model once the limit to the acceptable counting range is exceeded; and (3) that attempts to increase precision by increasing the number of plaques counted on each culture may be defeated by the resulting increase in the overlap bias, which may become as large as 40 %.

The effects of plaque morphology on counting

Morphological differences between the plaques produced by the two virus strains were slight but noticeable, the plaques produced by the LSc-2ab strain being less regular and less sharply defined. These differences might be expected to influence counting but, before attempting to determine the nature of this influence, the factors which the observer considers in attempting to resolve overlapping plaques must be identified.

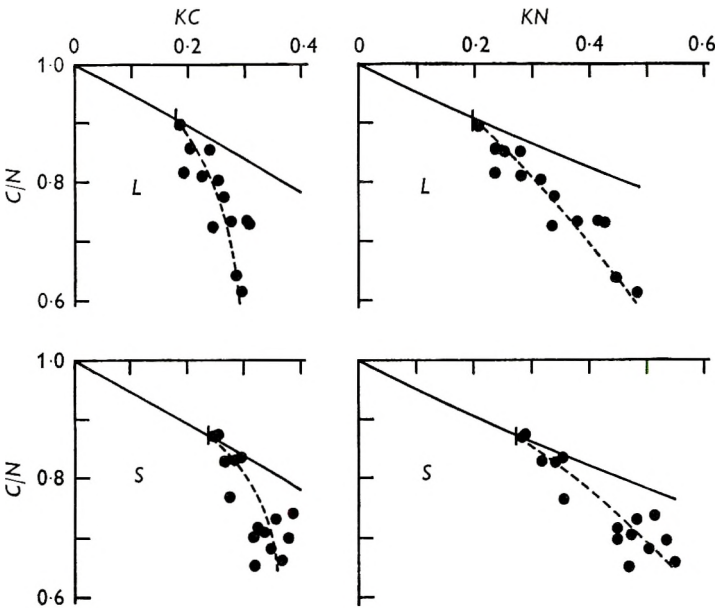

Fig. 2. Overlap biases beyond the limit to the acceptable counting range. True counts (N) for individual cultures were estimated by correcting the counts observed at the first counting time, and the overlap biases of counts (C) observed after plaques had increased in size are shown by the ratio C/N. Data falling within successive 0·01 increments in the range of values of KC were grouped, the mean value of KC was determined, and the corresponding value of KN calculated. Mean ratios were plotted against these values of KN, and the curves (interrupted lines) relating observed values of C/N and KN for the two virus strains LSc-2ab (L) and Saukett (S), were then converted into curves relating C/N and KC. These steps were applied only where values of KC for the later counts exceeded the counting limits, which are shown by the vertical bars.

Calculated overlap biases are shown by uninterrupted lines, and are exceeded once the limit to the acceptable counting range is passed.

Obviously the primary approach is through the analysis of contours and where plaques approach the ideal of sharply defined perfect circles of equal size this is all that is necessary. However, where plaques exhibit irregularities in contours, poor definition of margins and variation in size, resolution by means of contour analysis alone is insufficient. The observer must then attempt to establish a limit to the degree of irregularity which he will accept as representing something more than morphological variation, and must do this for various combinations of plaque size, bearing in mind that different combinations have different probabilities of

occurrence. For example, where it is difficult to decide whether an area of lysis represents one large irregular plaque, or four small ones of about the same size, the more probable interpretation will usually be accepted, provided the observer is familiar with the concept of probability. Here it is pertinent to note that, where this familiarity does not exist, it has been found that the less probable interpretation is often accepted, and marked 'overcounting', which is accompanied by increased errors, occurs.

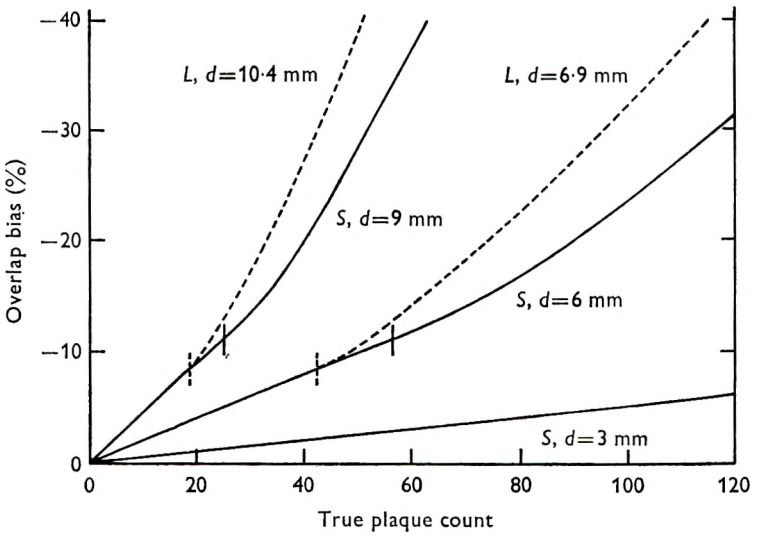

Fig. 3. The relationship between true plaque count and overlap bias for two strains of poliovirus. The single curve relating KN and the overlap bias (C/N) for each virus strain shown in Fig. 2 generates an infinite family of curves when the scale of values of KN is replaced by a scale of values of true counts N, there being one family of curves for each culture area. Three such curves for circular cultures of 60 mm. diameter, and for plaques of mean diameter (d) 3, 6 and 9 mm are shown for the Saukett strain by the solid lines (S), the limits to the acceptable counting ranges being shown by the solid vertical bars. Similar curves (interrupted lines, L) for the LSc-2ab strain, for mean plaque diameters chosen such that the bias of a given true count falling within the acceptable counting range is the same for the two strains, show that counting of LSc-2ab plaques deteriorates earlier (limits to acceptable counting range identified by vertical interrupted lines), so that once this limit is exceeded the overlap bias for a given true count is substantially greater for the LSc-2ab strain than for the Saukett strain.

In the present study the greater uncertainty of counting LSc-2ab plaques is attributed to the less satisfactory morphology of these plaques, and it is very probable that failure of correction procedures at a lower value of KC for this strain, which is shown more clearly in Fig. 3, is attributable to the same cause.

Even though theoretical considerations suggest that uncertain morphology should be accompanied by a deterioration in the ability of the observer to resolve overlapping plaques, the reverse effect was observed, within the acceptable counting range. The value of R was lower for LSc-2ab than for Saukett, which suggests that here increased uncertainty led to a bias towards overcounting.

Of these two effects, counting uncertainty is of greater practical importance, for

the experimentally determined value of R makes allowance for observer bias. Counting uncertainty can be countered either through improvement of plaque morphology or by eliminating consistently poor observers.

DISCUSSION

Simple models such as that advanced here, and the related models advanced by Dulbecco & Vogt (1954) and Cooper (1961), are inadequate since overlapping is treated in terms of the overlapping plaque pair, for which only the distance between centres is critical and orientation need not be considered. This simplification is acceptable for low plaque densities, but more complex forms of overlapping, in which plaques are obscured by the combined effects of two or more neighbours, assume increasing importance as the frequency of overlaps increases. Here the critical distance between centres is greater than that for plaque pairs, and varies both with the number and relative sizes of plaques and with their orientation relative to each other. It is inevitable therefore that such simple models will substantially underestimate the overlap biases of plaque counting.

A more complex model making allowance for this variation in the critical distance might be developed. However, such a model could be valid only if the vagaries of resolution were expressed in precise mathematical terms. At present this does not appear to be possible.

The remaining alternatives are either a wholly empirical approach in which the relationship between observed and true counts is fitted by equations of more or less appropriate form; or an empirical modification of a simple mathematical model to compensate for its theoretical and practical imperfections.

The first approach was adopted by Larsen & Reinicke (1965), who showed that it is feasible to select constants by trial and error. However, the method is too unwieldy to be of use in the routine correction of the overlap biases of observed counts.

The second approach was the one adopted in the present study. The deficiencies of the simple model were overcome by estimating the resolution factor, R, from observed changes in overlap bias which occur on increasing plaque size, instead of using the direct and theoretically correct method of measuring overlapping pairs of plaques at the limit of the resolving power of the observer. In effect, an empirical factor is thus defined which makes allowance for the neglect of the more complex forms of overlapping. Although this allowance cannot be exact, the expected residual biases due to overcorrection in the lower portion of the acceptable counting range, and undercorrection in the upper portion, were shown to be of no practical importance for a substantial part of the possible counting range.

This approach has several advantages over the first: (1) the limit to the number of plaques which may be counted can be determined objectively, and may be simply adjusted for each assay to make allowance for the variations which occur in the mean plaque diameter at the time of counting; (2) corrections for the biases of observed counts are simple to apply; (3) allowance can be made for the partial concealment of the errors of counts (Howes & Fazekas de St Groth, 1969); and

(4) it becomes possible to investigate the effects of plaque morphology and subjective factors on overlap bias and on other errors of plaque counting.

The practice of correction procedures has two stages. First, counting data must be analysed to estimate the value of the resolution factor and the limit to the acceptable counting range. These estimates are then adopted for all standard assays of a particular virus. The actual corrections are simple and routine computations which require only a table of correction factors or a nomogram, and a slide rule or desk calculator. (Howes & Fazekas de St Groth, in preparation).

Whether correction of the overlap biases should be used will depend on the magnitude of these biases and the level of precision required. They are unnecessary where plaques are very small relative to culture area, regular, and clearly defined; they are likely to be necessary where plaques are relatively large, irregular, or poorly defined. Even where plaques are of the latter type, correction procedures might not be needed if plaques are counted early, when still relatively small. However, small size allied with deficiencies in plaque morphology is likely to lead to greater uncertainty in counting and to a consequent loss of precision, while early counting can also lead to a substantial increase in the apparent heterogeneity of cultures with respect to their sensitivity to virus, with a further loss of precision (cf. Howes & Fazekas de St Groth, 1969).

Alternative solutions to the overlap problem have been used or proposed. The first, the 'additive' counting technique, is difficult to justify despite its frequent use. It is inefficient because the small plaques which appear after the first counting time are particularly readily obscured by the large plaques already present, and the resultant overlap biases are variable and difficult to predict. The second approach which could be adopted is to limit the overlap bias by severely restricting the number of plaques on any one culture. The limits which must be imposed in order to reduce the probability that a culture will contain an undetected plaque to less than 0·1 have been calculated by Lorenz & Zoeth (1966), but because no quantitative allowance could be made for the capacity of the observer to resolve overlapping plaques, these are very approximate values. From an economic point of view such low limits would make a return to quantal assay techniques preferable. This is shown in Table 5 where the limits necessary to avoid overlapping are compared with those required where the present correction procedures are used.

A more liberal approach to the choice of counting limits had been suggested by Cooper (1967), who proposed, as a guide, that 100 plaques per plate will give no significant loss by overlapping if the internal diameter of the plate is at least 25 times the average plaque diameter. The overlap bias at this limit, for plaques of 'good' morphology, e.g. the poliovirus plaques studied here, is 3·3 %, which varies in 'significance' with the number of replicate cultures used. Where this is 3, for example, the coefficient of variation for plaque-forming particles is 5·8 % and the overlap bias must be regarded as making a very substantial contribution to the total error.

Cooper's limit can also be used to estimate the practical value of correction procedures. For $R = 0{\cdot}1$ the value of KC corresponding to this limit is 0·065, but

where corrections are applied this can be raised to between 0·18 and 0·24, an approximately threefold increase in the acceptable counting range. Provided all counts within the counting range are equally represented, which is the expectation where data from many assays are considered, 11–12 % of the statistical information will be contained within the lower third of the counting range; 88–89 % in the upper two-thirds which is rejected when corrections are not applied. Correction procedures can therefore give as much as a ninefold increase in the statistical information provided by an assay, and a corresponding threefold increase in precision as measured by the coefficient of variation. The potential practical value of correction procedures is therefore very substantial.

Table 5. *Limits to observed counts for various combinations of plaque and culture diameters*

(Two limits to observed counts are given in each column: a = limit where corrections for overlap biases are applied; for limit $KC = 0{\cdot}2$ and $R = 0{\cdot}1$ (the overlap bias at $KC = 0{\cdot}2$ is 10·4 %). b = limit necessary to reduce probability that a culture will contain an obscured plaque to 0·1 (Lorenz & Zoeth, 1966).)

D† (mm.)		Mean plaque diameter, d (mm.)					
		1	2	3	4	5	6
30	*a*	450	112	50	28	18	12
	b	10	5	2	.	.	.
40	*a*	800	200	89	50	32	22
	b	13	7	4	2	.	.
50	*a*	1250	312	139	78	50	35
	b	17	9	6	4	2	.
60	*a*	1800	450	200	113	72	50
	b	22	10	7	5	4	.
100	*a*	5000	1250	556	312	200	139
	b	33	17	11	9	7	6

† Culture diameter.

Here it should be noted that, in the few previous studies in which counting data have been examined carefully for the presence of the overlap bias (Cooper, 1961, Berg, Harris & Chang, 1963, and Higgins, 1965, for polioviruses; and Larsen & Reinicke, 1965, for vaccinia virus), it has invariably been found. In the present study it has been effectively measured for the first time.

SUMMARY

The number of plaque-forming units is underestimated if plaques overlap. A simple model was developed to account for this bias, and tested by an extensive experimental analysis. It is shown that models of this type are inadequate, but can be modified to give objective methods for setting limits to the acceptable counting range, and for correcting the overlap bias of observed counts which fall within this range.

Where overlapping of plaques is a significant source of error, these methods will improve the efficiency of plaque assays and will render the statistical analysis of counting data more reliable.

REFERENCES

Armitage, P. (1949). An overlap problem arising in particle counting. *Biometrika* **36**, 257–66.
Berg, G., Harris, E. K., Chang, S. L. & Busch, K. A. (1963). Quantitation of viruses by the plaque technique. *J. Bact.* **85**, 691–700.
Bourdillon, R. B., Lidwell, O. M. & Lovelock, J. E. (1948). Studies in Air Hygiene. *Spec. Rep. Ser. med. Res. Coun.* no. 262.
Cooper, P. D. (1961). The plaque assay of animal viruses. *Adv. Virus Res.* **8**, 319–78.
Cooper, P. D. (1967). The plaque assay of animal viruses. In *Methods in Virology* (ed. Maramorosch and Koprowski), vol. III, p. 243. Academic Press.
Dulbecco, R. & Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167–82.
Higgins, P. D. (1965). A comparative study of a plaque and quantal method for assaying the neutralizing activity of antisera to type 1 poliovirus. *J. Hyg., Camb.* **63**, 311–25.
Howes, D. W. & Fazekas de St Groth, S. (1969). Overlap and the errors of plaque counting. II. The bias of the variance and the concealment of errors. *J. Hyg., Camb.* **67**, 335.
Irwin, J. G., Armitage, P. & Davies, C. N. (1949). Overlapping of dust particles on a sampling plate. *Nature, Lond.* **163**, 809.
Larsen, S. O. & Reinicke, V. (1965). Some statistical problems in relation to the use of the Poisson distribution in virus plaque assays. *Acta. path. microbiol. scand.* **65**, 84–92.
Lorenz, R. J. & Zoeth, B. (1966). An estimation of the overlap bias in plaque assay. *Virology* **28**, 379–85.
Roach, S. A. (1968). *The Theory of Random Clumping*. London: Methuen.
Thayer, J. (1965). The suitability of silvered leaf monkey (*Presbytis cristatus*) kidney cell cultures for testing poliovirus vaccines. *Aust. J. exp. Biol. med. Sci.* **43**, 803–8.

APPENDIX

Estimation of P by means of repeated counting of plaques

If we consider an ideal system for which a counting time may be chosen such that after plaques have once been counted, no new plaques appear, a decrease in the observed plaque count after an interval of time must be attributed to an increase in the number of undetected plaques, because the true count, N, is constant. This increased frequency of undetected plaques may be due either to an increase in plaque size, or to a change in the morphology of plaques which would result in a change in the value of the resolution factor P, or to both. However, in practice the plaque morphology of polioviruses does not change appreciably with time and any change in the frequency of undetected plaques is assumed to be due to a change in plaque size. From equation (6):

$$N = -\frac{1}{K}\log_e(1-KC) = \frac{1}{K}(KC+\tfrac{1}{2}K^2C^2+\tfrac{1}{3}K^3C^3+\ldots) \tag{10}$$

Since $KC < 0{\cdot}3$, in practice, $N = C+\frac{1}{2}KC^2$ with a maximal error of less than $2{\cdot}5\,\%$ usually less than $1\,\%$.

As N is the same at each time of counting

$$C_1+\tfrac{1}{2}K_1C_1^2 = C_2+\tfrac{1}{2}K_2C_2^2, \tag{11}$$

where the subscripts denote the values at the first and second time of counting. Substituting for K in terms of equation (5), we have

$$P^2 = \frac{D^2(C_1-C_2)}{2(C_2^2d_2^2-C_1^2d_1^2)} \tag{12}$$

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Overlap and the errors of plaque counting

II. The bias of the variance and the concealment of errors

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INTRODUCTION

Procedures for correcting overlap bias of plaque counts, described in the preceding paper (Howes, 1969), yield more reliable estimates of the numbers of plaque-forming units actually present in samples. However, to take full advantage of the method, it is also necessary to specify the errors associated with plaque counting.

Plaque numbers on replicate cultures are usually assumed to follow a Poisson distribution, but this assumption can be correct only where no overlaps occur. Deviations from this simplest model become of practical as well as theoretical importance where observed counts are noticeably biased by plaque overlap.

Because substantial overlapping is a feature of many plaque assay systems, a study of its effect upon the apparent errors of plaque counts was undertaken.

THEORETICAL

Derivation of equations

The fraction of plaques expected to become undetectable owing to overlapping is an exponential function of plaque number. High counts will therefore suffer a disproportionately greater reduction than low counts and the observed distribution will be negatively skewed. The effect on the variance is greater than that on the mean, so that the ratio of variance to mean for observed plaque numbers will be substantially less than the Poissonian expectation of 1. This is shown in Fig. 1 for two theoretical distributions.

The approximate relationship between the true and observed plaque numbers is

$$C = (1/K)\,(1 - e^{-KN}), \tag{1}$$

where C is the observed and N the true count, while K is the assay constant (as defined in the companion paper).

The expectation of C (= estimated mean C) is

$$E\{C\} = (1/K)[1 - E(e^{-KN})], \tag{2a}$$

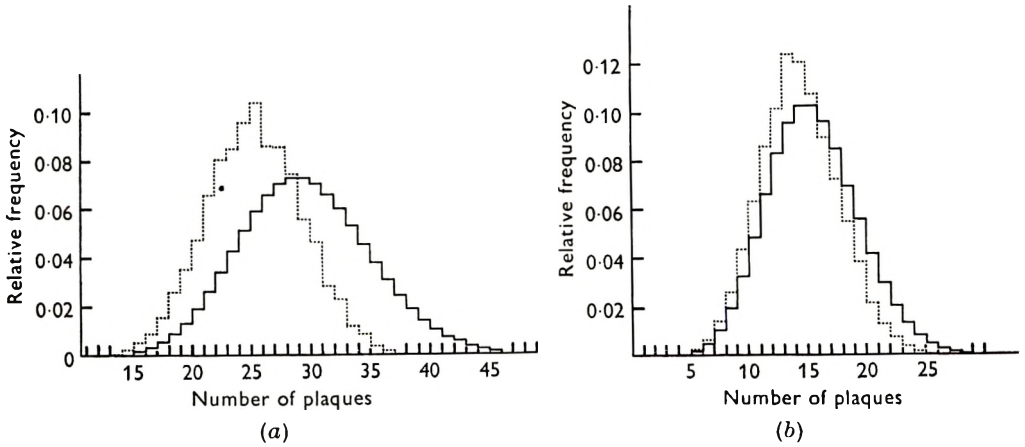

Fig. 1. Displacement and compression of hypothetical ideal plaque-count distributions by plaque overlapping. Assay constant, $K = 0{\cdot}01$. True count distributions shown by solid lines, with mean of 30 (*a*) and 15 (*b*) suffer displacement and compression by overlapping, becoming observed count distributions shown by interrupted lines.

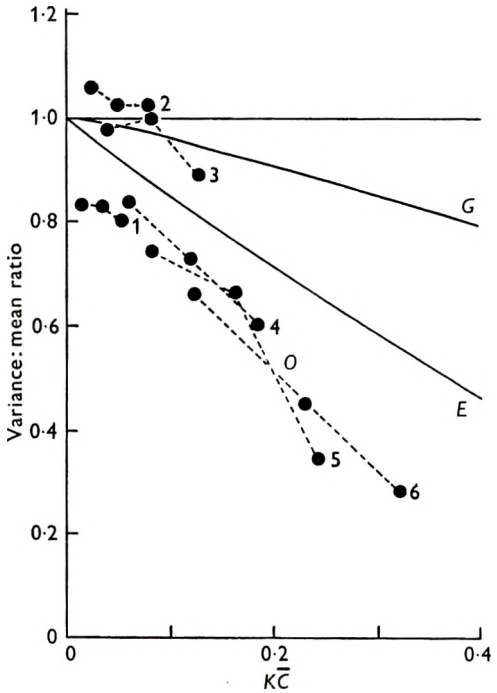

Fig. 2. Predicted and observed changes in the variance:mean ratios of plaque-count distributions produced by plaque overlapping. The predicted overlap biases of mean observed counts, for values of $K\bar{C}$ up to 0·4, are shown by the ratio of observed to true mean counts, line *G*. The variance to mean ratios predicted by equations (2b) and (3b) are shown by line *E*. Experimental values for observed variance to mean ratios are linked by broken lines, *O*. Each group of 30 replicate cultures inoculated with the Saukett strain of virus (Expt. 1) provided one value for each of the three counting times. The position of each group in the range of serial 1·6-fold dilutions is indicated by number, number 1 representing the most dilute inoculum.

which becomes

$$E\{C\} = (1/K)\{1 - \exp[-N(1-e^{-K})]\}. \tag{2b}$$

The variance of observed counts may be calculated as

$$V\{C\} = E\{C^2\} - [E\{C\}]^2,$$

and accordingly

$$V\{C\} = (1/K^2)\{\exp[-N(1-e^{-2K})] - \exp[-2N(1-e^{-K})]\}. \tag{3a}$$

Expanding the exponential terms, (3a) gives

$$V\{C\} = Ne^{-2KN} \tag{3b}$$

Variance $\{C\}/N$ is a single valued function of KN. Hence the variances of any two count distributions will be in the same proportion to their means if the value of $K\bar{C}$ is the same for both, even though the mean number, size, and morphology of plaques are different.

The progressive fall in the observed variance to mean ratio which is expected to occur as $K\bar{C}$ increases is shown in Fig. 2. This may be compared with the fall in the ratio C/N, which shows the expected progressive increase in the overlap biases as KC increases. Observed variances may be greater than those predicted by equation (3*b*) as, for a given true count, the numbers of plaques obscured by overlapping are also randomly distributed. However, this effect will be small relative to the random sampling error of true counts.

The variance of N may be derived from equation (3*b*) as

$$V(N) = e^{2KN}V(C) \tag{4}$$

Equation (4) might be used to prepare a table of correction factors corresponding to values of $K\bar{N}$ or $K\bar{C}$, and thus estimate the variance of true counts.

EXPERIMENTAL RESULTS

An experimental evaluation of the above relationship was carried out using the plaque counting data provided by the experiments described in the preceding paper.

Estimation of variances

The data from all experiments agreed with the expectation that, where some plaques become undetectable due to overlapping, the variance of a distribution of observed counts will be reduced by a larger factor than the mean count.

However, in only one of the four experiments (Expt. 1, Saukett) did the variance to mean ratio for low values of KC closely approach the ideal value of 1. In this experiment, observed decreases in the ratio paralleled those predicted by equation (3*b*) for an ideal system (Fig. 2). In the other experiment with this virus strain (Expt. 4) most of the initial variances of observed plaque count distributions were substantially lower than expected, while for the two experiments with the type 1 LSc-2ab strain, they were considerably higher than expected. In assessing *observed* variance to mean ratios for conformity with equation (3*b*), they were therefore expressed as a fraction of the estimated ratio for *true* counts, which was

calculated using the least biased data obtained at the first counting of each group of replicate cultures. This value was obtained by correcting the mean count by the methods described in the preceding paper, and by correcting the observed variance by means of equation (4). These normalized variance to mean ratios are shown in Fig. 3.

Although considerable scatter is evident, the adjusted ratios agree reasonably well with those predicted by equation (3*b*) for values of $K\bar{C}$ up to about 0·2. Above this value a trend towards lower values than those predicted was evident. This was to be expected since the counts were outside the range set for the validity of the correcting formula.

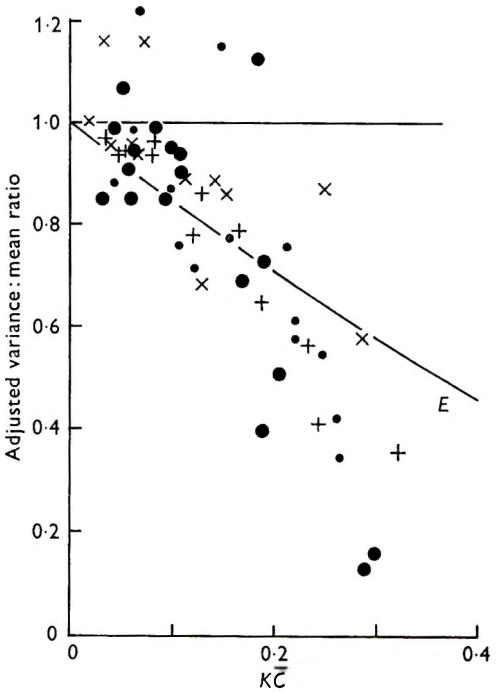

Fig. 3. 'Adjusted' variance: mean ratios for all experiments. Ratios, adjusted as described in the text, are plotted against $K\bar{C}$, and are compared with expected values shown by line *E*. ●, Expt. 2, LSc-2ab; •, Expt. 3, LSc-2ab; +, Expt. 1, Saukett; ×, Expt. 4, Saukett.

Diminution of observed variances with time

The progressive concealment of the true errors of plaque counting, which is the result of the drop in variance to mean ratios, is best illustrated by the unadjusted ratios shown in Table 1 for the LSc-2ab strain of virus (Expt. 2).

Repeated counting of the same plaques showed that, in all but one instance, an increase in the overlap bias caused by an increase in plaque size led to a fall in the variance to mean ratio. The data show that at or near the upper limit to the acceptable counting range ($KC = 0{\cdot}18$) overlapping may reduce the ratio by more than a half, and where the limit is greatly exceeded this reduction may be by as much as sevenfold.

Heterogeneity of cultures

It is of practical importance to find out whether the apparent heterogeneity of cultures in their sensitivity to virus, as indicated by the high variance to mean ratios at the first counting time, was due, partly or wholly, to counting plaques too early. This can be determined by examining the effect of adding the relatively small counts of late-appearing or 'new' plaques to the counts of early-appearing or 'original' plaques.

Table 1. *The overlap biases of variances of observed plaque-count distributions*

Observed variance: mean ratios for the LSc-2ab strain of virus (Expt. 2) demonstrate the fall in the observed variance: mean ratio which accompanies an increase in plaque size. Corrected ratios show that correction procedures will give more realistic if inexact estimates of the ratios for the true counts. Corrections become inadequate at about $K\overline{C} = 0{\cdot}18$. Mean plaque size for the three counting times were 2·8, 6·7, and 9·3 mm. respectively.

Rel. virus conc.	Counting time	$K\overline{C}$	Variance: Mean Ratios	
			Observed = $\frac{V(C)}{\overline{C}}$	Corrected = $\frac{V(N)}{\overline{N}}$
1·11	1	0·04	1·86	1·99
	2	0·20	1·01	1·42
	3	0·30	0·32	0·55
1·00	1	0·04	2·34	2·48
	2	0·19	0·98	1·35
	3	0·29	0·32	0·53
0·56	1	0·02	2·47	2·54
	2	0·10	2·38	2·81
	3	0·19	1·86	2·58
0·50	1	0·02	1·63	1·66
	2	0·09	1·48	1·71
	3	0·17	1·20	1·58
0·28	1	0·01	1·43	1·45
	2	0·06	1·32	1·44
	3	0·11	1·31	1·56
0·25	1	0·01	1·50	1·52
	2	0·05	1·62	1·75
	3	0·10	1·44	1·68

For a system in which there was neither heterogeneity nor overlap, the addition of new to original plaques would lead to identical increases in both the mean and the variance, and their ratio would remain constant at the value of 1. For a system free of heterogeneity but subject to overlap bias, new plaques would be more readily obscured because of their smaller size, and the ratio would fall slightly on adding the two sets of counts.

In practice there will be a tendency for this expected fall in the variance to mean ratios to be masked by the increased counting uncertainty which accompanies an increase in overlap bias, and by any increase in the heterogeneity of cultures with respect to late-appearing plaques. In the present study the Saukett strain

gave an example of such behaviour, and the addition of new to original plaques led to slight increases in the values of variance to mean ratios (Table 2).

The greater uncertainty associated with the counting of LSc-2ab plaques would be expected to lead to somewhat greater rises in variance to mean ratios on adding new to original plaques. However, pronounced falls were observed, which reflect a strong negative correlation between the counts of original and new plaques on

Table 2. *Changes in variance:mean ratios produced by adding new plaques to those previously present*

(Data used in the calculation of the variance: mean ratios shown in the body of the table were progressive total counts for each culture for two or three counting times. The mean diameter of the original plaques at each time is shown at the bottom of each column. New plaques present at the third counting time in Expts. 3 and 4 were not counted.)

Virus strain	Relative virus concentration	Counting time: 1st	2nd	3rd	1st	2nd
		Experiment 2 (15)*			Experiment 3 (15)	
LSc-2ab	1·39	—	—	—	2·56	1·25
	1·25	—	—	—	1·06	0·55
	1·11	1·86	1·49	1·54	1·41	0·90
	1·00	2·34	1·76	1·66	1·94	1·39
	0·56	2·47	1·79	1·74	1·33	1·05
	0·50	1·63	1·36	1·31	1·40	0·81
	0·28	1·43	1·00	1·04	2·11	1·51
	0·25	1·50	1·26	1·20	1·03	0·54
	0·14	1·15	1·10	1·10	0·88	0·92
	0·12	0·59	0·31	0·41	0·67	0·56
	Mean plaque Diam.	2·8 mm.	6·7 mm.	9·3 mm.	3·5 mm.	7·7 mm.
		Experiment 1 (30)			Experiment 4 (13-15)	
Saukett	0·50	0·66	0·71	0·70	—	—
	0·31	0·74	0·72	0·71	—	—
	0·28	—	—	—	0·56	0·69
	0·25	—	—	—	1·09	1·05
	0·20	0·84	1·00	0·99	—	—
	0·14	—	—	—	0·61	0·88
	0·12	0·98	0·84	0·84	0·71	0·86
	0·08	1·06	1·15	1·22	—	—
	0·06	—	—	—	0·50	0·68
	Mean plaque Diam.	4·7mm.	7·0 mm.	9·0 mm.	2·7 mm.	5·2 mm.

* Numbers in parentheses indicate the number of replicate cultures per group.

individual cultures (Table 2). This can only occur if a substantial part of the apparent heterogeneity in the sensitivities of cultures to virus, which was observed at the first counting time, was due to variation between cultures in the time of appearance of plaques. In such circumstances the use of early counting to avoid overlap bias will increase apparent heterogeneity and, although intended to decrease the total error, may actually increase it.

DISCUSSION

The reliability of plaque assays can be established only by repeatedly assaying the same virus suspension. Yet, in practice the determination of variance to mean ratios is often taken as a valid alternative. The latter approach is certainly simpler, but it has two disadvantages. Since it considers only some of the possible sources of experimental error, it will always give rather optimistic assessments of the reliability of a system. Even more damaging is the common assumption underlying this form of analysis, that the theoretical distribution of plaque numbers is Poissonian, and that the variance therefore equals the mean. This can hold only for counting techniques for which every object will always be counted even though overlapping occurs, and is therefore not true of plaque counting. In practice the adoption of the Poissonian model may be justified where the overlap bias of observed counts is very small. Where this is not so, allowance should be made for the disproportionate reduction of variances. Failing to do so can lead to gross underestimation of errors, and to statistically invalid and possibly misleading interpretation of data. The methods developed above permit compensation to be made for this bias and the estimation of true errors.

Additional requirements where true errors are being assessed are that cultures be identified by code numbers, and that inocula be allocated at random. Without such safeguards errors due to heterogeneity in materials or environmental conditions may be greatly underestimated. Even in carefully designed and conducted assays the heterogeneity of cultures may prove a major source of error. This was the case with the LSc-2ab strain of poliovirus analysed in this study. Heterogeneity could be attributed largely to variation in the time of appearance of plaques, and not to variation in the sensitivity of cultures to the initiating virus particles. This means that estimates of virus concentration provided by such an assay system will carry smaller errors if counting is delayed, and if the observed counts falling within the acceptable counting range are corrected for the overlap biases which are the consequence of delayed counting.

The approach to the analysis of errors used here, and the methods for correcting overlap bias should prove useful where the precision of other plaque assay systems is being evaluated. The conclusion of the present study, that variance to mean ratios for observed counts are unsatisfactory estimators of the true errors, is true for all systems. Such devices should never be used where the overlap biases of observed counts have not been studied.

SUMMARY

The overlapping of plaques compresses their distributions and reduces observed variances. For a given distribution the reduction of the variance is substantially larger than that of the mean. To derive the error of a plaque assay from the assumption that the variance equals the mean may therefore lead to serious overestimation of the precision of the assay.

Procedures for estimating the true error of plaque assays are developed, and their use is illustrated on experimental material.

REFERENCE

HOWES, D. W. (1969). Overlap and the errors of plaque counting. 1. The overlap biases of observed counts and their correction. *J. Hyg., Camb.* **67**, 317.

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Neutralization tests with varicella-zoster virus

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Varicella-zoster (V-Z) virus was first cultivated in tissue culture by Weller & Stoddard (1952). Subsequently the fluorescent antibody technique was used by Weller & Coons (1954) to demonstrate that antibodies to the virus are present in convalescent chickenpox and zoster sera, but few neutralization tests have been done with the agent because of the difficulty in obtaining virus in a cell-free state from most tissue culture systems. Weller & Witton (1958) showed that the ability of intact infected human embryonic fibroblasts to transmit virus to fresh tissue culture monolayers could be partially neutralized by antiserum if the antiserum was incorporated in the medium of the inoculated tissue cultures. The cytopathic effects of the virus were not suppressed completely but the number of foci of infection was greatly reduced and the foci themselves were altered in appearance. Taylor-Robinson (1959) described neutralization tests in which vesicle fluid taken directly from patients was used as a source of cell-free virus. This system gave satisfactory results but its application was limited by the difficulty of obtaining sufficient vesicle fluid. The production of cell-free virus from primary human thyroid cells (Caunt, 1963) provides a more convenient laboratory source of cell-free virus and this paper describes the method we have used for detecting V-Z virus neutralizing antibody in the sera of chickenpox and zoster patients and in pools of human gamma-globulin.

Kapsenberg (1964) and Ross, Subak Sharpe & Ferry (1965) showed some cross-reactions between *Herpes simplex* (HS) and V-Z virus in complement-fixation (CF) tests so we also tested most of our sera with HS virus to see whether any cross-neutralization could be demonstrated. The paired sera were also tested for CF antibody to both V-Z and HS antigens.

MATERIALS AND METHODS

Tissue cultures

V-Z virus was titrated either in primary human thyroid cells prepared as described by Pulvertaft, Davies, Weiss & Wilkinson (1959) or in Vero cells, a continuous line of vervet monkey kidney cells (Liebhaber, Riordan & Horstmann, 1967). In both cases the cells were grown in $6 \times \frac{5}{8}$ in. tubes. HS virus was titrated either in GMK-AH 1 cells, a continuous line of grivet monkey kidney cells (Gunalp, 1965), or in Vero cells.

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Media

Human thyroid cells were grown in 5% calf serum in Parker's 199 medium (Burroughs Wellcome Ltd.) and maintained in the same medium with the calf serum reduced to 2%. Vero cells were also grown in Parker's 199 medium but with 5% and 2% of foetal calf serum (Flow Laboratories) for growth and maintenance respectively. GMK-AH1 cells were grown in Eagle's minimum essential medium (M.E.M., Burroughs Wellcome Ltd.) with 5% and 2% of calf serum for growth and maintenance respectively.

Viruses

V-Z virus was prepared by the ultrasonic disruption of infected primary human thyroid cells as described by Caunt (1963). Several virus strains, all isolated from vesicle fluid of chickenpox or zoster patients, were used. Preliminary experiments showed no antigenic differences between these strains (Shaw, 1968). The virus preparations were stored at $-65°$ C. in small volumes and a fresh portion was thawed for each test.

One strain of HS virus isolated in human amnion cells and subsequently subcultured in continuous monkey kidney cell lines was used throughout. Virus preparations were made from infected tissue cultures in which all the cells showed cytopathic effects. The cells from two or three 12 oz. bottles were washed from the glass by pipetting, pooled in 3 ml. of maintenance medium and disrupted by ultrasonic treatment as used in the preparation of V-Z virus. The treated material was then diluted to 35 ml. with the medium harvested from the infected bottles, yielding a preparation with a titre of about 10^8 plaque-forming units (p.f.u.) per ml. which could be stored at 4° C. for 2 weeks with little loss of infectivity. Preparations were made from Vero and GMK-AH1 cells but were always titrated in the same cell system as that in which they were prepared.

Antigens for CF tests

V-Z antigen was prepared by the ultrasonic disruption of infected human thryoid cells (Caunt & Taylor-Robinson, 1964). HS antigen was prepared from infected BHK-21 cells by the method of Grist, Ross, Bell & Stott (1966).

Sera

Sera were stored at $-20°$ C. and were heated to 56° C. for 30 min. or 58° C. for 10 min. before use.

Human gamma-globulin

One specimen had been prepared by the Lister Institute and the other specimen, which was vaccinia immune globulin, by E. R. Squibb and Sons, New York.

Diluent for neutralization tests

Viruses and sera were diluted in phosphate buffered saline (Dulbecco & Vogt, 1954) to which 2% inactivated calf serum had been added. The final pH was 7·3.

Neutralization test techniques

V-Z virus

The virus preparation was diluted to contain 1–3 × 10^3 p.f.u./ml. and mixed with equal quantitites of serial dilutions of the serum under test or with 1/10 calf serum as a control. After incubation under various conditions, which are described in the experimental results, 0·2 ml. portions of the serum–virus mixtures were inoculated into each of two or three tubes of tissue culture from which the medium had been removed. The inoculated tubes were stoppered and left stationary at room temperature for 3½ hr. to allow un-neutralized virus to adsorb to the cell sheets before 1 ml. of maintenance medium was added to each tube. The tubes were incubated in stationary racks at 35° C. for 4–5 days until viral lesions were large enough to be counted. No secondary foci occurred within this time. The lesions were counted using a low-power microscope (magnification × 10) and dark-ground illumination.

HS virus

Neutralization tests were carried out by the method described by Ross *et al.* (1965) using the plaque assay method of Russell (1962). The inoculated cells were seeded into 1½ in. diameter glass Petri dishes using three dishes per serum–virus mixture, and these were incubated for 3 days at 35° C. in an atmosphere of 5 % CO_2 in air. The dishes were then drained, fixed in Bouin's fluid, washed under the tap and stained with carbol-fuchsin. The plaques could then be counted by the naked eye.

CF test technique

CF tests were performed by the method described in Public Health Monograph no. 74 (1965).

Table 1. *Comparison of various techniques for neutralization of V-Z virus*

	Number of plaques per tube with serum dilution				Virus control	
Condition of test	1/5	1/25	1/125	1/625	Plaque counts	Mean
Shaken at 35° C.	0, 0, 0	0, 0, 0	14, 7, 13	15, 20, 16	50, 90	70
Shaken at room temperature	0, 0, 0	1, 0, 0	54, 31, 29	59, 40, 63	60, 50	55
Stationary at 35° C.	0, 0, 0	6, 3, 11	51, 53, 67	72, 61, 74	100, 50	75

RESULTS

Comparison of techniques for neutralization tests with V-Z virus

A convalescent zoster serum was used for this test. The serum–virus mixtures were either incubated in stationary racks at 35° C. or were shaken slowly (40 strokes/min.) in a shaking water bath at room temperature or at 35° C. for 1 hr., in an attempt to increase the rate of reaction. The results of these tests are shown in Table 1. The tubes which were shaken at 35° C. gave the highest neutralization titre, with a virus control count not appreciably different from that resulting from

stationary incubation at 35° C. The virus has been shown not to suffer any detectable thermal inactivation at 37° C. in 1 hr. (Shaw, 1968). This technique was therefore used in all subsequent neutralization tests on V-Z virus. The titres recorded are the final dilutions which gave 50 % reduction in plaque count.

Table 2. *Paired sera from chickenpox cases tested for neutralizing and CF antibodies to V-Z and HS viruses*

Case	Duration of rash when serum taken	Serum dilution giving 50 % neutralization with		CF titre with	
		V-Z virus	HS virus	V-Z antigen	HS antigen
1	3 days	< 1/5	1/80	1/40	1/80
	14 days	1/40	1/80	1/1280	1/320
2	2 days	< 1/5	< 1/5	< 1/5	< 1/5
	6 days	1/20	< 1/5	1/1280	< 1/5
	21 days	1/20	< 1/5	1/1280	< 1/5
3	3 days	1/5	1/640	1/320	1/80
	18 days	1/40	1/640	1/2560	1/160
4	3 days	< 1/5	1/1280	1/10	1/20
	28 days	1/40	1/1280	> 1/2560	1/160
5	1 day	< 1/5	< 1/5	a/c	< 1/10
	16 days	1/20	< 1/5	1/1280	< 1/10
6	1 day	< 1/5	< 1/5	1/10	< 1/10
	14 days	1/20	< 1/5	1/320	< 1/10
7	1 day	< 1/5	< 1/5	1/40	< 1/10
	14 days	1/20	< 1/5	1/640	< 1/10

a/c = anticomplementary.

Neutralization and CF tests on paired sera from cases of chickenpox

The results of neutralization and CF tests on paired sera from seven cases of chickenpox are shown in Table 2. Up to 3 days after the appearance of the rash the titre of neutralizing antibody to V-Z virus did not exceed 1/5. In the only case tested at 6 days the titre had risen to 1/20 and did not subsequently rise further. The maximum titre of neutralizing antibody to V-Z attained by any of the cases was 1/40. There was no rise in titre of neutralizing antibody to HS virus in any of the cases.

The CF antibody titre with V-Z virus rose in all cases and where the patient was a herpetic the CF titre to HS rose also but this latter rise did not occur if there was no HS CF antibody in the acute stage serum.

Persistance of neutralizing antibody to V-Z virus after chickenpox

Sera were tested from nine adults who had had chickenpox many years previously, but who had not had zoster, to see how long neutralizing antibodies persisted. The results are shown in Table 3. Six of these cases had titres of 1/10 or less and three had titres of 1/20, 1/40 and 1/160 respectively. A second sample from this last case taken 10 years after the first still showed a titre of 1/160.

Sera from two adults who had never had chickenpox were also tested and no antibody could be detected at a dilution of 1/5.

No correlation between neutralizing antibody titres to V-Z and HS viruses could be shown.

Table 3. *Sera from adults who had had chickenpox but not zoster tested for neutralizing antibodies to V-Z and HS viruses*

Case	Time since chickenpox (in years)	Serum dilution giving 50 % neutralization with	
		V-Z virus	HS virus
8	40	1/20	< 1/10
9	35	1/10	1/320
10	30	< 1/5	< 1/10
11	30	1/40	1/160
12a	20	1/160	NT
12b	30	1/160	< 1/10
13	28	< 1/5	1/80
14	25	< 1/5	1/100
15	20	1/5	1/160
16	20	1/10	< 1/10
17	not had CP	< 1/5	< 1/10
18	not had CP	< 1/5	> 1/50

NT = not tested.

Table 4. *Paired sera from cases of zoster tested for neutralizing and CF antibodies to V–Z and HS viruses*

Case	Duration of rash when serum taken	Serum dilution giving 50 % neutralization with		CF titre with	
		V-Z virus	HS virus	V-Z antigen	HS antigen
19	3 days	< 1/5	NT	1/10	NT
	9 days	1/320	1/640	> 1/2560	1/320
20	2 days	< 1/5	1/640	1/10	1/160
	23 days	1/40	1/640	1/320	1/160
21	4 days	1/5	< 1/10	1/10	1/80
	15 days	> 1/320	< 1/10	1/2560	1/80

NT = Not tested. Insufficient serum.

Neutralization and CF tests on paired sera from patients with zoster

Paired sera from three cases of zoster were tested for neutralizing and CF antibodies to V-Z and HS viruses and the results are shown in Table 4. It can be seen that although the titre of neutralizing antibody to V-Z virus in the acute stage sera was low or absent, as in the acute stage of chickenpox, two of the convalescent sera show titres of 1/320. No rise in titre of neutralizing antibody to HS occurred in any of the patients.

The titres of CF antibody to V-Z antigen rose in all three cases but no rise in CF titre to HS antigen was seen.

Persistence of neutralizing antibody to V-Z virus after zoster

Single sera from nine patients who had had zoster at some time were tested for neutralizing antibodies to V-Z and HS viruses and the results are shown in Table 5. A high level of neutralizing antibody to V-Z virus appears to be common after zoster and to persist for many years, although sera from patient 28 showed some fall in titre after 2 years.

Neutralizing antibody titres to HS virus varied and again showed no correlation with those to V-Z virus.

Table 5. *Sera from patients who had had zoster tested for neutralizing antibodies to V-Z and HS viruses*

Case	Time from onset of rash when serum taken	Serum dilution giving 50 % neutralization with V-Z virus	Serum dilution giving 50 % neutralization with HS virus
22	5 days	1/160	1/320
23	14 days	> 1/160	NT
24	18 days	1/40	1/40
25	18 days	1/80	< 1/10
26	1 month	1/80	1/40
27	3 months	> 1/640	1/40
28a	2 months	> 1/160	> 1/320
28b	2 years	1/80	NT
29	15 years	1/160	1/40
30	40 years	1/160	1/80

NT = not tested.

Table 6. *Neutralization tests with V-Z virus on paired sera from contacts of chickenpox or zoster*

Case	Time from contact when serum taken	Serum dilution giving 50 % neutralization with V-Z virus
31	0 days	1/80
	32 days	1/80
32	1 day	< 1/10
	41 days	< 1/10
33	5 days	1/40
	49 days	1/40
34	5 days	1/20
	4½ months	1/20
35	2 days	< 1/10
	29 days	1/80

Neutralization tests with V-Z virus on sera from close contacts of chickenpox and zoster

Hope-Simpson (1965) suggested that adults who had had chickenpox in childhood might become reinfected on contact with chickenpox cases and show an antibody response to the virus even though they did not develop clinical illness.

In order to test this hypothesis paired sera from five close household contacts of chickenpox or zoster were supplied to us by Dr Hope-Simpson. The first serum in each pair was taken on the day of contact or very shortly afterwards. The results of these tests are shown in Table 6. The only rise in neutralizing antibody to V-Z virus occurred in case 35, a mother whose three children developed chickenpox. Her first serum was taken 2 days after the first child developed the disease. Cases 31 and 32 were also the parents of children who developed chickenpox. Case 33 was a boy whose mother had chickenpox and case 34 was a boy whose mother had zoster

The sera from case 35 were tested for CF antibodies to V-Z. The first serum showed no fixation and the second one was anticomplementary.

Neutralization tests against V-Z virus on pooled human gamma-globulin

The gamma-globulin solution was first diluted 1/10 and then serial twofold dilutions were made. Up to 1/40 both batches were toxic to the tissue culture cells but at higher dilutions neutralization occurred and the 50% end-point was 1/160 for the Lister Institute specimen and 1/320 for the other.

DISCUSSION

The results presented in Table 2 show that the neutralizing antibody response immediately after primary varicella infection is low and the maximum titre demonstrated was 1/40. Among people who had had chickenpox many years previously six of nine tested had neutralizing antibody titres of 1/10 or less which would be consistent with a gradual decline from an original low level. Two of the others had titres of 1/20 and 1/40 which could be explained by a variation in the original antibody response, but case 12, with a neutralizing titre of 1/160, is more difficult to explain in this way and will be discussed more fully below. The results in Table 4 show that zoster arises in people who have low or undetectable amounts of V-Z neutralizing antibodies but a high level of such antibody develops rapidly and, from the evidence in Table 5, it persists for many years. This typical secondary antibody response in zoster supports the theory that zoster represents a recurrence or second experience of V-Z virus. A similar secondary response in CF antibody in zoster cases was reported by Weller & Witton (1958) and Taylor-Robinson & Downie (1959). If it does represent a recurrence of a latent infection then clearly the mechanism of latency is different from that in recurrent *Herpes simplex* infection where a high level of neutralizing antibody persists after the primary attack and local clinical disease recurs despite the presence of circulating antibody. Hope-Simpson (1965) postulated that, after chickenpox, V-Z virus becomes latent in the sensory root ganglia of the spinal nerves in a 'pro-virus' state or in some way sequestered from the reticulo-endothelial system so that it does not provide a continuous antigenic stimulus and as a result the host's immunity falls to a negligible level. In such a carrier of latent virus reversions to virulence of the virus might occur from time to time. If the patient still possessed circulating, neutralizing antibody then no disease would occur but a rise in antibody titre would result. If the reversion took place when the antibody titre was below a critical level, however,

clinical zoster would result. The low titres of antibody in the acute stage of the paired zoster sera we have tested support this view and the high titre of neutralizing antibody in case 12 (Table 3), who had not had zoster, might have arisen because of a reversion to virulence of endogenous virus.

Another possible reason for this high neutralizing titre in the absence of zoster is that reinfection might occur from an external source and result in a rise in the level of antibody. This possibility was also proposed by Hope-Simpson (1965) and it was tested using paired sera from close contacts of chickenpox or zoster. In one case the titre rose from < 1/10 to 1/80, thus showing that this mechanism can operate. However, case 12 was unaware of any contact with chickenpox or zoster about the time when the serum samples were taken and CF tests using V-Z antigen proved negative, thus making it unlikely that he had had recent experience of the virus. If the high level of antibody in case 12 is the result of reinfection by contact with chickenpox or zoster or of recrudesence of latent virus then either of these events must have occurred sufficiently long ago for the CF antibody to have waned although the neutralizing antibody persists.

Ross *et al.* (1965) showed a rise in CF antibody to HS in 48 % of their chickenpox patients and in 26 % of their zoster patients. They also showed slight rises (less than fourfold) in neutralizing antibody to HS in some chickenpox cases. They argued that concurrent HS infection could be discounted because in recurrent HS there is no rise in CF antibody and the presence of neutralizing antibodies in the acute stage sera eliminates the possibility of primary HS.

We have confirmed the rise in CF antibody to HS occurring during the course of chickenpox in patients who are already herpetics but we could not show it during zoster in the two patients tested although they did have HS antibodies. No rise in titre of neutralizing antibody to HS was shown in any of our chickenpox patients, even in those who showed a rise in CF antibody to HS during their illness.

There does not appear to be any relationship between neutralizing antibodies to HS and V-Z in the sera we have tested and we could not attribute low levels of neutralizing antibody to V-Z virus to the possession of high titres to HS virus. We have not been able to examine the effect of primary HS infection on levels of neutralizing antibody to V-Z virus.

There have been many conflicting reports about the value of human gamma-globulin in the prevention of chickenpox. Abrahamson (1944) used plasma from a convalescent zoster patient and Trimble (1957) used pooled human gamma-globulin to protect susceptible contacts of chickenpox from the disease. Ross (1962) claimed that the use of gamma-globulin produced significant modification of chickenpox in family contacts. However, Schaffer (1965) stated that gamma-globulin was ineffective in chickenpox when used in the same dosage as that which is effective against measles, hepatitis and poliomyelitis and Shaw & Grossman (1966) also found gamma-globulin unreliable in protecting susceptible contacts of chickenpox in a hospital ward.

One of the possible explanations for these discrepancies is that many adults have very low levels of neutralizing antibody to V-Z virus in their serum and if a gamma-globulin pool contained only such sera then it would be ineffective in the

prophylaxis of chickenpox but other batches of gamma-globulin might have a much higher titre and be correspondingly more effective. We tested two batches of human gamma-globulin and found neutralizing antibody titres of 1/160 and 1/320 respectively. The first batch had been used in the treatment of severe chickenpox in a child whose immunological responses had been impaired by radiotherapy and drugs and it was thought to have made an important contribution to her recovery (D. Mainwaring, personal communication). It is now possible to test batches of gamma-globulin for neutralizing antibodies to V-Z virus and select, if necessary, those with high enough titres to be useful in the prevention or treatment of chickenpox.

SUMMARY

A technique for neutralization tests using varicella-zoster virus propagated in primary human thyroid cells is described. The level of neutralizing antibody following chickenpox does not usually exceed a titre of 1/40 and in adults many years after infection it may be very low. After zoster a much higher and more persistent antibody response occurs. Contact with chickenpox also produced a rise in neutralizing antibody in one out of the five patients tested. One case who had had chickenpox but not zoster had a high level of neutralizing antibody and the possible reasons for this are discussed. No cross-neutralization with *Herpes simplex* virus was demonstrated but the rise in titre of complement-fixing antibody to HS occurring in herpetic subjects with chickenpox (Ross *et al.* 1965) was confirmed. Two samples of human gamma-globulin were shown to have high levels of neutralizing antibody to V-Z virus and one was known to have been found effective clinically.

We are indebted to Dr R. E. Hope-Simpson for the paired contact sera and also for many valuable discussions in the course of this work. We would also like to thank Dr D. Taylor-Robinson who collected the paired zoster sera, Dr R. M. Rawcliffe and Dr F. Whitwell who arranged the supply of thyroid tissue, and Dr G. C. Turner and Dr J. Eller for the samples of human gamma-globulin. We are grateful to Mrs M. Stewart for valuable technical assistance.

REFERENCES

Abrahamson, A. W. (1944). Varicella and herpes zoster: an experiment. *Br. med. J.* i, 812–13.

Caunt, A. E. (1963). Growth of varicella-zoster virus in human thyroid tissue cultures. *Lancet* ii, 982–3.

Caunt, A. E. & Taylor-Robinson, D. (1964). Cell-free varicella-zoster virus in tissue culture. *J. Hyg., Camb.* **62**, 413–24.

Dulbecco, R. & Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167–82.

Grist, N. R., Ross, C. A. C., Bell, E. J. & Stott, E. J. (1966). *Diagnostic Methods in Clinical Virology*, p. 65. Oxford: Blackwell.

Gunalp, A. (1965). Growth and cytopathic effect of rubella virus in a line of green monkey kidney cells. *Proc. Soc. exp. Biol. Med.* **118**, 85–90.

Hope-Simpson, R. E. (1965). The nature of herpes zoster: a long-term study and a new hypothesis. *Proc. R. Soc. Med.* **58**, 9–20.

Kapsenberg, J. G. (1964). Possible antigenic relationship between varicella-zoster virus and herpes simplex virus. *Arch. ges. Virusforsch.* **15**, 67–73.

LIEBHABER, H., RIORDAN, J. T. & HORSTMANN, D. M. (1967). Replication of rubella virus in a continuous line of African green monkey kidney cells (Vero). *Proc. Soc. exp. Biol. Med.* **125**, 636–43.

PUBLIC HEALTH MONOGRAPH No. 74 (1965). U.S. Department of Health, Education and Welfare, Public Health Service Publication no. 1228. Washington.

PULVERTAFT, R. J. V., DAVIES, J. R., WEISS, L. & WILKINSON, J. H. (1959). Studies on tissue cultures of human pathological thyroids, *J. Path. Bact.* **77**, 19–32.

ROSS, A. H. (1962). Modification of chickenpox in family contacts by administration of gamma-globulin. *New Engl. J. Med.* **267**, 369–76.

ROSS, C. A. C., SUBAK SHARPE, J. H. & FERRY, P. (1965). Antigenic relationship of varicella-zoster and herpes simplex. *Lancet* ii, 708–11.

RUSSELL, W. C. (1962). A sensitive and precise plaque assay for herpes virus. *Nature, Lond.* **195**, 1028–29.

SCHAFFER, A. J. (1965). *Diseases of the Newborn*, 2nd ed., p. 737. London: Saunders.

SHAW, D. G. (1968). Laboratory studies on varicella-zoster virus. Ph.D. thesis, University of Liverpool.

SHAW, E. B. & GROSSMAN, M. (1966). Viral contagious infections. In *Pediatric Therapy*, 2nd ed. (ed. H. C. Shirkey), ch. 55, p. 410. St Louis: Mosby.

TAYLOR-ROBINSON, D. (1959). Chickenpox and herpes zoster. III. Tissue culture studies. *Br. J. exp. Path.* **40**, 521–32.

TAYLOR-ROBINSON, D. & DOWNIE, A. W. (1959). Chickenpox and herpes zoster. I. Complement fixation studies. *Br. J. exp. Path.* **40**, 398–409.

TRIMBLE, G. X. (1957). Attenuation of chickenpox with gamma-globulin. *Can. med. Ass. J.* **77**, 697–9.

WELLER, T. H. & COONS, A. H. (1954). Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*. *Proc. Soc. exp. Biol. Med.* **86**, 789–94.

WELLER, T. H. & STODDARD, M. B. (1952). Intranuclear inclusion bodies in cultures of human tissue inoculated with varicella vesicle fluid. *J. Immun.* **68**, 311–9.

WELLER, T. H. & WITTON, H. M. (1958). The etiologic agents of varicella and herpes zoster. Serologic studies with the viruses as propagated in vitro. *J. exp. Med.* **108**, 869–90.

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Antibody against influenza A2 virus neuraminidase in human sera

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Surveys of antibody to influenza A viruses in the human population have frequently relied upon the haemagglutination-inhibition technique and this method has also been widely used in the serological diagnosis of influenza infections. Haemagglutination-inhibition tests have the advantage of rapidity and simplicity and they make it possible to detect antibody reacting specifically with different subtypes of influenza virus. In addition the titre of haemagglutination-inhibiting antibody is related to the capacity of a serum to neutralize virus infectivity and the presence of such antibody has been correlated with the susceptibility of the individual to influenza virus infection (Bell *et al.* 1957; Tyrrell, 1967). However, it is now well established that haemagglutinin is but one of the two principal viral antigens present on the surface envelope of the influenza A virus; the other virus-coded antigen being the enzyme neuraminidase. The neuraminidase of influenza A virus has been purified and separated from haemagglutinin (Laver, 1964; Laver & Kilbourne, 1966). Anti-neuraminidase antisera have enzyme-inhibiting capacity but do not inhibit haemagglutination and anti-haemagglutinin antisera do not inhibit enzyme activity (Webster & Laver, 1967; Seto & Rott, 1966). Because of the immunological independence of haemagglutinin and neuraminidase it seems likely that the measurement of haemagglutination-inhibiting antibody alone might give an incomplete picture of the antibody response in human influenza infections. We have therefore studied the development of neuraminidase-inhibiting antibody for influenza A2 viruses in man following natural influenza A2 infections and following vaccination with killed influenza A2 virus.

MATERIALS AND METHODS

Virus strains

Human influenza viruses A1/FM1, A2/Singapore/1/57, A2/England/2/64, A2/England/76/66 and B/England/5/66 were from the stocks of this laboratory. A0/BEL was kindly supplied by Drs W. G. Laver and R. G. Webster of the John Curtin School of Medical Research, Canberra, Australia.

FPV-A2 (R4) was a laboratory recombinant virus derived from fowl plague and A2/Singapore/1/57 (Tumova & Pereira, 1965). The neuraminidase of this strain has been shown to be immunologically identical with that of the A2 parent whilst the haemagglutinin is identical with that of fowl-plague virus (Easterday, Laver,

Pereira & Schild, in preparation). Influenza A/Turkey/Massachusetts/65 (Pereira *et al.* 1966) is a virus of avian origin which contains a neuraminidase immunologically identical with that of A2/Singapore/1/57 and a haemagglutinin unrelated to that of human A2 virus (Pereira, Tumova & Webster, 1967; Webster & Pereira, 1968).

Virus purification and concentration

The viruses used in neuraminidase inhibition tests were grown in the allantoic cavity of 10-day embryonated eggs. The allantoic fluids were harvested 1–3 days after infection and the virus was purified by adsorption onto and elution from fowl erythrocytes followed by differential centrifugation and one or two cycles of rate zonal centrifugation on a linear 10–40 % sucrose gradient. The final yield of purified virus from 100 eggs was suspended in 0·5 ml. saline. These preparations contained between 500 and 5000 units of neuraminidase activity per 0·1 ml.

Human sera

Acute and convalescent serum samples taken on the first or second day of illness and three to four weeks later were obtained from adults with clinical influenza between December 1967 and February 1968. These sera were collected during the course of a clinical trial of an antiviral compound carried out under the auspices of the College of General Practitioners, London. However, in the present study the sera used were from donors who received an inactive placebo.

Pre- and post-immunization serum specimens were obtained from adult volunteers who received formalin-inactivated influenza virus vaccines containing A2/Singapore/1/57 virus. Sera were tested from six volunteers who received a vaccine incorporating a vegetable-oil adjuvant (A65) in a vaccine trial carried out by the Medical Research Council at R.A.F. Halton. (Unpublished report to the M.R.C. Committee on Influenza and other Respiratory Virus Vaccines, 1967). A further seven serum pairs from volunteers receiving a mineral-oil adjuvant (drakeol-arlacel) vaccine in a trial carried out by Evans Medical Ltd., Liverpool, were used.

Rabbit antisera

The preparation of a rabbit antiserum against purified A neuraminidase has been described (Webster & Laver, 1967) The source of the purified neuraminidase was X7 (F1) virus (Kilbourne *et al.* 1967), a recombinant virus which contains neuraminidase derived from its A2(R1/5) (1957) parent. This serum had no haemagglutinin-inhibiting antibody for A2 virus. For use in neuraminidase inhibition tests antisera against ultraviolet-inactivated, purified influenza virus preparations with Freund's complete adjuvant were prepared in rabbits as described by Webster & Laver (1967). All sera were stored at $-20°$ C.

Haemagglutinin estimations

Influenza virus haemagglutinin and the measurements of haemagglutinin-inhibiting antibody in serum specimens were carried out by standard methods (W.H.O. Expert Committee on Influenza, 1953). Virus and antiserum were left in contact for 1 hr. before addition of erythrocytes.

Preparation of purified neuraminidase.

Purified and concentrated FPV-A2(R4) virus (Tumova & Pereira, 1965) was disrupted by sodium dodecyl sulphate (1 %) and the proteins separated by electrophoresis on cellulose acetate strips (Laver, 1964). The protein band containing the enzymic activity was eluted with water and the enzyme freed of sodium dodecyl sulphate by precipitation of the detergent with saturated potassium chloride solution.

Estimation of neuraminidase activity and neuraminidase-inhibiting antibody

The neuraminidase activity of virus preparations was estimated by incubating serial dilutions of virus (0·05 ml.) for 1 hr. at 37° C. with 0·1 ml. volumes of buffered fetuin solution (12 mg./ml. in phosphate buffer, pH 5·9). The release of *N*-acetyl neuraminic acid was assayed by a modification of the Warren method (Webster & Laver, 1967) employing optical density determinations on the Unicam SP500 at 549 μ.

For the estimation of neuraminidase-inhibiting antibody a modification of the technique described by Laver & Kilbourne (1966) was used. Dilutions of purified influenza virus containing 1–2 units of enzyme (Webster & Laver, 1967) in 0·05 ml were incubated at 4° C. for 15 hr. with serial 10–fold or 3·3-fold dilutions of serum. Fetuin solution (0·1 ml.) in buffered saline (pH 5·9) was added and the mixture incubated at 37° C. for 1 hr. Control samples of enzyme were incubated with equivalent dilutions of normal human or rabbit serum. The neuraminidase-inhibition titre of a serum was expressed as the reciprocal of the serum dilution which produced 50 % inhibition of enzyme activity. This value was obtained from the plot of serum dilution against the percentage inhibition of enzyme activity.

RESULTS

Neuraminidase antibody in natural A2 infections

The development of neuraminidase-inhibiting antibody was studied in paired sera from twenty adults with serologically confirmed influenza A2 infections. For each serum pair, serological evidence of influenza A2 infection was obtained both in complement-fixation tests with influenza A soluble antigen and in haemagglutination-inhibition tests with A2/Singapore/1/57 and A2/England/10/67. The latter is a virus strain isolated at the time of the epidemic during which the paired sera were collected. Representative results are shown in Table 1. Neuraminidase-inhibiting antibody for A2/Singapore/1/57 virus was detected in only three of 20 (15 %) of the acute serum samples and the titres were low, ranging from 1/10 to 1/50. In 18 of the 20 serum pairs studied neuraminidase-inhibiting antibody for A2/Singapore/1/57 was acquired during the course of the infection or showed an increase in titre. The neuraminidase-inhibiting antibody titres in the convalescent sera ranged from 1/50 to 1/3000 and the arithmetic mean titre in the convalescent sera was 1/420. In the remaining two serum pairs no neuraminidase-inhibiting antibody was detected in the acute or convalescent serum. When A0/BEL and

Table 1. *Development of neuraminidase-inhibiting antibody in adults with serologically confirmed influenza A 2 infections**

Serum pair		Complement-fixation antibody titres (PR 8-S antigen)	Haemagglutination-inhibition antibody titres with		Neuraminidase-inhibiting antibody titres† with various influenza virus strains:			
			A2/Singapore/1/57	A2/England/10/67	A0/BEL	A1/FM1	A2/Singapore/1/57	B/England/5/66
2B	*a*‡	10	480	48	<	<	<	<
	c	320	1280	192	<	<	450	<
3B	*a*	<§	160	<	<	<	<	15
	c	> 320	> 1280	96	<	<	75	15
5A	*a*	<	30	<	<	<	30	<
	c	320	1280	192	<	<	500	<
14A	*a*	<	<	<	<	<	<	<
	c	320	36	24	<	<	130	<
16A	*a*	20	10	40	<	<	<	<
	c	320	640	96	<	<	3000	<
18D	*a*	<	240	24	<	<	<	<
	c	> 320	1280	> 384	<	<	750	<
26A	*a*	<	240	24	<	<	<	<
	c	> 320	960	96	<	<	450	<
20A	*a*	20	60	<	<	<	<	10
	c	240	> 1280	192	<	<	50	120

* Infections occurred in the period December–February 1968 in England. † Reciprocal of serum dilution producing 50 % inhibition of neuraminidase activity. ‡ *a*, Acute serum specimen; *c*, convalescent serum specimen 2–4 weeks after onset of illness. § < = titros less than 1/10.

A1/FM1 viruses were used, none of the sera showed detectable neuraminidase-inhibiting antibody. In one of the 20 serum pairs (20A) there were simultaneous increases in neuraminidase-inhibiting antibody against influenza A2 and influenza B/England/5/66. It seems likely that these sera were from an individual with simultaneously occurring influenza A2 and B infections, although haemagglutination-inhibiting tests indicated an antibody response to A2 virus but not B/Engand/5/66.

Table 2. *Development of neuraminidase-inhibiting antibody in individuals with influenza B infections**

Serum pair	Haemagglutination-inhibiting antibody titres	Neuraminidase-inhibiting antibody titres with	
	B/England/5/66	B/England/5/66	A2/Singapore/1/57
767 *a*†	<‡	<	20
c	320	75	20
769 *a*	<	<	10
c	160	10	10
770 *a*	<	<	<
c	160	30	<

* Infections occurred in 1966 in England.
† *a*, Acute serum specimen;
c, denotes convalescent serum taken 2–4 weeks after onset of illness.
‡ < = titres less than 1/10.

Table 2 shows the results of similar studies with paired sera from individuals with serologically confirmed influenza B infections. In all three serum-pairs neuraminidase-inhibiting antibody to influenza B/England/5/66 virus was acquired during the infection. However, the antibody titres for influenza B neuraminidase in the convalescent sera were much lower than those detected for A2 virus in convalescent sera from individuals with influenza A2 infections. There was no simultaneous rise in antibody for the neuraminidase of influenza A2/Singapore/1/57 virus.

To extend these observations, a random collection of sera from adults obtained in the period January to November 1967, before the onset of the 1967–8 influenza A2 epidemic, was tested for frequency and titre of neuraminidase-inhibiting and haemagglutination-inhibiting antibody. In Table 3 the relationship between the antibody titres of the random sera in both types of test is compared with that found in a collection of convalescent sera. Neuraminidase-inhibiting antibody was detected in 30% of the random serum samples and the titres were low, in no case exceeding 1/50. This neuraminidase antibody was more frequent in sera which had high titres of haemagglutination-inhibiting antibody. Thus, 77% of random serum samples with haemagglutination-inhibition titres of 1/640 or greater had neuraminidase antibody whilst only 8% of those with haemagglutination-inhibition titres of 1/160 or less had antibody for neuraminidase. For the collection of random serum samples the ratio of the mean neuraminidase-inhibiting antibody titre and the mean haemagglutination-inhibiting antibody titres were low (< 0·05). However,

Table 3. *Relationship between the neuraminidase-inhibition and haemagglutination-inhibition antibody titres for influenza A 2/Singapore/1/57 virus in pre-epidemic and convalescent sera from adults*

	Haemagglutination-inhibiting antibody titre	No. of sera with the stated haemagglutination inhibition titre	No. and % of sera with neuraminidase-inhibiting antibody at 1/5 or greater	Mean neuraminidase-inhibiting antibody titre in positive sera	Ratio: $\frac{\text{mean neuraminidase-inhibition titre}}{\text{mean haemagglutination-inhibition titre}}$
Random (pre-epidemic) serum specimens	< 10	24	0 (0 %)	—	—
	80–160	24	2 (8·3 %)	1/5	0·04
	⩾640	26	20 (77 %)	1/17	0·02
	All pre-epidemic sera	74	22 (30 %)	< 1/17	0·05
Convalescent serum specimens	⩾640	15	15 (100 %)	1/520	0·54

for the collection of convalescent sera (Table 3) neuraminidase-inhibiting antibody was detected in all samples and the ratio of the mean neuraminidase-inhibition to haemagglutination-inhibition antibody titres was at least ten-fold higher (0·54). Indeed for four of 15 convalescent serum samples the neuraminidase-inhibiting antibody titre was higher than the haemagglutination-inhibition antibody titre with A2/Singapore/1/57.

Table 4. *Persistence of neuraminidase-inhibiting antibody after human influenza A 2 infections*

Patient		Pre-infection serum*	Post-infection serum samples† 1st	2nd	3rd	4th
11/1	NI‡	<§	200	10	<	<
	HI	<	1280	160	120	80
	CF	<	> 320	> 80	> 80	⩾ 80
11/2	NI	<	250	<	<	<
	HI	<	1280	320	80	160
	CF	<	> 320	⩾ 80	⩾ 80	⩾ 80
15/1	NI	<	75	<	<	<
	HI	<	20	20	15	10
	CF	<	80	> 80	> 80	> 80
19/2	NI	<	150	20	<	<
	HI	<	480	640	320	320
	CF	<	⩾ 320	⩾ 80	⩾ 80	⩾ 80

* Pre-infection serum samples were obtained in June 1957. † 1st post infection serum samples were obtained in November 1957; 2nd in March 1958; 3rd in June 1958; 4th in June 1959. ‡ NI = Antibody titres detected in neuraminidase-inhibition tests using A2/Singapore/1/57 virus. HI = Haemagglutination-inhibition antibody titres using A2/Singapore/1/57 virus. CF = Antibody titres in complement-fixation tests with influenza A soluble antigen. § < denotes titres of less than 1/10.

Persistence of neuraminidase antibody after influenza infections

One interpretation of the above findings is that antibody to neuraminidase, as detected in neuraminidase-inhibition tests, persists for shorter periods of time after influenza A2 infection than does antibody to haemagglutinin. To test this possibility, serial serum samples obtained from four individuals at intervals after they were infected with influenza A2 in 1957 were tested for neuraminidase-inhibiting antibody (Table 4). For two of the four persons antibody could no longer be detected 5 months after infection and for two individuals it was present in much reduced titres. None of the four individuals had detectable antibody 9 months after infection. In two of the four individuals a decrease in haemagglutination-inhibiting antibody was detected in the first 5 months after infection but thereafter the antibody levels remained relatively constant over a 2-year period. In two individuals the haemagglutination-inhibiting antibody titres did not decrease significantly.

Table 5. *Specificity of the neuraminidase-inhibition antibody response in human influenza A 2 infections*

Human serum pair*	Haemagglutination-inhibiting antibody titres with		Neuraminidase-inhibiting antibody titres with influenza A2 neuraminidase from various sources:					
	A2/Singapore/1/57	A/turkey/Mass/65	Purified A2 enzyme	A/turkey/Mass/65 virus	FPV-A2 (R4) virus	A2/Sing/1/57 virus	A2/Eng/12/64 virus	A2/Eng/76/66 virus
3A *a*	480	<†	<	<	<	<	<	<
c	> 1280	<	300	750	200	250	50	100
5A *a*	30	<	10	40	20	30	5	5
c	1280	<	600	1200	500	500	150	75
16A *a*	10	<	<	<	<	<	<	<
c	640	<	1500	4500	700	3000	750	500
18C *a*	240	<	5	20	5	20	<	<
c	1280	<	200	750	250	350	75	25
20B *a*	48	<	<	<	<	<	<	<
c	960	<	100	120	50	50	350	400

* *a* denotes acute serum specimen, *c* denotes convalescent serum 2–4 weeks after onset of illness. † < denotes titres less than 1/10.

Table 6. *Serological relationships between the neuraminidases of various influenza A 2 virus strains and A/turkey/Massachusetts/65 in neuraminidase-inhibition tests using rabbit antisera*

Source of neuraminidase / Rabbit antisera ...	Anti purified A2/57 neuraminidase	Anti A2/Singapore/1/57	Anti A2/England/12/64	Anti A/turkey/Massachusetts/65	Anti A0/BEL
Purified A2 neuraminidase (source A2/57)	**3,000***	250	20	400	<
A2/Singapore/1/57 virus	2,500	**300**	30	350	<
A2/England/12/64 virus	1,500	<	**3,500**	40	<
A2/England/76/66 virus	1,680	<	**3,500**	20	<
A/turkey/Massachusetts/65 virus	10,000	120	800	**2,510**	<
A0/BEL virus	<	<	<	<	**3,000**
A1/FM1 virus	<	<	<	<	450

* Reciprocal of serum dilution producing 50 % inhibition of neuraminidase activity.

Specificity of neuraminidase antibody

It was important to establish that the neuraminidase-inhibiting activity detected in convalescent sera was due to antibody reacting specifically with neuraminidase. Neuraminidase-inhibition tests carried out with sera from hyperimmunized rabbits (Paniker, 1968; Easterday, Laver, Pereira & Schild, in preparation) have indicated that high titres of antibody to neuraminidase may produce apparent neutralization of neuraminidase when intact virus particles are used as source of neuraminidase. Such effects are probably dependent upon antibody to haemagglutinin coating the virus surface and producing steric hindrance of the access of substrate to the enzyme. Thus, a number of acute and convalescent human sera were retested against a purified enzyme preparation obtained by the electrophoretic separation of influenza A2 virus after sodium dodecyl-sulphate treatment. In addition, influenza A/turkey/Massachusetts/65 virus and FPV-A2 (R4) virus were used since these viruses are known to contain influenza A2 neuraminidase but not influenza A2 haemagglutinin. It was also of interest to compare neuraminidase-inhibiting antibody titres with A2 strains other than A2/Singapore/1/57.

In five serum-pairs tested against purified A2 neuraminidase and FPV-A2 (R4) the antibody rises observed were of the same order as those found using intact A2/Singapore/1/57 (Table 5). When A/turkey/Massachusetts/65 was used neuraminidase-inhibiting antibody titres in the convalescent sera were in some cases higher than with A2/Singapore/1/57 although there was no haemagglutination-inhibiting antibody for the turkey influenza virus in these sera. It was concluded from these results that the neuraminidase-inhibiting activity of the human convalescent sera was due to antibody directed against virus neuraminidase.

When virus strains A2/England/12/64 and A2/England/76/66 were used as sources of neuraminidase all five serum pairs showed significant rises in neuraminidase-inhibiting antibody. Four of the five convalescent sera had titres lower than those detected with A2/Singapore/1/57 and for the fifth convalescent serum the neuraminidase-inhibition antibody titres with the A2/64 and A2/66 viruses were higher than with the A2/57 virus.

Studies with rabbit antisera

The results obtained with the convalescent human sera suggested that the neuraminidases present in the A2/64 and A2/66 viruses might differ immunologically from that present in A2/Singapore/1/57. This possibility was investigated in tests with immune animal sera (Table 6). A rabbit antiserum prepared against purified A2/57 neuraminidase reacted with all A2 virus strains tested and with A/turkey/Massachusetts/65. However, rabbit antiserum prepared against intact A2/Singapore/1/57 virus reacted only with purified A2 neuraminidase and with the neuraminidases of intact A2/Singapore/1/57 and A/turkey/Massachusetts/65 virus but not with A2/England/12/64 or A2/England/76/66. The failure of the antiserum prepared against A2/Singapore/1/57 virus to react with the A2/64 and A2/66 viruses probably reflects the fact that this antiserum had a lower homologous neuraminidase-inhibition titre than did the antiserum against purified A2/57

neuraminidase. Antiserum against A/turkey/Massachusetts/65 virus reacted with higher titres to A2/Singapore/1/57 than with the A2/64 and A2/66 virus strains. Antiserum for A2/England/12/64 virus reacted to high titres with the neuramini-

Table 7. *Neuraminidase antibody response in man after immunization with killed influenza A2 virus*

Serum sample	Haemagglutination-inhibition antibody titre, A2/Singapore/1/57	Neuraminidase-inhibiting antibody titre using: A2/Singapore/1/57	Neuraminidase-inhibiting antibody titre using: A/turkey/Massachusetts/65
1 *a**	384	10	< 5
b	384	10	< 5
c	1536	50	30
4 *a*	12	< 5	< 5
b	768	120	75
c	1536	175	150
6 *a*	192	10	< 5
b	1536	30	50
c	1536	120	150
10 *a*	192	5	< 5
b	1536	35	30
c	1536	200	150
13 *a*	24	< 5	< 5
b	96	< 5	< 5
c	1536	< 5	< 5
17 *a*	48	< 5	< 5
b	1536	50	75
c	1536	150	200
21 *a*	96	< 5	< 5
b	1536	20	35
c	1536	20	50
3 *a*†	24	< 5	< 5
b	576	20	10
12 *a*	48	< 5	< 5
b	384	< 5	< 5
55 *a*	192	15	< 5
b	768	40	30
65 *a*	36	< 5	< 5
b	768	75	50
66 *a*	9	< 5	< 5
b	768	30	50
72 *a*	9	< 5	< 5
b	192	< 5	10

* Persons receiving drakeol-arlacel adjuvant vaccine containing 3500 haemagglutinating units of A2/Singapore/1/57 and A2/England/1/66 virus inactivated with 1/10,000 formalin. *a*, pre-immunization serum; *b*, 1 month post-immunization; *c*, 3 months post-immunization.

† Persons receiving A65 vegetable-oil adjuvant vaccine containing 3500 haemagglutinating units of A2/Singapore/1/57 inactivated with 1/10,000 formalin. *a*, Pre-immunization serum; *b*, 5–6 weeks post-immunization.

dase of the homologous virus and with A2/England/76/66 but poorly with A2/Singapore/1/57 neuraminidase.

These results indicate that amongst the influenza A2 viruses there are two immunologically distinguishable, but related types of neuraminidase. These are represented by the neuraminidases present in A2/Singapore/1/57 and in A2/England/12/64 virus. The neuraminidase of A/turkey/Massachusetts/65 seemed to resemble that of A2/Singapore/1/57 more than that of A2/England/12/64. This latter finding is in agreement with the conclusions of Pereira *et al.* (1967), who compared the turkey virus with human A2 strains in strain specific complement fixation tests.

No cross-reactions were detected between the neuraminidases of the A2 viruses and examples of the A0 or A1 subtypes of human influenza virus, although the neuraminidases of the A0 and A1 viruses were themselves immunologically related. Similar findings in regard to the cross-reactions between the neuraminidases of A0 and A1 virus have been reported (Paniker, 1968).

Neuraminidase antibody induced by vaccination

We wondered whether neuraminidase-inhibiting antibody appeared after immunization with killed influenza virus. The sera from individuals who had received a single dose of two different influenza virus vaccines were therefore titrated using A2/Singapore/1/57 and A/turkey/Massachusetts/65 (Table 7). One vaccine preparation contained formalin-inactivated influenza virus with vegetable oil (A65) adjuvant (Woodhour *et al.* 1964), the other contained a preparation of formalinized virus with mineral-oil adjuvant. Neuraminidase-inhibiting antibody was detected in only a proportion of individuals who received the vegetable-oil adjuvant vaccine and the titres were low although the same vaccines stimulated high titres of haemagglutination-inhibiting antibody. In individuals receiving the mineral-oil adjuvant vaccine the titres of neuraminidase-inhibiting antibody (and also of haemagglutination-inhibiting antibody) were higher than with the vegetable-oil adjuvant vaccine but not as high as those developing after natural influenza A2 infections.

DISCUSSION

The biological properties of antibody prepared specifically against influenza neuraminidase have been studied in *in vitro* systems by a number of workers. Such antibody has potent enzyme-inhibiting activity but it does not inhibit virus haemagglutination (Seto & Rott, 1966; Laver & Kilbourne, 1966; Webster & Laver 1967). Another activity of antineuraminidase antiserum is to reduce virus plaque size (Seto & Rott, 1966; Jahiel & Kilbourne, 1966) and to cause apparent virus neutralization by inhibiting virus release from infected cells (Webster & Laver 1967). Elution of virus after adsorption to erythrocytes is also inhibited (Brown & Laver, 1968). These biological properties of antineuraminidase antibody suggest that such antibody if stimulated by natural influenza infections, might play a role in the development of immunity to influenza in man.

In the present investigation 18 of 20 individuals studied developed neuramini-

dase-inhibiting antibody after natural influenza A2 infections. However, it should be emphasized that the paired sera used in the study were from individuals with clinical influenza in which the diagnosis was confirmed by the demonstration of significant antibody rises both in complement fixation tests with influenza A soluble antigen and in haemagglutination-inhibition tests. It is possible that the frequency and titre of neuraminidase-inhibiting antibody would have been lower had the study included individuals for which the serological evidence of infection was less complete, or if individuals with subclinical infections were included in the study.

The relevance of our findings in the development of immunity to influenza infections in man requires further study. A suitable experimental model might be the study of experimental influenza virus infections in animals specifically immunized against neuraminidase.

It is of interest that the studies with rabbit antisera indicated two immunologically distinguishable types of neuraminidase among the influenza A2 viruses which were compared. The strains A2/Singapore/1/57 and A2/England/12/64 are examples of viruses containing the two different types of enzyme. These findings suggest that antigenic variation among human influenza A virus strains of a single subtype may reflect changes in neuraminidase as well as in the haemagglutinin. However, both the studies of Paniker (1968) and those reported here indicate that the neuraminidase of the human influenza viruses of A0 and A1 subtypes are closely related immunologically although their haemagglutinins are different. It seems therefore that antigenic variation in neuraminidase and in haemagglutinin occur independently. The human convalescent sera were in general more broadly reactive than the immune rabbit sera and frequently these sera had similar neuraminidase-inhibition titres irrespective of whether A2/Singapore/1/57 or A2/England/12/64 was used as a source of neuraminidase.

The failure of neuraminidase-inhibiting antibody to persist longer than 5 months after infection in the four individuals from whom serial specimens were available is of interest. Before the significance of this finding can be determined further studies are required on the nature of neuraminidase-inhibiting antibodies in convalescent sera.

SUMMARY

1. High titres of neuraminidase-inhibiting antibody were detected in convalescent human sera following natural influenza A2 infections.

2. Such antibody was encountered infrequently in acute serum samples. Antibody persisted only 5–6 months after infection in the four individuals from whom serial serum specimens were available.

3. Following immunization with killed influenza virus vaccines (with adjuvant) neuraminidase inhibiting antibody was detected in human sera. The titres were in general lower than those detected in convalescent human sera.

4. The specificity of the neuraminidase-inhibiting antibody in human and animal antisera was studied. Tests with convalescent human sera using purified neuraminidase preparations and with a recombinant virus containing A2 neuraminidase

and haemagglutinin distinct from that of human influenza A viruses enabled the conclusion that the antibody detected was specific for influenza A 2 neuraminidase.

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REFERENCES

Bell, J. A., Ward, T. G., Kapikian, A. Z., Shelokov, A., Reichelderfer, T. E.&Huebner, R. J. (1957). Artificially induced Asian influenza in vaccinated and unvaccinated volunteers. *J. Am. med. Ass.* **165**, 1366.

Brown, J. & Laver, W. G. (1968). The effect of antineuraminidase antibody on the elution of influenza virus from cells. *J. gen. Virol.* **2**, 291.

Jahiel, R. I. & Kilbourne, E. D. (1966). Reduction in plaque size and reduction in plaque number as differing indices of influenza virus antibody reactions. *J. Bact.* **92**, 1521.

Kilbourne, E. D., Lief, F. S., Schulman, J. L., Jahiel, R. I. & Laver, W. G. (1967). Antigenic hybrids of influenza and their implications. *Perspectives in Virology* **5**, 87.

Laver, W. G. (1964). Structural studies on the protein subunits from three strains of influenza virus. *J. molec. Biol.* **9**, 109.

Laver, W. G. & Kilbourne, E. D. (1966). Identification in a recombinant virus of structural proteins derived from both parents. *Virology* **30**, 493.

Paniker, C. K. J. (1968). Serological relationships between the neuraminidases of influenza viruses. *J. gen. Virol.* **2**, 385.

Pereira, H. G., Lang, G., Olesiuk, O. M. Snoeyenbos, G. M., Roberts, D. H. & Easterday, B. C. (1966). New antigenic variants of avian influenza A viruses. *Bull. Wld Hlth Org.* **35**, 799.

Pereira, H. G., Tumova, B. & Webster, R. G. (1967). Antigenic relationship between influenza A viruses of human and avian origin. *Nature, Lond.* **215**, 982.

Seto, J. T. & Rott, R. (1966). Functional significance of sialidase during influenza virus multiplication. *Virology* **30**, 731.

Tumova, B. & Pereira, H. G. (1965). Genetic interaction between influenza A viruses of human and animal origin. *Virology* **27**, 253.

Tyrrell, D. A. J. (1967). Serological studies on infections by respiratory viruses of the inhabitants of Tristan da Cunha. *J. Hyg., Camb.* **65**, 327.

Webster, R. G. & Laver, W. G. (1967). Preparation and properties of antibody directed specifically against the neuraminidase of influenza A virus. *J. Immun.* **99**, 49.

Webster, R. G. & Pereira, H. G. (1968). A common surface antigen in influenza viruses from human and avian sources. *J. gen. Virol.* **3**, 201.

W.H.O. Expert Committee on Influenza (1953). *Tech. Rep. Ser. Wld Hlth Org.* no. 64.

Woodhour, A. F. Metzgar, D. P., Stim, T. B., Tytell, A. A. & Hillemann, M. R. (1964). New metabolizable immunologic adjuvant for human use. I. Development and animal immune response. *Proc. Soc. exp. Biol. Med.* **116**, 516.

J. Hyg., Camb. (1969), **67**, 367
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A minerals-modified glutamate medium for the enumeration of coliform organisms in water

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

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INTRODUCTION

In a previous paper (P.H.L.S., 1968) Gray's improved modification of Folpmers' medium (Gray, 1964) was recommended as a superior alternative to MacConkey broth for the detection of coliform organisms in water by the multiple-tube method. For the experiments described in that paper an Oxoid dehydrated version of Gray's medium was used. This differed somewhat in mineral composition from the original in order to prevent the phosphates of calcium, iron and magnesium forming a precipitate which was removed by filtration in Gray's original method. It was also found on analysis that the mineral salts remaining in solution, using Gray's method, varied widely on different occasions. To avoid this variation a medium was designed at the Metropolitan Water Board Laboratories (Windle Taylor, 1965–6) with a mineral composition more nearly resembling the average composition of the medium as prepared by Gray and which could be prepared in dehydrated form. The present paper describes the results obtained in ten laboratories in which this minerals-modified dehydrated medium was compared with the original Oxoid dehydrated medium and with liquid medium prepared by Gray.

The mineral composition of the glutamate media is given in Table 1. In the original Oxoid formula as used in the previous series of experiments, the iron and calcium were retained in solution by considerably lowering the phosphate content. This was undesirable since a low phosphate concentration could greatly reduce bacterial growth as well as buffering capacity. The maximum phosphate concentration found on analysis of the medium prepared by Gray's method was therefore incorporated in the new medium. Much more calcium than magnesium was lost by precipitation and in order to retain more than minimal calcium the lowest concentration of magnesium found on analysis (0·2 g./l. of $MgSO_4 . 7H_2O$) was used. The concentration of iron was considered to be more important than that of

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calcium, especially since many water samples contain considerable amounts of calcium. In preparing the dehydrated medium the maximum amount of iron that could be used without forming a precipitate was 0·02 g./l. of ferric citrate, a figure well within the upper range found on analysis. The highest concentration of calcium chloride that could then be added without precipitation occurring was 0·015 g./l. of $CaCl_2$.

Table 1. *Mineral composition of glutamate media (double strength, in g./l.)*

	Minerals originally added by Gray (1964)	Minerals remaining in solution after precipitation by Gray's method	Minerals incorporated in the minerals-modified medium	Minerals incorporated in original Oxoid dehydrated medium
K_2HPO_4	2	1·4–1·8	1·8	0·6
$MgSO_4.7H_2O$	0·4	0·2–0·4	0·2	0·2
Ferric citrate	0·2	0·001–0·05	0·02	0·2
$CaCl_2$	0·4	0·02–0·15	0·015	0·2

MATERIALS AND METHODS

Media

The complete double-strength minerals-modified dehydrated glutamate medium used in the current trials therefore had the following formula: L(+) glutamic acid sodium salt, 12·7 g.; lactose, 20 g.; L(−) cystine, 0·04 g.; L(−) aspartic acid, 0·048 g.; L(+) arginine monohydrochloride, 0·04 g.; thiamin (aneurin hydrochloride), 2 mg.; nicotinic acid, 2 mg.; pantothenic acid, 2 mg.; K_2HPO_4, 1·8 g.; $MgSO_4.7H_2O$, 0·2 g.; ferric ammonium citrate, 0·02 g.; $CaCl_2.2H_20$, 0·02 g.; sodium formate, 0·5 g.; bromcresol purple (1 % ethanolic solution), 2 ml; NH_4Cl, 5 g.; distilled water to 1000 ml.; pH after sterilization, 6·7. Ferric ammonium citrate was used instead of ferric citrate because of its greater ease of solution, the iron content of the two compounds being approximately the same. As in previous trials, the ammonium chloride was not included in the dehydrated product but was added when making up. The medium was sterilized either at 116° C. for 10 min. or in the steamer at 100° C. for 30 min. on three successive days. The pH falls on sterilization and the extent of the fall depends on the sterilization equipment used. If the same equipment and procedure are always used the pH change should be constant, so that the pH should be adjusted, if necessary, before sterilization to achieve a pH of 6·7 after sterilization.

The original Oxoid dehydrated glutamate medium was prepared in a similar manner with pH adjustment if necessary. Medium prepared by Gray's original method was distributed in bulk to each of the ten participating laboratories (nine Public Health Laboratories and the Metropolitan Water Board). The three glutamate media were also compared with MacConkey broth made from the same batch of Oxoid dehydrated medium as was used in the previous trials.

Water samples

A wide range of samples was used, similar to that used in the previous trials, including marginally chlorinated samples, using the method previously described (P.H.L.S., 1968).

Methods of recording results

All samples were set up using one 50 ml., five 10 ml., and five 1 ml. volumes with each of the four media. When necessary, samples were diluted in order to give some negative tubes in such a series. The statistical significance of differences in numbers of positive tubes is much more easily assessed using these quantities throughout. The tubes were incubated at 37° C. without prior warming. A simplified system of recording the amount of acid and gas after 18, 24 and 48 hr. was adopted; otherwise the procedure was as in the previous trial. At 18 and 24 hr., cultures were regarded as positive if they produced any amount of acid and gas, however small. At 48 hr. cultures were not regarded as positive unless there was sufficient gas to fill the concavity of the Durham tube.

RESULTS

Methods of comparison

Comparisons based on the numbers of positive tubes obtained in all laboratories are given in Table 2. This involves a bias in favour of laboratories which examined the greatest number of samples. Results from individual laboratories have therefore been discussed separately but detailed results have not been presented. As all positive results have been counted there is also a bias in favour of samples giving large numbers of positive tubes. Furthermore, although differences between the media are revealed there is no indication of the magnitude of these differences. To overcome these two factors, some results have also been compared in Table 3 on the basis of mean and median ratios. As it is important for the water bacteriologist to know the effect of a change of method on existing standards of bacterial quality and as these are normally applied to 48 hr. results, it is only the 48 hr. results that have been compared by mean and median ratios.

Comparison by numbers of tubes

From Table 2 it can readily be seen that the differences between the three glutamate media were much less than the differences between the glutamate media and MacConkey broth. Comparison of each pair of corresponding figures for coliform organisms and *Esch. coli* shows that at 18 hr. two of the glutamate media were superior to MacConkey broth for *Esch. coli* in unchlorinated water but all were inferior in the other comparisons. At 24 hr. all the glutamate media were superior to MacConkey broth in all comparisons, except for Gray's laboratory-prepared medium for coliform organisms in chlorinated samples. At 48 hr. all the glutamate media were superior to MacConkey broth in all comparisons.

Comparing the results given by glutamate media, one or other of the dehydrated media was superior to Gray's laboratory-prepared glutamate medium in all the

Table 2. *Comparison of glutamate media and MacConkey broth by numbers of positive tubes*

	Number of tubes yielding								
	False positive reactions			Coliform organisms			*Esch. coli*		
	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
	Unchlorinated samples (no. of tubes examined 2079)								
Oxoid MacConkey broth	17	37	100	625	806	1060	467	528	582
Original dehydrated glutamate medium	1	11	87	459	810	1180	408	686	791
Modified dehydrated glutamate medium	2	20	97	557	858	1175	503	707	764
Gray glutamate medium	2	17	81	538	848	1180	488	670	761
	Chlorinated samples (no. of tubes examined 715)								
Oxoid MacConkey broth	4	19	49	125	216	315	77	121	128
Original dehydrated glutamate medium	1	5	31	49	220	389	40	148	211
Modified dehydrated glutamate medium	0	1	37	59	223	395	39	144	203
Gray glutamate medium	0	3	44	44	202	361	22	127	165

comparisons, except one where results were equal. These results indicate that Gray's laboratory-prepared medium has no advantage over the dehydrated media.

Comparing the two dehydrated glutamate media, the modified medium was superior to the original dehydrated medium at 18 and 24 hr. in six out of eight comparisons, and was slightly inferior at 48 hr. in three out of four comparisons. The modified medium therefore gave earlier positive results and thus overcame the major disadvantage in the original dehydrated medium.

Variations between individual laboratories

The results so far quoted are from the sum of results for all ten laboratories. There was some variation between individual laboratories but detailed figures are not shown. The superior results with glutamate media in 18 hr. for *Esch. coli* in unchlorinated waters were obtained in four laboratories, and one laboratory obtained higher 18 hr. results for *Esch. coli* with all glutamate media than with MacConkey broth also in chlorinated waters. At only one laboratory was MacConkey broth at 24 hr. superior to all the glutamate media in all comparisons. In all laboratories one or more of the glutamate media were superior at 48 hr. to MacConkey broth in all comparisons.

The only laboratory in which Gray's laboratory-prepared medium differed markedly in performance from the general pattern was in Gray's own laboratory. He obtained better results with his medium than with the other glutamate media in all comparisons. He did not, however, examine any chlorinated samples.

Comparing the two dehydrated media, the 18 and 24 hr. results showed a remarkably similar pattern in all laboratories. One laboratory showed lower and one showed higher results at 18 hr. with the modified medium in all comparisons. All other laboratories had some results higher and some lower. The inter-laboratory variation was therefore not very great.

Comparison by ratios

In order to determine what difference in results may be expected from the use of the modified dehydrated glutamate medium, the mean and median ratios of the most probable number (MPN) results obtained with this medium to those obtained with MacConkey broth were determined (Table 3). These ratios confirm what has always been found with glutamate media, that the increased counts are due mainly to improved detection of *Esch. coli*.

Whether the difference is considered to be greater with chlorinated than with unchlorinated water depends on whether mean or median ratios are taken as the criterion. It must be emphasized, however, that these ratios indicate only the sort of results which may be expected. Individual ratios varied widely around these figures, and it must be borne in mind that when calculating MPN ratios, duplicate samples examined by identical methods could give a ratio of approximately 9:1 within the 95% confidence limits.

It is of interest to note, however, that the highest single ratio obtained in the whole series was 50:1 for an *Esch. coli* result on an unchlorinated sample, and the

highest count obtained with a corresponding negative result with MacConkey broth was 25/100 ml., again for an *Esch. coli* result on an unchlorinated sample.

The highest single inverse ratio was 1:14, also for an *Esch. coli* result on an unchlorinated water. The highest count given by MacConkey broth with a negative result in the corresponding modified glutamate medium was 5 *Esch. coli* per 100 ml. in a chlorinated sample.

Table 3. *Ratios of modified dehydrated glutamate results to MacConkey broth results in 48 hr.*

	Unchlorinated		Chlorinated	
	Coliform organisms	*Esch. coli*	Coliform organisms	*Esch. coli*
Mean ratio	2·0	3·2	1·5	2·9
Median ratio	1·0	2·7	1·2	5·0

Comparison between dehydrated and laboratory-prepared media of the same composition

At the Metropolitan Water Board Laboratories where the mineral modifications were formulated in conjunction with Oxoid Ltd., an additional series of comparisons was made with medium prepared from separate ingredients in the laboratory in the normal manner, having the same composition as the dehydrated minerals-modified glutamate medium. The results, based on numbers of positive tubes, are presented in Table 4. This shows that the general pattern of results compared with MacConkey broth was similar to the complete series for all laboratories given in Table 2. It also shows that there was very little difference between the results with the dehydrated and laboratory-prepared media. This confirms that the process of manufacturing the dehydrated medium did not produce any adverse effect.

DISCUSSION

These trials confirm the conclusions reached in the previous report (P.H.L.S., 1968) that Gray's glutamate medium is superior to MacConkey broth for the detection of coliform organisms in water, and that the difference between them increases with incubation time up to 48 hr. One disadvantage of glutamate media has been that growth is sometimes slower than in MacConkey broth, so that incubation for 24 hr. is required to achieve equivalent or better results. Although the 48 hr. results with the minerals-modified medium were slightly lower than with the original dehydrated medium, they were still significantly higher than results with MacConkey broth, which was the previously accepted standard. The differences in favour of the minerals-modified medium at 18 and 24 hr. were generally greater than those in favour of the original dehydrated medium at 48 hr. As under most circumstances early results are important so that remedial action can be taken quickly, the minerals-modified glutamate medium described in this paper is recommended. The results show that the differences between these

Table 4. *Comparison of dehydrated and laboratory-prepared minerals-modified glutamate*

	Numbers of tubes yielding								
	False positive reactions			Coliform organisms			*Esch. coli*		
	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	24 hr.	18 hr.	24 hr.	48 hr.
	Unchlorinated samples (no. of tubes examined 187)								
Oxoid MacConkey broth	1	3	13	107	124	138	85	87	87
Modified dehydrated glutamate medium	0	1	2	99	127	141	97	106	107
Modified laboratory-prepared glutamate medium	0	0	0	102	124	146	94	102	105
	Chlorinated samples (no. of tubes examined 187)								
Oxoid MacConkey broth	1	6	12	8	24	42	7	13	15
Modified dehydrated glutamate medium	0	0	5	0	23	61	0	20	39
Modified laboratory-prepared glutamate medium	0	0	1	0	29	61	0	27	42

modifications in glutamate media are very small compared with the difference between any one of them and MacConkey broth. It is interesting to note that the minerals-modified medium is closer in composition to the medium originally described by Gray.

The recommendation in this paper applies solely to the use of glutamate medium for enumeration of coliform organisms in water. The conclusions reached must not be interpreted as being applicable to milk or foods. The selectivity of the medium depends on the use of nutrients with a limited availability. It contains no inhibitory substances. The introduction of additional non-selective nutrients with the sample could be expected to change its growth characteristics.

SUMMARY

Oxoid dehydrated MacConkey broth was compared with laboratory-prepared Gray's improved formate lactose glutamate medium and with two dehydrated versions of it, by participants in ten laboratories. A variety of chlorinated and unchlorinated water samples was used. The superiority of the glutamate media over MacConkey broth for the detection of coliform organisms in water was again confirmed. The minerals-modified version, either dehydrated or laboratory-prepared, gave the best results and is the medium recommended for use in water examination. Care should be taken to ensure that the final pH after sterilization is 6·7.

REFERENCES

GRAY, R. D. (1964). An improved formate lactose glutamate medium for the detection of *Escherichia coli* and other coliform organisms in water. *J. Hyg. Camb.* **62**, 495–508.

PUBLIC HEALTH LABORATORY SERVICE STANDING COMMITTEE ON THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES (1968). Comparison of MacConkey broth, Teepol broth and glutamic acid media for the enumeration of coliform organisms in water. *J. Hyg., Camb.* **66**, 67–82.

WINDLE TAYLOR, E. (1965–6). Progress with glutamic acid media. *Rep. Results chem. bact. Exam. Lond. Wat.* **42**, 11–14.

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