J. Hyg., Camb. (1964), **62**, 127
Printed in Great Britain

Influenza haemagglutination-inhibition antibodies in Sydney

By Y. E. COSSART*
Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Sydney, Australia

(*Received* 15 *December* 1962)

Virus strains have been isolated from each major outbreak of influenza since 1933, and it has been found that group A strains have been responsible for almost all widespread epidemics. A sequence of variants has been obtained, each strain causing clinical influenza in many countries simultaneously, then disappearing with the emergence of the next strain. The strains fall into three subgroups; classical A strains persisted from the initial isolation of the strain PR8 until the first A′ strains emerged in 1946, A′ strains were then obtained until the A_2 (Asian) pandemic in 1957, and A_2 strains are still being isolated. A few representative strains are shown in Table 1.

Table 1. *Representative strains of influenza A*

Group	Type	Year isolated	Country of origin
A	PR8	1934	Puerto Rico
A′	CAM	1946	Australia
	FM1	1947	U.S.A.
	Ned/36	1956	Netherlands
A_2	Jap 305	1957	Japan
	PAR	1957	Singapore

Influenza B, on the other hand, gives rise to localized outbreaks of infection, exhibits less antigenic variation and, on the whole, behaves as an endemic pathogen. A and B groups are distinguished serologically on the basis of a soluble complement-fixing antigen. The type-specific antigens, however, are closely bound to the virus particle and are most easily detected by the haemagglutination-inhibition technique (von Magnus, 1954).

Measurement of the haemagglutination-inhibition (HI) titres in normal subjects in the United States between 1943 and 1951 (Hilleman, Werner & Gauld, 1954) showed that, at least since 1943, almost every individual had antibodies to influenza A, and that high titres persisted even in 1951, although significant isolations of this strain had not been obtained since about 1946. Influenza A′ antibodies were present in an increasing proportion of the population between 1946 and 1951, and the average titre was also rising.

* Present address: Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9.

If the antibody levels of different age groups be determined, it is found that young children have high levels only to the current strain, while adults maintain high levels to the strain prevalent in their youth, irrespective of their reaction to the current strain (Davenport & Hennessy, 1956; Hilleman *et al.* 1958). The response to infection or to vaccination with monovalent vaccines shows that, while all age groups give the greatest response to the infecting or vaccinating strain, infants and young children show little or no heterologous antibody response. Adults show a response to several strains, the heterologous rise being greatest for the strains they first encountered (Hennessy & Davenport, 1958; Culver, Lennette, Navarre & Donahue, 1958; Lief & Henle, 1960). These considerations have led Davenport, Hennessy & Francis (1953) to advance the theory that, in his heterologous antibody response to a new stimulus with influenza A, an individual recapitulates his past experience with A antigens.

After vaccination the increases induced in the levels of both homologous and heterologous antibody may be detected for about 2 or 3 years (Davis *et al.* 1961). After natural infection, antibodies probably persist for a similar period of time and this is thought to explain the 2-year cycles of influenza epidemics (Jensen, Dunn & Robinson, 1958). The maintenance of persistently high titres of A antibody long after the disappearance of the strain may be attributed to recurrent stimulation with the antigenic components common to both A and A′ strains. It should be noted, however, that experiments with guinea-pigs have shown that, in these animals at least, continued exposure to monovalent influenza vaccine gives rise to a broadening of the serological response so that heterologous antibodies may be detected (Robinson, 1961).

The A_2 (Asian) strains are distinct from the A′ group. This is shown by the lack of protection afforded by A′ vaccination against infection with A_2, by the failure of A or A′ antibody levels to rise in response to infection or vaccination with A_2 strains (Hobson & Pearson, 1961), by the great and irregular sensitivity of the virus to non-specific inhibitor, and by the irregular antibody response elicited by the virus strains (PQR variation) (Levy & Wagner, 1958).

It was therefore thought relevant to follow the changes in antibody pattern elicited in a general community following the introduction of the new A_2 strain in 1957.

Three different groups of healthy adult subjects were studied: the first in February 1958, the second in April 1961, and the third in October 1961. Influenza of the A_2 type first appeared in Sydney during July 1957, when a large number of clinical cases was seen, and several A_2 strains were isolated in this laboratory. A second wave of influenza was seen in 1959, and A_2 strains were again isolated. No widespread outbreak has since occurred in Sydney, although an A_2 strain was obtained from a fatal case which occurred in a country boarding school during April 1961.

MATERIALS AND METHODS

Sera

Sera were obtained through the courtesy of the New South Wales Red Cross Blood Transfusion Service, from consecutive blood donors in February 1958, April 1961, and October 1961. They were stored at −20° C. for up to 2 months before testing. Immediately before use they were inactivated at 56° C. for 30 min. and then periodated (Burnet & Lind, 1954).

Antigens

The antigens were manufactured from pooled allantoic fluids according to the directions of the W.H.O. Expert Committee on Influenza (W.H.O. Technical Report Series, 1953). The strains used were PR 8, FM 1, and Lee (W.H.O. Reference Strains) and PAR, an A_2 strain which was isolated in Singapore in 1957, and kindly made available by Dr E. L. French, Walter and Eliza Hall Institute, Melbourne. The same pools were used throughout, and positive control sera included with each batch of tests gave constant readings over the entire period.

Haemagglutination-inhibition tests

HI tests were performed in plastic plates, using a dropping technique. Each antigen was used at a strength of four minimal haemagglutinating doses in a volume of 0·2 ml. Each serum was used in doubling dilutions from 1/5 to 1/2560, in volumes of 0·2 ml.

A 1 % suspension of triple-washed erythrocytes drawn from a young capon was employed, and 0·85 % sodium chloride was used as the diluent.

The tests were performed thus: Doubling dilutions of the sera were made directly in the plates, in 0·2 ml. volumes, 0·2 ml. antigen was added, and mixed by tapping the sides of the plates, which were then covered and incubated at 37° C. for 1 hr. Then 0·4 ml. of the fowl erythrocyte suspension was added to each cup, and the contents again mixed. The red cells were allowed to settle at room temperature, and the results read at 30 min. The end-point was taken as 50 % inhibition of haemagglutination. Saline, antigen and positive serum controls were included with each batch.

RESULTS

Table 2 gives the actual values obtained. Some difficulty was experienced in the statistical analysis of these results since some of the individual series were badly skewed. The 'Maximum Likelihood' method of Tallis & Young (1962) was followed for the 1958 and April 1961 series. The means and variances so obtained are given in Table 3.

DISCUSSION

A_2 *antibody*

In 1958 8 % of persons showed some haemagglutination-inhibiting antibody to the A_2 strain. Compared with the results of other surveys, this value is low for the post-epidemic period. For example, Versteeg (1961) found 17 % of healthy persons

had antibodies to A_2 3 weeks after the height of the epidemic in Holland, while 85% of persons with clinical respiratory disease during the outbreak yielded A_2 antibodies after the 3 weeks interval. Similarly Jensen *et al.* (1958) found that, 3 months after the epidemic, 50% of a group of American high school students who had not been ill showed HI titres to A_2 of 1/10 or more.

Table 2. *Results of haemagglutination-inhibition tests*

Titre	1/5 or less	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280 or higher	Total
					Influenza A (PR8)					
1958	5	0	3	11	21	25	17	15	3	100
April 1961	2	5	14	22	27	15	4	4	1	93
October 1961	1	8	16	29	18	17	1	6	4	100
					Influenza A′ (FM1)					
1958	4	1	7	24	35	15	6	6	2	100
April 1961	6	7	18	22	18	16	3	3	0	93
October 1961	1	14	29	36	11	8	1	0	0	100
					Influenza A_2 (PAR)					
1958	92	1	6	1	0	0	0	0	0	100
April 1961	38	13	23	10	3	1	2	2	1	93
October 1961	16	13	19	23	4	11	8	1	5	100
					Influenza B (Lee)					
1958	0	0	3	26	25	32	13	1	0	100
April 1961	52	24	12	2	1	1	1	0	0	93
October 1961	91	8	1	0	0	0	0	0	0	100

Table 3. *Statistical analysis of results*

Strain	Mean	Variance	Square of standard deviation	Mean ± 2 × standard error
PR8 (1958)	215·7	1183·826674	180873·51	216 ± 68·8
PR8 (1961A)	77·59	116·950666	12729·7919	78 ± 21·6
FM1 (1958)	100·6	208·872453	23819·64	101 ± 28·8
FM1 (1961A)	65·21	115·113583	12557·6559	65 ± 21·4
PAR (1958)	1·840	0·24456112	27·5544	1·8 ± 1·0
PAR (1961A)	25·72	15·186286	6283·4816	26 ± 7·8
Lee (1958)	91·33	65·833391	6238·8311	91 ± 16·4
Lee (1961A)	8·067	1·825152079	192·723511	8·1 ± 2·6

However, Hilleman *et al.* (1958) found only 2·7% of persons with A_2 HI antibody titres greater than 1/5 6–12 months after the initial outbreak. The low values obtained in the present study might be attributed to a low attack rate, but there is no evidence to support this suggestion. The local strains may, however, have been poor antibody producers since A_2 strains show very marked PQR variation, and some strains isolated in Sydney at this time were in the Q phase.

Another factor which may influence the antibody titres obtained is the avidity of the testing strain for antibody. Different A_2 strains have been shown (Fukumi, 1959) to give widely different HI titres with individual sera. In the present study

the same antigen pools were used for all the tests, so this difficulty has been avoided for the purpose of comparing the 2 years.

In 1961, a little over 3 years after the pandemic, both the percentage of people with antibodies and the mean titre have increased, and the difference is highly significant. This means that the A_2 strain has persisted in the community to supersede A′ as the current strain, and may be contrasted with the observations of Versteeg (1961) who found that in Holland in 1960 only 16 % of hospital admissions showed A_2 antibodies.

Heterologous response

During the period of study both the A and A′ HI levels have fallen by a small but statistically significant amount. This is different in principle from the heterologous stimulation of A antibody reported during A′ outbreaks. This may be interpreted to mean that, while the group-specific soluble antigen is shared by A, A′ and A_2 strains, A and A′ share common type-specific antigen(s) not possessed by A_2. However, antigenic analysis of strains by the use of adsorption techniques on doubly immunized ferrets (Harboe, 1960) demonstrates some relationship between A′ and A_2 strains.

The results of surveying the healthy adult population may be compared with those obtained by Hobson & Pearson (1961) who studied the heterologous A and A′ response produced by infection and by monovalent vaccination with A_2. They also found little evidence of the recall effect previously described in response to stimulus with influenza antigens. It may be that immunological memory of the A and A′ epidemic is now fading and may disappear as completely as traces of the 1918 pandemics have vanished.

B *antibody*

Both the average titre and the percentage of persons with influenza B (Lee) HI antibodies in 1958 correspond to the levels recorded in other communities (Brown, Oligschlaeger, Legier & Schmidt, 1960; Hilleman *et al.* 1958). However, the decline observed in Sydney between 1958 and 1961 is striking and contrasts with the findings of these other observers.

The 3-year period studied is roughly similar to the time suggested for the disappearance of A antibody after infection and vaccination. The absence of any evidence of stimulation of B antibodies during this time is in striking contrast to the A_2 findings and it can be inferred that influenza B strains have been absent from the community for at least 3 years.

Further, this disappearance of B antibodies may indicate susceptibility of the community to an epidemic of influenza B during the coming winter. It is interesting that *The Lancet* of 20 January 1962 reported a widespread outbreak of influenza B in England and Wales during the preceding week.

SUMMARY

Haemagglutination-inhibiting antibodies against A, A′ and A_2 strains of influenza virus and against one strain of influenza B were estimated in sera from blood donors in Sydney in February 1958 and in April and October 1961.

The A_2 antibody increased substantially while both A and A′ antibodies declined slightly. There was a great decline in influenza B antibody during the same period.

Thanks are due to Dr Phyllis M. Rountree who suggested this study. Miss Patricia Taylor gave excellent technical assistance in the early part of this work.

REFERENCES

BROWN, G., OLIGSCHLAEGER, D., LEGIER, M. & SCHMIDT, R. (1960). Studies of the epidemiology of influenza as demonstrated by serum pools. *Arch. int. Med.* **106**, 496.

BURNET, F. M. & LIND, P. E. (1954). Recombination of influenza viruses in the de-embryonated egg. *Aust. J. exp. Biol. med. Sci.* **32**, 145.

CULVER, J., LENNETTE, E., NAVARRE, G. & DONAHUE, G. (1958). The recall phenomenon in the antibody response to influenza vaccines. *J. Immunol.* **81**, 452.

DAVENPORT, F. M. & HENNESSY, A. V. (1956). A serologic recapitulation of past experience with influenza A: antibody response to monovalent vaccine. *J. exp. Med.* **103**, 85.

DAVENPORT, F. M., HENNESSY, A. V. & FRANCIS, T. J. (1953). Epidemic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. *J. exp. Med.* **98**, 641.

DAVIS, D. J., PHILIP, R. N., BELL, J. A., VOGEL, J. E. & JENSEN, D. V. (1961). Epidemiologic studies on influenza in familial and general population groups, 1951–1956. III. Laboratory observation. *Amer. J. Hyg.* **73**, 138.

FUKUMI, H. (1959). Studies on the P–Q phase of A/Asia/57 influenza virus. *Bull. Wld Hlth Org.* **20**, 421.

HARBOE, A. (1960). Heterologous antibody absorption of sera from ferrets cross infected with influenza virus of the A_2-type and other A-types. *Acta path. microbiol. scand.* **49**, 493–8.

HENNESSY, A. V. & DAVENPORT, F. J. (1958). Epidemiologic implication of the distribution by age of antibody response to experimental influenza virus vaccine. *Immunology*, **80**, 114.

HILLEMAN, M., FLATLEY, F., ANDERSON, D., LEVEKING, M. & LEVINSON, D. (1958). Distribution and significance of Asian and other influenza antibodies in the human population. *New Engl. J. Med.* **258**, 969.

HILLEMAN, M., WERNER, J. & GAULD, R. (1954). Influenza antibodies in the population of the U.S.A. *Wld Hlth Org. Monogr. Ser.* No. 20, p. 25.

HOBSON, N. W. & PEARSON, E. (1961). Serological response to Asian influenza virus in man—a comparison of haemagglutination-inhibition and complement-fixing methods. *Brit. J. exp. Path.* **42**, 53.

JENSEN, K. E., DUNN, F. L. & ROBINSON, R. O. (1958). Influenza 1957. A variant and the pandemic. *Progr. med. Virol.* **1**, 165.

Lancet (1962). i, 168.

LEVY, A. H. & WAGNER, R. R. (1958). Increased avidity of antibody for Baltimore 1957 strains of Asian influenza virus (P–Q–R variation). *Proc. Soc. exp. Biol., N.Y.*, **98**, 357.

LIEF, F. S. & HENLE, W. (1960). Antigenic analysis of influenza virus by complement-fixation test. IV. Implication of age distribution of antibodies to heterologous strains following infection and vaccination. *J. Immunol.* **85**, 494.

ROBINSON, R. O. (1961). Strain characterization of Asian influenza virus. *Amer. J. resp. Dis.* **83**, 105.

TALLIS, G. M. & YOUNG, S. S. Y. (1962). Maximum likelihood estimation of parameters of the normal, log-normal, truncated normal and bi-variate distribution from grouped data. *Aust. J. Statistics*, **4**, 49.

VERSTEEG, J. (1961). Some observations on antibody formation in influenza A_2 (A/Asian) virus infection. *Ned. Tijdschr. Geneesk.* **105**, 980.

VON MAGNUS, P. (1954). The influenza virus—its morphology, immunology and kinetics of multiplication. *Wld Hlth Org. Monogr. Ser.* No. 20, p. 55.

W.H.O. EXPERT COMMITTEE ON INFLUENZA (1953). *Tech. Rep. Wld Hlth Org.* No. 64.

J. Hyg., Camb. (1964), **62**, 133
Printed in Great Britain

The use of ceiling temperature and reactivation in the isolation of pox virus hybrids

By K. R. DUMBELL and H. S. BEDSON
Department of Bacteriology, University of Liverpool

(*Received* 19 *November* 1963)

INTRODUCTION

Genetic recombination among the mammalian pox viruses was first described in 1958 when it was observed by Fenner and Comben in a study of mixed infection with two different strains of vaccinia. It has since been demonstrated between the white mutants of rabbit pox (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960) and between a number of different viruses of the variola–vaccinia group (Woodroofe & Fenner, 1960). In the latter instance where different viruses and not separate strains of the same virus were involved, it was suggested that the new viruses produced should be called *hybrids* rather than recombinants.

In this paper we describe a method for the clonal isolation of hybrid pox viruses which differs from those used previously. Our method introduces some degree of selection, though we have not achieved a selective system in which the growth of both parental types is suppressed. The novel feature of our method is that it employs a temperature of incubation which has been raised sufficiently to inhibit one parental component while yet permitting the growth of the other parental component. It is based on the observation that viruses of the variola–vaccinia group differ in the temperatures at which their growth on the chick chorioallantois (C.A.M.) is inhibited (Bedson & Dumbell, 1961). The maximum temperature at which lesions are produced (ceiling temperature) is a constant character of each of the viruses tested. We are concerned here with two properties of virus inhibited by raised temperature. The inhibited virus will grow again if the temperature is lowered, and, in its inhibited state, the virus behaves as a 'reactivating agent' (Joklik, Woodroofe, Holmes & Fenner, 1960).

We propose to describe, as 'heat-tethered', virus which, although not inactivated, has been prevented from growth on the C.A.M. by a raised temperature of incubation. This will avoid confusion when describing experiments which also involve virus which has been inactivated by heating at 55° C. *in vitro*. The term 'heat-tethered' connotes restraint and reversibility and is appropriate to virus in this particular condition.

The preliminary data on heat-tethered virus are given in the first part of this paper. There follows a short account of the use of reactivation with heat-tethered virus to obtain clones of presumptive hybrids. Alastrim has been crossed with rabbit pox virus, and variola major with cowpox virus. The detailed characters of these hybrid viruses are given in the two accompanying papers (Bedson & Dumbell, 1964*a*, *b*).

MATERIAL AND METHODS

Virus strains

The viruses used were the international reference strains of alastrim (Butler), variola major (Harvey), cowpox (Brighton) and rabbit pox (Utrecht) which were listed by Fenner & Burnet (1957). In addition to these, a few experiments were made with vaccinia (Connaught Labs.) and with white variants of the strains of cowpox and rabbit pox mentioned above. The characters of these additional viruses are given by Fenner (1958).

Eggs

Inoculations were made on the C.A.M.S of 12-day chick embryos, prepared for inoculation by the technique of McCarthy & Dumbell (1961). After inoculation the eggs were incubated at the required temperatures as previously described (Bedson & Dumbell, 1961).

Virus suspensions

Heavily infected C.A.M.S were harvested after 3 days' incubation at 35° C., shaken with glass beads in McIlvaine's phosphate–citrate buffer (0·004 M-phosphate, pH 7·2) and centrifuged at 2000 r.p.m. to deposit cellular debris. The supernatant was mixed with an equal volume of sterile glycerol and stored at − 20° C. for use as stock suspension.

Heat-inactivated virus suspensions

The low-speed supernatant of an extract of virus-infected C.A.M.S was centrifuged at 8000 r.p.m. for 30 min. in a Spinco Model L centrifuge (Rotor No. 40) to deposit the virus. The pellet was resuspended in 0·1 M-sodium chloride in McIlvaine's phosphate–citrate buffer (0·01 M phosphate pH 7·2). This suspension was sealed in a thin glass ampoule and completely immersed in a water bath at 55° C. for $2\frac{1}{2}$ hr. to inactivate the virus. The inactivation was done on the same day that the suspension was prepared (Woodroofe, 1960). The suspension was tested after heating to ensure that no residual active virus was present. Two successive passes on the C.A.M. at 35° C. produced no lesions (Joklik *et al.* 1960).

Isolation of virus clones

The methods used to prepare clones of virus were essentially those of Fenner (1959). Initial mixtures of viruses were inoculated on the C.A.M. at a dilution giving discrete pocks. These pocks were sampled with finely drawn Pasteur pipettes and the material thus obtained was again inoculated on the C.A.M. at 35° C. at a dilution to give not more than 30 pocks. If these latter pocks were all alike in appearance, a single pock was excised, extracted with buffer and inoculated on the C.A.M. of 2–3 eggs at 35° C. Stock suspensions in 50 % glycerol buffer were prepared from these C.A.M.S.

Reversibility

PROPERTIES OF HEAT-TETHERED VIRUS

Alastrim virus produces no lesions on the C.A.M. of eggs incubated at or above 38° C. (Bedson & Dumbell, 1961). This inhibition is removed if, 16 hr. after inoculation, the temperature is lowered to 35° C. Growth curves have been determined for alastrim virus in eggs incubated at 35, 38·5 and 40° C., and also in eggs incubated at 38·5° C. for the first 16 hr. after inoculation and then transferred to 35° C. In these experiments the inoculum for each egg was 10^3 pock-forming units (p.f.u.) and three membranes were harvested for each sample. The results are plotted in Fig. 1. There was no evidence of progressive multiplication at either 38·5 or 40° C. In the controls at 35° C., an exponential increase in titratable virus was found from the 10th hr. onwards. When eggs were transferred from 38·5 to 35° C. after 16 hr. incubation at the higher temperature, virus almost immediately began to increase at approximately the same rate as in the controls.

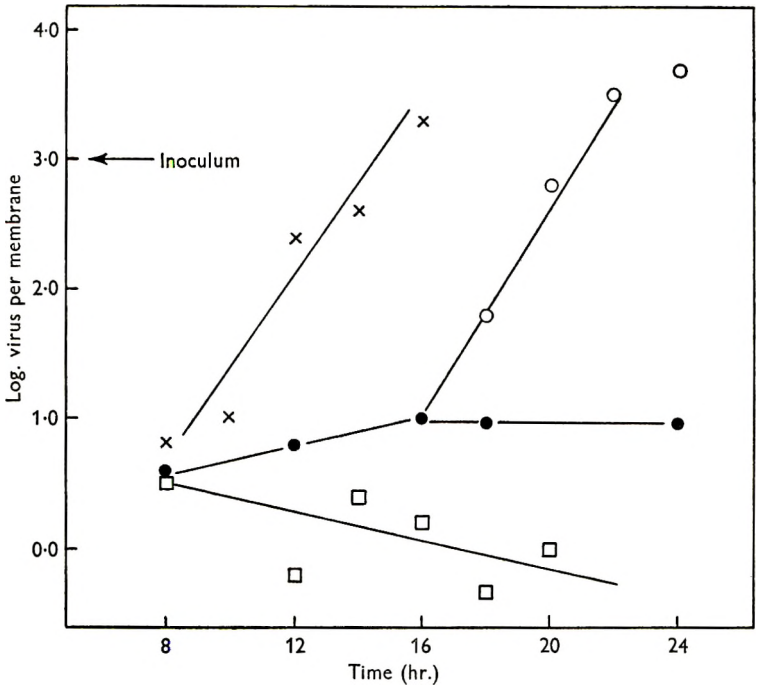


Fig. 1. Growth of alastrim virus in chick chorioallantois at 35° C. (×—×), at 38·5° C. (●—●), at 40° C. (□—□) and at 35° C. after 16 hr. at 38·5° C. (○—○).

Experiments were also made with variola major virus. Hahon, Ratner & Kozikowski (1958) had shown that the multiplication of variola major in the C.A.M. was reduced at 37° C. and inhibited at 39° C. We have found that variola major does not multiply in the C.A.M. at 39·5° C. If eggs were incubated at this temperature for 16 or 18 hr. after inoculation and then transferred to 35° C., a rapid increase in virus began almost immediately.

The inhibition imposed on the growth of variola minor virus at 38·5° C., and on variola major at 39·5° C., thus appears to be reversible, at least within certain limits. The reversibility of the inhibition shows that the growth potential of the inhibited virus was not seriously affected by the amount of restraint imposed in these experiments.

The short interval between the lowering of an inhibitory incubator temperature and the increase of titratable virus suggested that the inhibition occurred at a fairly late stage in the intracellular cycle of virus development. Reactivation is associated with early stages in the virus growth cycle (Fenner, 1962; Joklik, 1962). This encouraged us to hope that heat-tethered virus might behave as a reactivating agent.

Reactivation by heat-tethered virus

In the first series of experiments the Utrecht strain of rabbit pox was chosen as the virus to be reactivated, because it had the highest ceiling temperature of those tested by Bedson & Dumbell (1961).

A heat-inactivated suspension of rabbit pox was prepared as described above. Eggs were inoculated with mixtures of the heated rabbit pox suspension and 10^3–$10^{4\cdot5}$ p.f.u. of active alastrim, variola major or cowpox. The eggs were incubated above the ceiling temperature of the active virus chosen. Controls showed that none of the active viruses produced lesions on the C.A.M. when used at the same dose and temperature as in the mixed inoculations. Every mixture tested produced lesions, most of which resembled those produced by rabbit pox. A small proportion of atypical lesions was found; these will be discussed later. The results of these experiments are set out in Table 1A. A second series of experiments, listed in Table 1B, showed that heat-tethered alastrim virus reactivated heated suspensions of vaccinia, cowpox and variola major, and also white variants of cowpox and rabbit pox. These heated suspensions were prepared in a manner similar to that used for the rabbit pox suspension already mentioned. All were tested by serial passage on the chick C.A.M. and produced no lesions.

Heat-tethered virus may be used to titrate heat-inactivated suspensions. Table 1C illustrates the linear increase in pock count as the concentration of heat-inactivated virus is raised. An advantage of the use of heat-tethered virus in studies on reactivation is that there are no background pocks, so that all pocks that appear have arisen following reactivation. In genetic work this means that one parent is apparently inhibited at a late stage in its developmental cycle. The intracellular growth of the other parent is possible only after it has been reactivated by the heat-tethered parent. The virus progeny from such a cell, and later from the resulting pock, might contain a proportion of hybrids. A higher proportion of hybrid viruses could be expected from pocks whose appearance was unlike that of either parent virus. This supposition was put to practical test in the experiments now to be described.

Table 1. *The reactivation of heated pox virus suspensions by heat-tethered pox viruses*

	Inactivated virus			Active virus				
	Virus	log p.f.u. per egg*	Ceiling temperature† (° C.)	Virus	log p.f.u. per egg	Ceiling temperature† (° C.)	Temperature of incubation‡ (° C.)	Average no. reactivated pocks§
A	Rabbit pox	7·0	> 41	Alastrim	3·0	37·5	38·5	89
	Rabbit pox	7·0	.	Alastrim	3·0	37·5	40	5
	Rabbit pox	7·0	.	Variola major	3·0	38·5	40	70
	Rabbit pox	7·0	.	Cowpox	4·5	40	41·3	10
B	Rabbit pox W.	6·7	> 41	Alastrim	4·0	37·5	38·5	100
	Vaccinia	6·6	41	Alastrim	3·0	.	38·5	51
	Cowpox	6·0	40	Alastrim	5·0	.	38·5	15
	Cowpox W.	5·7	40	Alastrim	4·0	.	38·5	34
	Variola major	6·7	38·5	Alastrim	5·0	.	38·5	3
C	Vaccinia	4·1	41	Alastrim	4·5	37·5	38·5	9
	Vaccinia	4·4	.	Alastrim	4·5	.	38·5	16
	Vaccinia	4·7	.	Alastrim	4·5	.	38·5	33
	Vaccinia	5·0	.	Alastrim	4·5	.	38·5	64

* Titre of heat-inactivated virus given as the equivalent number of pock-forming units before inactivation.
† Data from Bedson & Dumbell (1961) and unpublished data.
‡ Incubator temperature controlled to ± 0·25° C.
§ Pock count given as arithmetic mean of total counts on 3–6 membranes.

THE PRODUCTION OF HYBRID STRAINS

Crosses between alastrim and rabbit pox

Eggs were inoculated on the C.A.M. with mixtures of $10^{3\cdot4}$ p.f.u. of active alastrim virus (ceiling temperature, 37·5° C.) and heat-inactivated rabbit pox (ceiling temperature > 41° C.) equivalent to 10^6 p.f.u. before inactivation. The eggs were incubated at 40° C. for 3 days and then examined. There were altogether 32 pocks, of which 20 looked like rabbit pox and 12 were white. The white pocks were themselves not uniform in appearance. Some were ulcerated and others smaller, whiter and domed. Material from two white pocks, one of each kind, was sampled. The material from the ulcerated pock was passed on eggs incubated at 38·5° C. The resulting pocks were well separated and clones were developed from three of them. These were labelled AR1, AR2 and AR3. Material from the non-ulcerated pock was passed at 35 and 38·5° C. Three clones, AR4, AR5 and AR6, were developed from pocks in the eggs incubated at 35° C. One clone, AR7, was grown from a pock appearing at 38·5° C. The properties of these seven clones will be described in a subsequent paper (Bedson & Dumbell, 1964*a*).

Crosses between variola major and cowpox

Active variola major virus (C.T. 38·5° C.) was used to reactivate heat-inactivated cowpox virus (C.T. 40° C.). This was carried out on the C.A.M. of eggs incubated at 39·5 °C.

Several eggs were inoculated with approximately 10^4 p.f.u. of variola virus and heated cowpox, equivalent to 10^6 p.f.u. before inactivation. After incubation at 39·5° C. for 3 days, there were about 20–40 pocks on each membrane. These varied in appearance and were almost all unlike those of either cowpox or variola major; the majority were white and ulcerated and some had a small central area of haemorrhage. Material was collected from six of the pocks for the isolation of virus clones. During the preparation of these, it became apparent that at least five of the six pocks had originally contained a mixture of viruses. From the six samples, 16 clones of virus were prepared. These were selected on the basis of their pock appearance. Two had haemorrhagic, ulcerated pocks, like those of cowpox, but the remaining 14 were chosen because their pocks differed from those of cowpox. The properties of these clones will be described in a subsequent paper (Bedson & Dumbell, 1964*b*).

DISCUSSION

The experiments reported here show that hybrid pox viruses may be isolated with relatively little labour. The method used ensures that growth of virus is initiated only in cells which have been infected with both parental types. This is achieved without technical difficulty. Temperature has been used to prevent the growth of one parental type but the method is not doubly selective. This is because the other parental virus can grow freely as soon as it has been reactivated.

The C.A.M. has some advantages over a cell monolayer for picking out variants. Pock appearance allows for more individual variation than plaque type and also the sampling process is much easier. The clones whose isolation has been described

are presumed to be hybrids on the basis of pock appearance alone. Their detailed characterization for several markers is presented in the accompanying papers (Bedson & Dumbell, 1964*a*, *b*).

The novel feature of our method of obtaining hybrids has been the use of virus in the heat-tethered state. Two properties of heat-tethered virus have been described. The reversibility of the state of inhibition suggests that the virus genome is not damaged by constraint of this degree and kind. The rapidity with which new virus appears when the inhibition is released suggests that the block occurs at a late stage of the viral developmental cycle. As yet we have not attempted to determine directly whether any viral DNA synthesis occurs in the heat-tethered state. The ability of the heat-tethered genome to take part in genetic mixing would suggest that it underwent replication but we cannot exclude the possibility that the other parental virus is able, after it is itself reactivated, to release in some way the state of inhibition of the heat-tethered parent. It is obvious that heat-tethered virus is sufficiently interesting to warrant further study. A more detailed report of this phenomenon is in preparation. Here we have been concerned only with those aspects which relate directly to experiments with pox virus hybrids.

SUMMARY

A simple method for the isolation of pox virus hybrids on the C.A.M. has been described. One parental virus was used as a heat-inactivated suspension. The other parent was used in the active state, but at a temperature higher than its ceiling temperature. Under these conditions the inactive parent was reactivated so that pocks resulted only from the cells infected with both parental viruses. Many of these pocks were unlike those of either parent. Such lesions were found to contain a high proportion of hybrids. In these experiments, alastrim was crossed with rabbit pox and variola major with cowpox.

The term 'heat-tethered' has been used to describe virus whose intracellular cycle of development has been arrested by incubation at too high a temperature. Heat-tethered virus has interesting properties and two of these have been described. When the temperature is lowered, heat-tethered virus will start to grow again. Its reactivating potential has been mentioned above. A more detailed account of the properties of heat-tethered virus is being prepared.

REFERENCES

Bedson, H. S. & Dumbell, K. R. (1961). The effect of temperature on the growth of pox viruses in the chick embryo. *J. Hyg., Camb.*, **59**, 457.

Bedson, H. S. & Dumbell, K. R. (1964*a*). Hybrids derived from the viruses of alastrim and rabbit pox. *J. Hyg., Camb.*, **62**, 141.

Bedson, H. S. & Dumbell, K. R. (1964*b*). Hybrids derived from the viruses of variola major and cowpox. *J. Hyg., Camb.*, **62**, 147.

Fenner, F. (1958). The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. *Virology*, **5**, 502.

Fenner, F. (1959). Genetic studies with mammalian poxviruses. II. Recombination between two strains of vaccinia virus in single HeLa cells. *Virology*, **8**, 499.

Fenner, F. (1962). The Leeuwenhoek Lecture. Interactions between pox viruses. *Proc. roy. Soc.* B, **156**, 388.

FENNER, F. & BURNET, F. M. (1957). A short description of the poxvirus group (vaccinia and related viruses). *Virology*, **4**, 305.

FENNER, F. & COMBEN, B. M. (1958). Genetic studies with mammalian poxviruses. I. Demonstration of recombination between two strains of vaccinia virus. *Virology*, **5**, 530.

GEMMELL, A. & CAIRNS, J. (1959). Linkage in the genome of an animal virus. *Virology*, **8**, 381.

GEMMELL, A. & FENNER, F. (1960). Genetic studies with mammalian poxviruses. III. White (*u*) mutants of rabbitpox virus. *Virology*, **11**, 219.

HAHON, N., RATNER, M. & KOZIKOWSKI, E. (1958). Factors influencing variola virus growth on the chorioallantoic membrane of embryonated eggs. *J. Bact.* **75**, 707.

JOKLIK, W. K. (1962). The multiplication of poxvirus DNA. *Cold Spr. Harb. Symp. quant. Biol.* **27**, 199.

JOKLIK, W. K., WOODROOFE, G. M., HOLMES, I. H. & FENNER, F. (1960). The reactivation of poxviruses. I. Demonstration of the phenomenon and techniques of assay. *Virology*, **11**, 168.

MCCARTHY, K. & DUMBELL, K. R. (1961). Chorioallantoic inoculation of eggs. *Virology*, **14**, 488.

WOODROOFE, G. M. (1960). The heat inactivation of vaccinia virus. *Virology*, **10**, 379.

WOODROOFE, G. M. & FENNER, F. (1960). Genetic studies with mammalian poxviruses. IV. Hybridization between several different poxviruses. *Virology*, **12**, 272.

J. Hyg., Camb. (1964), **62**, 141
Printed in Great Britain

Hybrids derived from the viruses of alastrim and rabbit pox

By H. S. BEDSON and K. R. DUMBELL
Department of Bacteriology, University of Liverpool

(*Received* 19 *November* 1963)

INTRODUCTION

In the preceding paper (Dumbell & Bedson, 1964) an account was given of the isolation of presumptive hybrid clones from the viruses of alastrim and rabbit pox. We report here the results of a more detailed examination of the seven clones of virus in question. As with the variola major–cowpox hybrids considered in the succeeding paper (Bedson & Dumbell, 1964) the main object of the work has been the investigation of the ceiling temperature character and its possible relationship to virulence among the pox viruses.

TESTS FOR MARKER CHARACTERS

The virus strains used and the methods of inoculating eggs and preparing stock suspensions were those described in the preceding paper (Dumbell & Bedson, 1964). Where appropriate the tests for marker characters have been made using the methods developed by Fenner (1958).

(1) *Pock morphology*

The size, colour and degree of ulceration of discrete pocks was noted after 2 and after 3 days' incubation at 35° C. Those of rabbit pox were large, red and grey in colour and were ulcerated. The pocks of alastrim were smaller, white and not ulcerated.

(2) *Ceiling temperature*

Tests were made to determine the presence or absence of pocks at 40° C., using inocula which would produce 50–100 pocks at 35° C. Under these conditions rabbit pox produced obvious lesions and alastrim produced none.

(3) *Haemagglutinin*

Extracts of heavily infected chorioallantoic membranes (C.A.M.S) were diluted 1/5, 1/20 and 1/80 in saline and were tested for haemagglutinin with a 1% suspension of vaccinia-agglutinable fowl cells containing 1% normal rabbit serum. The mixtures were shaken and incubated at 37° C. for 20 min., shaken again and incubated for a further hour. Readings were taken after the cells had been allowed to settle for 10 min. at room temperature. Rabbit pox gave no haemagglutination, whereas alastrim did, at each of the dilutions tested. The ability to induce the production of anti-haemagglutinin in rabbits (Fenner, 1958) was not tested.

(4) *Thermal stability*

Freshly prepared virus suspensions were sealed in thin glass ampoules and immersed in a water bath at 55° C. for 20 min. The reduction in titre of the heated suspension was determined by titration on the C.A.M. after storage at 4° C. overnight. With rabbit pox the fall in titre was 1·0 log unit or less, while the comparable figure for alastrim was in the region of 5·0 log units.

(5) *Type of plaque in chick embryo tissue culture*

Monolayers of chick embryo fibroblasts were prepared in 5 cm. Petri dishes using a modification of Dulbecco's method (Dulbecco, 1952). The growth medium was based on that of Porterfield & Allison (1960). It contained 5 % inactivated calf serum, 0·25 % lactalbumin hydrolysate, and was buffered with *tris* (hydroxymethyl) aminomethane. Each dish received approximately $10^{7\cdot7}$ cells suspended in 4 ml. of growth medium, and was incubated at 35° C. for 2 days before use.

Inoculations were made with virus diluted appropriately in Hanks's phosphate buffered saline, 0·2 ml. being applied to each dish after removal of the growth medium. After adsorption at room temperature for 1 hr., 4 ml. of Eagle's medium (Eagle, 1955) containing 1 % calf serum and triple strength bicarbonate was added. The dishes were incubated at 35° C. in a humidified atmosphere of 5 % CO_2 in air for periods of 2, 3 and 4 days. The medium was then removed and the cell sheets stained with strong carbol fuchsin (Postlethwaite, 1960). With rabbit pox, plaques were present at 2 days. In appearance they were trabeculated and similar to those of cowpox depicted by Bedson & Dumbell (1964; Plate 1A). With alastrim, the monolayers appeared normal at 2 days. The characteristic heavy-rimmed plaques were not present until 4 days. The appearances in this case were indistinguishable from those described for variola major (Bedson & Dumbell, 1964; Plate 1C, D).

(6) *Rabbit virulence*

Virulence for the rabbit was assessed by the type of lesion which resulted from the intradermal inoculation of a standard dose of 10^5 pock forming units (p.f.u.) of virus. Inspections were made daily in the week following inoculation. By the 5th day, rabbit pox lesions showed extensive haemorrhage and necrosis with surrounding oedema. Alastrim produced only trivial lesions, which, by the 5th day, had almost completely regressed.

(7) *Mouse virulence*

The virulence of a virus for the mouse was determined from the mortality rates in 1-month-old mice inoculated by the intracerebral route. A group of five mice was used for each virus. Inocula were 0·01 ml. and contained 10^5 p.f.u. of virus. Under these conditions, rabbit pox was uniformly lethal, the mice dying within 5 days of inoculation, whereas alastrim produced no deaths.

ANALYSIS OF THE ALASTRIM-RABBIT POX CLONES

Seven clones of virus developed from the alastrim–rabbit pox system were subjected to characterization in detail. In the following account they will be referred to as AR viruses 1–7. The results of applying tests for the seven marker characters to these AR viruses are presented in Table 1. The characters of the parent viruses are included in the table for ease of comparison. In most instances the individual characters of the AR viruses were of one or other parental type but some intermediate results were obtained. None of the viruses had all the characters of alastrim but there was one (AR 5) which was indistinguishable from rabbit pox. The other six differed from the parent viruses and also amongst themselves. Each had its own distinct combination of parental characters. There were thus six separate new types of virus.

The genetic stability of these new viruses has not been investigated specifically. In the course of the tests for marker characters the virus clones have been passed on the C.A.M. at 35° C., and several hundred pocks have been examined in tests for ceiling temperature and thermal stability. In all these experiments each of the six clones has maintained the uniformity of its pock type. It was felt that this evidence was sufficient for the purposes of the present study. A more detailed investigation has been made of the stability of the variola major–cowpox hybrids (Bedson & Dumbell, 1964).

Although the AR viruses had been derived from mixed infections and had been shown to exhibit combinations of the appropriate parental characters, it is important to consider in greater detail the question of whether they may be accepted as hybrid viruses. Upon this point rests the validity not only of the claim, made in the preceding paper (Dumbell & Bedson, 1964), that temperature may be used selectively to facilitate the recovery of hybrids, but also of some of the deductions made in that paper about the properties of 'heat-tethered' virus.

The only reasonable alternative explanation is that the AR viruses are mutants derived from the reactivated rabbit pox virus. This virus is known to produce a wide variety of white mutants (Gemmell & Fenner, 1960), but the frequency with which it does this, although appreciable, would not seem to be nearly as great as in our reactivation experiments. In particular, it is to be noted that the six new AR viruses came from only two pocks.

A further point against their being mutants is the fact that practically all the characters of the other virus concerned (alastrim) have been found amongst them. Although this argument has *general* validity, it should be noted that it is much less convincing for an *individual* virus, because of the very wide range of characters shown by the white variants of rabbit pox (Gemmell & Fenner, 1960).

There remains the possibility that the heat-inactivation of rabbit pox may have damaged the genetic material and led to an increase in the frequency of mutation. This suggestion runs counter to current concepts of heat-inactivation and reactivation which, for the pox viruses, is regarded as a 'non-genetic' process (Fenner, 1962). In any case a high frequency of mutation does not seem to have been observed when reactivation has been carried out with an active virus of another

Table 1. *Characters of rabbit pox, alastrim and hybrids developed from them (AR 1–7)*

Virus	Pock type	Pocks at 40° C.	Haem-agglutinin	Thermal stability*	Plaque type	Plaques appear (days)	Rabbit virulence†	Mouse virulence‡
Rabbit pox	Large, red, ulcerated	+	0	0·8	Trabeculated	2	+ +	+
Alastrim	Small, white, non-ulcerated	0	+	5·0	Heavy-rimmed	4	0	0
AR 1	Small, white, ulcerated	+	+	0·8	Heavy-rimmed	3	+	0
AR 2	Small, white, ulcerated	+	+	0·9	Trabeculated	2	+	±
AR 3	Large, white, ulcerated	+	+	0·7	Heavy-rimmed	3	±	0
AR 4	Small, white, non-ulcerated	0	0	3·9	Trabeculated	4	0	0
AR 5	Large, red, ulcerated	+	0	1·1	Trabeculated	2	+ +	+
AR 6	Small, white, non-ulcerated	0	0	0·7	Trabeculated	4	0	+
AR 7	Large, white, ulcerated	+	0	1·0	Trabeculated	3	0	+

* Figures represent the reduction in titre ($\log_{10}$ units) after heating at 55° C. for 20 min.

† + +, Large lesion with haemorrhage and necrosis; +, papule with central necrosis but no haemorrhage; ±, papule without haemorrhage or necrosis; 0, insignificant lesion.

‡ +, 100 % mortality; ±, some mortality; 0, no mortality.

Table 2. *Analysis of pairwise crosses of individual marker characters amongst the AR clones*

	Rabbit pox markers								
	Pocks on C.A.M.					Plaques			
Alastrim markers	Large	Ulcerated	At 40° C.	No haem-agglutinin	High thermal stability	Trabecular	2 days	Rabbit virulent	Mouse virulent
Pocks small	−	(+)	(+)	+	(+)	+	+	+	+
Pocks non-ulcerated	0	−	0	+	(+)	+	0	0	+
No pocks at 40° C.	0	0	−	+	(+)	+	0	0	+
Haemagglutinin	+	+	+	−	+	(+)	+	+	+
Low thermal stability	0	0	0	+	−	+	0	0	0
Plaques rimmed	+	+	+	0	+	−	0	+	0
No plaque 2 days	+	(+)	(+)	+	(+)	(+)	−	(+)	(+)
Not rabbit virulent	+	(+)	(+)	+	(+)	+	0	−	+
Not mouse virulent	+	+	+	+	(+)	(+)	0	+	−

+, Pairwise cross and its reciprocal present; (+), pairwise cross but not reciprocal present; 0, pairwise cross absent.

subgroup (Fenner & Woodroofe, 1960). Had white mutants occurred with increased frequency it would hardly have been possible for these workers to have made the observation that hybrids do not occur in this type of system.

It seems therefore extremely probable from all points of view that the six new AR viruses are hybrids. If this is accepted, it becomes of interest to examine the behaviour of the individual marker characters in segregation. The pairwise crosses between markers that have been found among the AR viruses are shown in Table 2. From the table it will be seen that, where necessary, markers have been arbitrarily redefined in such a way as to class intermediate results with one or other parent. It will also be seen that the number of markers considered has been increased to 9 by regarding pock size, degree of ulceration of pocks, plaque type and speed of plaque formation, all as separate characters. The colour of the pocks has been omitted from the analysis because selection of potential hybrids was based on this character and clones with red pocks were in general avoided. With nine characters, the number of possible pairings is 72 and 53 of these were shown by the six AR hybrids. In 18 instances the pairwise cross and its reciprocal cross were present and in only one case was neither of the two possible crosses for a pair of markers found. Considering the very few hybrids which have been examined the proportion of pairwise crosses encountered is surprisingly high. The evidence strongly suggests, therefore, that each of the markers used is capable of segregating independently. This point has, of course, a particular significance in relation to virulence characters, but a more extensive discussion of this aspect of the work will be made in the following paper after the properties of the variola–cowpox hybrids have been described (Bedson & Dumbell, 1964).

SUMMARY

The characters of seven clones of virus derived from mixed infections with alastrim and rabbit pox have been described. One clone was shown to behave as rabbit pox in respect of all its markers. The other six were found to be distinct new types of virus, each having a different combination of the parental characters. The reasons for accepting these viruses as hybrids have been discussed. An analysis of the pairwise crosses between individual markers suggested that each of the nine markers was capable of segregating independently.

REFERENCES

Bedson, H. S. & Dumbell, K. R. (1964). Hybrids derived from the viruses of variola major and cowpox. *J. Hyg., Camb.*, **62**, 147.

Dulbecco, R. (1952). Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. nat. Acad. Sci., Wash.*, **38**, 747.

Dumbell, K. R. & Bedson, H. S. (1964). The use of ceiling temperature and reactivation in the isolation of pox virus hybrids. *J. Hyg., Camb.*, **62**, 133.

Eagle, H. (1955). The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. *J. exp. Med.* **102**, 37.

Fenner, F. (1958). The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. *Virology*, **5**, 502.

Fenner, F. (1962). The Leeuwenhoek Lecture. Interactions between pox viruses. *Proc. roy. Soc.* B, **156**, 388.

FENNER, F. & WOODROOFE, G. M. (1960). The reactivation of poxviruses. II. The range of reactivating viruses. *Virology*, **11**, 185.

GEMMELL, A. & FENNER, F. (1960). Genetic studies with mammalian poxviruses. III. White (*u*) mutants of rabbitpox virus. *Virology*, **11**, 219.

PORTERFIELD, J. S. & ALLISON, A. C. (1960). Studies with poxviruses by an improved plaque technique. *Virology*, **10**, 233.

POSTLETHWAITE, R. (1960). A plaque technique for the titration of vaccinia virus in chick embryo cells and some features of vaccinial infection in this system. *Virology*, **10**, 466.

J. Hyg., Camb. (1964), **62**, 147
With 1 plate
Printed in Great Britain

Hybrids derived from the viruses of variola major and cowpox

By H. S. BEDSON and K. R. DUMBELL
Department of Bacteriology, University of Liverpool

(*Received* 19 *November* 1963)

INTRODUCTION

The isolation of presumptive hybrids from the viruses of variola major and cowpox has already been described (Dumbell & Bedson, 1964). We report here the detailed characterization of the 16 clones of virus obtained. As with the alastrim–rabbit pox hybrids considered in the previous paper (Bedson & Dumbell, 1964), attention has been paid to the behaviour of the ceiling temperature character in recombination and to the possible relationship it and other markers might have to the virulence of these viruses. Both sets of hybrids may also be regarded as making a minor contribution to pox virus genetics, in that variola viruses have now been shown to form hybrids with other members of the variola–vaccinia group.

TESTS FOR MARKER CHARACTERS

Three of the seven marker characters used in the study of the alastrim–rabbit pox hybrids have not been used for the variola major–cowpox hybrids. The markers excluded were haemagglutinin production, thermal stability and mouse virulence. The first two were not considered to offer worthwhile differences in the present system, while mouse virulence was omitted for reasons of personal convenience.

Tests for pock morphology, type of plaque in chick embryo tissue culture and rabbit virulence have been made as described for the alastrim–rabbit pox hybrids. Details of the tests for ceiling temperature and for three additional markers are given below.

Ceiling temperature

In initial tests the ceiling temperatures were determined as described by Bedson & Dumbell (1961). The presence or absence of pocks was noted at the temperatures 38·5, 39, 39·5 and 40° C. using an inoculum giving an average of 50–100 pocks per membrane at 35° C.

A second series of tests was performed to determine more precisely the reduction in pock-forming efficiency at the temperature concerned. In these tests, counts were made of the pocks present in groups of three to four eggs incubated at each of the relevant temperatures and in a control group at 35° C. Inocula larger than 100 pock-forming units of virus were used if the temperature was such that very few or no pocks were to be expected. The extent to which pocks were reduced at any temperature was taken as: $\log_{10}$ pock titre at 35° C. $- \log_{10}$ pock titre at the temperature concerned. For each virus the values obtained were then plotted

against temperature. In the experiments 35° C. was used as the control temperature as a matter of convenience.

Cytoplasmic inclusions

Portions of infected C.A.M. were fixed in 10% formal-zenker for 1 hr., washed and embedded in paraffin. Sections were stained with haematoxylin and eosin.

Egg virulence

Virulence for the chick embryo was determined from the mortality rates of 12-day embryos inoculated on the C.A.M. and incubated at 35° C. For each virus three separate doses of inoculum were used, a group of six eggs being inoculated with each dose. The D4 value—i.e. the log dose of virus giving a mean survival time of 4 days—has been calculated from the results as previously described (Bedson & Dumbell, 1961).

Diffusible LS antigen

Extracts of heavily infected C.A.M.S were examined for the presence of a diffusible LS antigen in agar-gel precipitation tests using an anti-serum supplied by Dr C. J. M. Rondle. This had been prepared in a rabbit by the intravenous injection of a preparation of LS antigen made from vaccinia-infected rabbit material by the method of Shedlovsky & Smadel (1942). Reservoirs 4 mm. in diameter with centres 5 mm. apart were made in a layer of agar 1 mm. deep on a microscope slide. The agar was at a 1% concentration in isotonic phosphate-buffered saline pH 7·3 containing 0·01% merthiolate. The antigen extracts were made in isotonic phosphate-buffered saline pH 7·3, 1 ml. per membrane.

PROPERTIES OF THE VARIOLA MAJOR–COWPOX CLONES

In the following account it will be convenient to refer to the 16 clones of virus prepared from the variola major–cowpox system as VC clones 1–16. Not all 16 were independent, for VC2, 3 and 4 were taken deliberately from a single clone in order to provide an internal check on the methods used and on the stability of the cloned viruses. Similarly VC10 and 15 came from the same clone, but in this case there was at first a suggestion from the pock appearance that the clone might not be pure.

The results of applying tests for the seven marker characters to the VC viruses and to the parent viruses are presented in Table 1. These results are discussed in greater detail in the following sections which deal with each marker character separately.

Pock morphology

The pocks of cowpox are large, ulcerated and conspicuously red from haemorrhage while those of variola are smaller, not ulcerated and white. Despite these well-marked differences pock appearance has not been a particularly useful marker in classifying the VC clones. This may have been in part due to its use at the onset to exclude viruses with pocks of the cowpox type, but the chief difficulty was that

Table 1. *Characters of cowpox, variola major and hybrids developed from them (VC 1–16)*

Virus	Pock type	Cyto-plasmic inclusions	Egg virulence (D4 value)*	Diffusible LS antigen	Ceiling temperature (° C.)	Plaque type	Plaques appear (days)	Rabbit virulence†
Cowpox	Red, ulcerated	+	3·5	0	40	Trabeculated	2	+
Variola major	White, non-ulcerated	0	4·5–5·5	+	38·5	Heavy-rimmed	4	0
VC 1	Red, ulcerated	+	.	0	40	Trabeculated	2	+
VC 2	Intermediate, ulcerated	+	5·6	0	40	Trabeculated	2	+
VC 3	Intermediate, ulcerated	+	.	0	40	Trabeculated	2	+
VC 4	Intermediate, ulcerated	+	.	0	40	Trabeculated	2	+
VC 5	Intermediate, ulcerated	0	2·3	0	38·5	Trabeculated	3	0
VC 6	White, non-ulcerated	+	.	0	40	Trabeculated	3	0
VC 7	Intermediate, ulcerated	+	2·5	0	40	Trabeculated	3	+
VC 8	White, ulcerated	0	7·0	+	38·5	Heavy-rimmed	4	±
VC 9	Red, ulcerated	+	3·4	0	40	Trabeculated	2	+
VC 10	White, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 11	Red and white, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 12	White, ulcerated	+	3·9	0	40	Heavy-rimmed	3	±
VC 13	Intermediate, ulcerated	+	4·2	0	39	Trabeculated	2	+
VC 14	Intermediate, ulcerated	+	3·2	0	40	Trabeculated	3	+
VC 15	White, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 16	White, ulcerated	0	.	0	40	Trabeculated	2	±

* Log dose of virus giving harmonic mean survival time of 4 days.

† +, Papule with haemorrhage and necrosis; ±, papule without haemorrhage or necrosis; 0, insignificant lesion.

of analysing the very wide variety of appearance in terms of the parental viruses. Two viruses (VC1 and 9) had pocks indistinguishable from those of cowpox. One (VC 6) had pocks like those of smallpox, but perhaps rather smaller than is typical of this virus. The rest were all either white or grey and ulcerated to a varying extent. A number had some degree of central haemorrhage and these have been recorded in Table 1 as being of intermediate colour.

After 3 days' incubation at 35° C. the pocks of many of the VC clones resembled those produced by different strains of vaccinia after 2 days. Thus VC8, 10, 12, 15 and 16 had pocks very similar to those of dermo-vaccinia, VC2, 3, 4 and 14 had pocks like neurovaccinia and VC7 and 13 gave pocks more like those of the Utrecht strain of rabbit pox. Such descriptions are, of course, only approximations and take little account of minor distinguishing features.

Cytoplasmic inclusions

The histology of cowpox-infected C.A.M. is characterized by the presence of very numerous compact or 'solid' eosinophilic cytoplasmic inclusion bodies (Downie, 1939), whereas C.A.M. infected with variola virus shows only granular cytoplasmic inclusion material. Sections were examined from both confluent infections and discrete pocks for each VC virus and there were only three (VC5, 8 and 16) which did not show the solid inclusions characteristic of cowpox.

Egg virulence

In previous work (Bedson & Dumbell, 1961) it was established that cowpox differs from variola major in its virulence for the chick embryo and that this difference could be expressed quantitatively in terms of the log dose giving a mean survival time of 4 days (the D4 value). Cowpox is the more virulent and has therefore a lower D4 value (3·5) than that of variola major (4·5–5·5). D4 values for eight of the VC clones were determined. Among these there were two like cowpox (VC9 and 14), two of intermediate virulence (VC12 and 13) and one like variola major (VC2). Perhaps the most interesting finding was that there were also clones with values outside the parental range. Both VC5 and 7 appeared significantly more virulent than cowpox, while VC8 was definitely less virulent than variola major.

Diffusible LS antigen

It has been shown by Rondle & Dumbell (1962) that antigen component *f* cannot normally be demonstrated in agar gel-diffusion analysis of extracts of cowpox-infected tissue. They suggest that this component, which appears to be a part of the LS antigen complex, is present in such extracts in a non-diffusible state. In extracts of variola-infected tissue this component is readily demonstrable. Extracts of C.A.M. infected with cowpox, variola major and VC clones 1–16 have been examined for the presence of antigen *f* using an antiserum prepared by Dr C. J. M Rondle which reacts specifically with this antigen in gel-diffusion tests. Only variola major and VC8 gave a line of precipitation with this serum used both unconcentrated and after fivefold concentration.

Ceiling temperature

The values shown in Table 1 were obtained from the initial tests in which small doses of virus were used and in which only the presence or absence of pocks was noted. Despite their limitations these tests revealed a considerable variety amongst the VC clones. There were 10 clones which behaved like cowpox, giving pocks at 40 but not at 40·5° C. The latter point established their difference from strains of vaccinia which gave clear-cut lesions in parallel tests at 40·5° C. Three of the clones (VC10, 11 and 15) formed pocks at 39·5 but not at 40° C., while the remaining three did not appear to form pocks at 39·5° C. One (VC13) gave pocks at 39 but not at 39·5° C. and the other two (VC5 and 8), like variola major, formed pocks at 38·5 but not at 39° C.

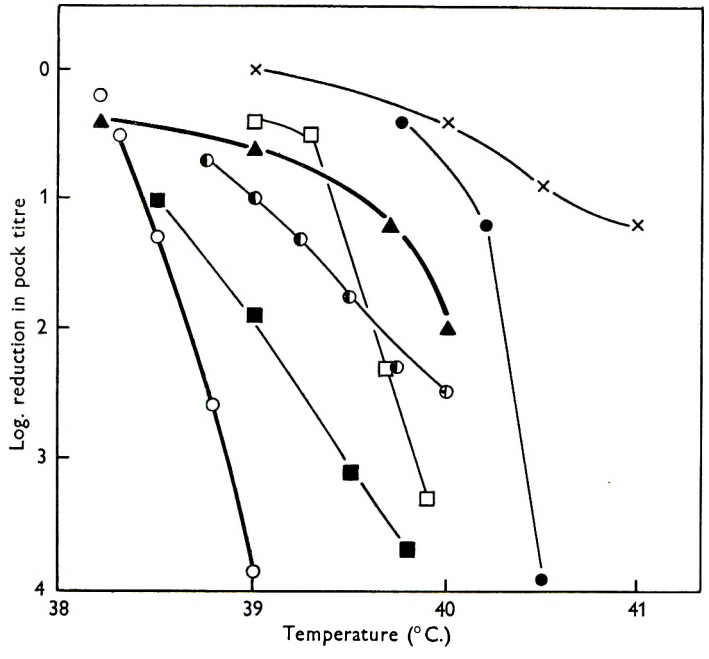


Fig. 1. Plots of log. reduction in pock titre, based on titre at 35° C., against temperature for variola major (○—○), VC8 (■—■), VC13 (◐—◐), cowpox (▲—▲), VC10 (□—□), VC16 (●—●) and vaccinia (×—×).

These last results were surprising, for it appeared that three of the VC clones would not grow at 39·5° C. although the original reactivation had taken place at this temperature. A second series of tests was therefore made in order to determine more precisely the decline in pock-forming efficiency with rising temperature. Each of the VC clones was examined and log values for the reduction in pock titre at the relevant temperatures were obtained. These values were then plotted against temperature. Some examples of the plots are shown in Fig. 1. The parent viruses, cowpox and variola major, and also vaccinia, are included for comparison. Among the VC clones five different types of plot were recognized. The first corresponds closely to that of cowpox and was seen with seven of the clones (VC1, 2, 3, 4, 6, 7 and 9). The remaining four types are shown by the four examples given in Fig. 1.

Of the viruses not shown in the figure, VC12 and 14 were like VC16, VC11 and 15 were like VC10, and VC5 was like VC8.

Although these results do not conflict with those of the initial tests, they affect their interpretation. In the first place they showed that none of the VC clones was exactly like variola major and that none of them was completely incapable of growth at 39·5° C. They also showed that three clones (VC12, 14 and 16) had slightly higher ceiling temperatures than cowpox, even though none of them reached the range of vaccinia. But perhaps the most interesting point to emerge was that not all the viruses reacted to rising temperature in the same way. With some viruses, especially variola major, VC10 and 16, there came a point at which pock-forming efficiency declined abruptly. At this stage there was a fall of about 2 log units within a range of 0·5° C. With these viruses the value obtained for the ceiling temperature is practically independent of the dose of virus used in the test. However, other viruses showed a much more gradual decline in pock-forming efficiency. This type of response was shown by VC5, 8 and 13 and also to some extent by cowpox. For these viruses, the dose of virus used in the test becomes of critical importance. Indeed, for them, the ceiling temperature of pock formation can only be defined in terms of some arbitrarily chosen level of reduction in pock numbers. It will be seen from Fig. 1 that the ceiling temperatures recorded in Table 1 correspond in most cases to a temperature at which there is a reduction in pock-forming efficiency of 1 log unit.

Plaques in chick embryo monolayers

The major difference between the plaques of cowpox and variola major was in the speed of their formation but there was also a difference in the type of plaque produced. Those of cowpox were present at 2 days and were trabecular in appearance (Pl. 1A). After 3 days they had become much larger and secondary plaques were usually visible (Pl. 1B). With variola major the monolayers appeared normal at 2 days and definite plaques with a characteristic heavy rim were not seen until 4 days (Pl. 1C). Specific lesions were in fact visible at 3 days but at this stage they were present only as densely staining dots (Pl. 1D). It was thought that the plaques developed from these foci by central necrosis and detachment of cells. In some dishes in which development was uneven, lesions in various stages of transition were observed. An attempt was made to gain a better understanding of the changes involved using both phase contrast microscopy of lesions in tube cultures and conventional microscopy of monolayers fixed and stained with haematoxylin and eosin. Although there were often small giant cells in the bases of plaques there was no massive syncytial formation and it appeared that both the densely staining dots and the heavy rims of plaques were attributable to the more intense staining of cells undergoing degeneration.

Among the VC clones there were 10 that gave plaques of the cowpox type present at 2 days. Only one (VC8) behaved like variola, giving heavy-rimmed plaques at 4 days. The other five were intermediate in that plaques were first seen at 3 days. In four cases (VC5, 6, 7 and 14) the plaques were of the trabeculated type. Two examples—VC14 at 3 days and VC7 at 4 days—are shown in Pl. 1E and F,

respectively. The fifth (VC12) gave heavy-rimmed plaques. The lesions of this virus at 3, 4 and 5 days are shown in Pl. 1G, H and J. The target-like appearance at 5 days is reminiscent of the 'ring-zone' phenomenon observed with fowl pox virus (Mayr & Kalcher, 1961).

Rabbit virulence

In tests of virulence in the rabbit skin there were three main kinds of response. First, there were the papules which proceeded to extensive central haemorrhage and necrosis, usually accompanied by considerable oedema. This type of result was given by cowpox and eight of the VC clones. In Table 1 this has been recorded as rabbit virulence +. VC7 appeared to differ slightly from the others in that the lesion was always slower to evolve and did not develop haemorrhage or necrosis until after the 5th day. At the other extreme was the response given by variola major and by VC 5 and 6. With these viruses there were either no lesions at all or at best small soft papules which began to regress after 3 days and had practically disappeared by 5 days. This type of response is shown in Table 1 as rabbit virulence 0. Intermediate between these were the six VC clones, shown in Table 1 as having rabbit virulence ±, which gave well-developed firm papules which did not progress to haemorrhage or necrosis.

ANALYSIS OF RESULTS

From the results presented in Table 1, it is clear that there are many different kinds of virus amongst the VC clones. Two (VC1 and 9) were indistinguishable from cowpox but none had all the characters of variola major. Of the others, VC11 should be excluded from consideration because its mixed pocks suggested that it may not have been a pure clone. VC3 and 4 can also be excluded for they were indistinguishable from VC2, and VC15 was exactly like VC10. This leaves 10 separate new kinds of virus, each of which differed from the parent viruses and from the other nine.

The stability of these new viruses was next considered. Some evidence on this point is contained in the results already presented, e.g. the lack of any difference between VC2, 3 and 4 and between VC10 and 15. Nevertheless the point was thought of sufficient importance to warrant further experiments. Each of the 10 new types of virus was passed twice on the C.A.M. at high concentration. Fresh stocks were prepared from the membranes of the second pass. These were tested for pock morphology, type of plaque, diffusible LS antigen, rabbit virulence and for ceiling temperature. In no instance was a change found in the characters of the new stock. Additional evidence of stability was obtained for VC12. A heat-inactivated preparation of this virus was reactivated on the C.A.M. at 39·5° C. with variola major. Several clones of virus were derived from the resulting pocks and each proved similar to VC12 in respect of pock and plaque type, ceiling temperature and the absence of diffusible LS antigen.

It must also be considered whether these new viruses are to be accepted as hybrids or whether they are white variants of cowpox (Downie & Haddock, 1952). In answer to this question it may be said that all the arguments advanced in the

case of the alastrim–rabbit pox (AR) hybrids (Bedson & Dumbell, 1964) apply with at least equal force to the VC viruses. Thus it is to be noted that 6 of the 10 new types of virus were developed from a single reactivation pock. There is also the point that, whereas rabbit pox has been shown to produce a very wide variety of white variants (Gemmell & Fenner, 1960), a corresponding variety has not been seen amongst the white variants derived from the nine strains of cowpox virus extensively studied in our department. The difference between rabbit pox and cowpox in this respect is confirmed by the difficulty we have experienced in obtaining wild cowpox by crossing its white variants (unpublished observations) although this can readily be achieved with rabbit pox (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960).

As with the AR hybrids, it is possible to study the segregation of individual marker characters by examining the pairwise crosses that have been encountered. These are shown in Table 2. From the table it will be seen that some of the markers have been arbitrarily redefined in such a way as to class intermediate results with one or other of the parental types. The number of markers has also been increased to eight by considering the speed of plaque formation and type of plaque as separate characters. In the scheme shown, there are 56 possible pairwise crosses and 44 of them were present among the 10 new VC viruses. For every pair of characters at least one of the two possible crosses was found and in 16 instances both the pairwise cross and its reciprocal were present. There is, therefore, good evidence that each of the markers chosen is capable of segregating independently. Although a high proportion of the pairwise crosses has been found among the VC viruses, it must be noted that we have studied only a very small number of clones in relation to the large number of hybrids which potentially exist. Even neglecting intermediate results, as in Table 2, there are 254 possible new types of virus.

GENERAL DISCUSSION OF VC AND AR HYBRIDS

In this discussion reference will be made not only to the results of the present work but also to the alastrim–rabbit pox (AR) hybrids described in the preceding paper (Bedson & Dumbell, 1964).

Four of the markers which have been used in these studies have not previously been used in genetic work with the pox viruses. That involving the LS antigen would seem to be ideal for this kind of work because of the ease of testing large numbers of clones. It is a pity that the prospect of developing further antigenic markers of this type does not at present appear more hopeful. Both virulence for the chick embryo and the presence or absence of solid inclusions are too laborious for large-scale use, but the fourth new marker—ceiling temperature—does not suffer from this defect. The recovery of hybrids with intermediate ceiling temperatures has shown that it is a graded character and the more detailed study of the VC hybrids has revealed some of its complexities. Nevertheless, simple tests are all that have been required for its use as a marker with both sets of hybrids.

The behaviour of the viruses in chick tissue culture is also of interest. Variola major and alastrim have not previously been shown to produce plaques in chick

Table 2. *Analysis of pairwise crosses of individual marker characters amongst the VC clones*

	Cowpox markers							
Variola major markers	Pocks on C.A.M. ulcerated	Cyto-plasmic inclusions	Egg virulent (D4 < 4·0)	Diffusible LS antigen absent	Ceiling temperature ⩾ 39·5° C.	Plaques: Trabecular	Plaques: 2 days	Rabbit virulent (+ in Table 1)
Pocks non-ulcerated	—	+	0	+	+	+	0	0
Cytoplasmic inclusions absent	+	—	+	(+)	+	+	+	0
Not (D4 > 4·0) egg virulent	(+)	+	—	(+)	+	+	+	+
Diffusible LS present	+	0	0	—	0	0	0	0
Ceiling temperature < 39·5° C.	+	+	+	(+)	—	+	+	+
Plaques rimmed	+	+	+	(+)	+	—	0	0
No plaques at 2 days	(+)	+	+	(+)	+	(+)	—	+
Not rabbit virulent (0 or ± in Table 1)	(+)	(+)	+	(+)	+	(+)	+	—

+, Pairwise cross and its reciprocal present; (+), pairwise cross but not reciprocal present; 0, pairwise cross absent.

tissue culture and at first difficulty was experienced in obtaining plaques from these viruses. It was, in fact, only after a study of the intermediate forms of plaques and particularly those of VC12, that the behaviour of variola was fully appreciated. It must be stressed that we did not use an agar overlay, because in our experience an overlay considerably delays the evolution of plaques. Our findings cannot therefore be said to conflict with those of Mika & Pirsch (1960), who claimed that variola did not produce plaques in chick embryo monolayers, but they used an agar overlay system and ceased observations on the 4th day.

It was recognized early in the study of the genetics of pox viruses that virulence characters were complex and unsuited for genetic analysis (Fenner & Comben, 1958). They have, however, continued to be of practical importance, and this has been shown yet again by the three virulence markers used in the present study. As in previous studies, hybrids of intermediate virulence have been relatively common, suggesting that each virulence character is controlled by multiple factors. In no case has this been more clearly demonstrated than with the VC hybrids and their virulence for the chick embryo. The drawbacks of this marker have already been discussed but it does have the advantage that it allows a comparative measure of virulence. It was this which made it possible to show quite clearly that some hybrids had a virulence which was outside the parental range.

The hybrids have also been of use in analysing the extent to which certain characters are interdependent. An example of this is shown by the VC hybrids and the characters chick virulence, speed of plaque formation and ceiling temperature. These three characters all relate to facets of the chick embryo host and it might not be unreasonable to expect some connexion between them. Indeed, this is the conclusion suggested by an examination of the 'natural' pox viruses, for, as is shown in Table 3, the three characters appear to be closely correlated. The data for chick virulence and ceiling temperature have been taken from Bedson & Dumbell (1961) and those for plaque formation from unpublished observations made in our department in collaboration with Mr D. P. McHugh. Yet, when one examines the VC hybrids (Table 1) neither ceiling temperature nor speed of plaque formation are correlated with chick virulence, and the pairwise crosses (Table 2) show quite convincingly that these markers segregate independently. In the same way rabbit and mouse virulence have been shown to be independent among the AR hybrids. It follows that correlations between single characters and virulence are always likely to be fortuitous, and that one should not expect to find strict correlations of this kind in attempts to determine the characters which underly virulence.

One further point which may be considered in the light of the VC hybrids is the often disputed question of the origin of vaccinia. Derivations from both smallpox and cowpox have been claimed but the histories of the many strains now in use are too obscure to be of any value. The drawback to both claims is that attempts to repeat the process have usually been completely unsuccessful and in the few instances in which success has been claimed the work has always been open to serious criticism (for discussion see Downie & Dumbell, 1956). There is, however, the possibility that vaccinia may have originated as a hybrid of variola and

cowpox. These viruses between them possess all the characters that we at present recognize in vaccinia, and the study of the VC hybrids has shown how readily viruses resembling vaccinia may be produced. It is most unlikely that the necessary mixed infection will have occurred purely by chance, but it is stated that in some quarters on the continent there has been an accepted practice of mixing variola with vaccine virus from time to time, in order to enhance its potency (Pettenkofer, Stöss, Helmbold & Vogel, 1962). Such an act performed at an earlier stage in the history of vaccinia could have provided the necessary conditions. It may be objected that none of the VC hybrids so far examined corresponds to vaccinia in all its characters, but only relatively few strains have been studied and there seems no reason why a hybrid with such properties should not eventually be found.

Table 3. *Virulence for the chick embryo, ceiling temperature and speed of plaque formation of the natural pox viruses*

Virus	Virulence (D4 value)*	Ceiling temperature (° C.)*	Speed of plaque formation (hr.)†
Rabbit pox	0·3	>41	36
Vaccinia	0·8–2·2	41	36
Cowpox	3·2–3·5	40	42
Monkey pox	3·3–3·7	39	72
Ectromelia	3·3–3·6	39	72
Variola major	4·6–5·5	38·5	96
Alastrim	6·8	37·5	96

* Data from Bedson & Dumbell (1961).

† Unpublished data obtained in collaboration with D. P. McHugh; the time given is in each case the optimum for counting primary plaques in the system without agar overlay.

SUMMARY

The characterization of 16 clones of virus derived from mixed infections with variola major and cowpox has been described. This work has involved the description of the plaques produced by variola major in chick embryo monolayers, and a more detailed study of the effects of raised temperature on pock production by pox viruses on the C.A.M.

Two of the variola major–cowpox clones were found to correspond to cowpox virus, while the other 14 shared various combinations of parental characters. Among them there were 10 distinct new types of virus. Evidence of the stability of these viruses has been presented.

Analysis of the combination of characters encountered among the hybrids has given good evidence that all the eight marker characters used segregate independently. A particular instance of this was the character of chick embryo virulence which, among the hybrid viruses, was shown to be unrelated either to ceiling temperature or speed of plaque formation.

The significance of these findings in relation to the general problem of virulence has been briefly discussed. The possibility that vaccinia may have originated as a hybrid of variola and cowpox has lso been considered.

REFERENCES

BEDSON, H. S. & DUMBELL, K. R. (1961). The effect of temperature on the growth of pox viruses in the chick embryo. *J. Hyg., Camb.*, **59**, 457.

BEDSON, H. S. & DUMBELL, K. R. (1964). Hybrids derived from the viruses of alastrim and rabbit pox. *J. Hyg., Camb.*, **62**, 141.

DOWNIE, A. W. (1939). A study of the lesions produced experimentally by cowpox virus. *J. Path. Bact.* **48**, 361.

DOWNIE, A. W. & DUMBELL, K. R. (1956). Pox viruses. *Annu. Rev. Microbiol.* **10**, 237.

DOWNIE, A. W. & HADDOCK, D. W. (1952). A variant of cowpox virus. *Lancet*, i, 1049.

DUMBELL, K. R. & BEDSON, H. S. (1964). The use of ceiling temperature and reactivation in the isolation of pox virus hybrids. *J. Hyg., Camb.*, **62**, 133.

FENNER, F. & COMBEN, B. M. (1958). Genetic studies with mammalian poxviruses. I. Demonstration of recombination between two strains of vaccinia virus. *Virology*, **5**, 530.

GEMMELL, A. & CAIRNS, J. (1959). Linkage in the genome of an animal virus. *Virology*, **8**, 381.

GEMMELL, A. & FENNER, F. (1960). Genetic studies with mammalian poxviruses. III. White (*u*) mutants of rabbitpox virus. *Virology*, **11**, 219.

MAYR, A. & KALCHER, K. (1961). Plaque-bildung bei den geflügelpockenviren. *Arch. ges. Virusforsch.* **11**, 307.

MIKA, L. A. & PIRSCH, J. B. (1960). Differentiation of variola from other members of the poxvirus group by the plaque technique. *J. Bact.* **80**, 861.

PETTENKOFER, H. J., STÖSS, B., HELMBOLD, W. & VOGEL, F. (1962). Alleged causes of the present-day world distribution of the human ABO blood groups. *Nature, Lond.*, **193**, 445.

RONDLE, C. J. M. & DUMBELL, K. R. (1962). Antigens of cowpox virus. *J. Hyg., Camb.*, **60**, 41.

SHEDLOVSKY, T. & SMADEL, J. E. (1942). The LS-antigen of vaccinia. II. Isolation of a single substance containing both L- and S-activity. *J. exp. Med.* **75**, 165.

EXPLANATION OF PLATE 1

Examples of the plaques produced in chick embryo monolayers by variola major, cowpox and various hybrid (VC) viruses.

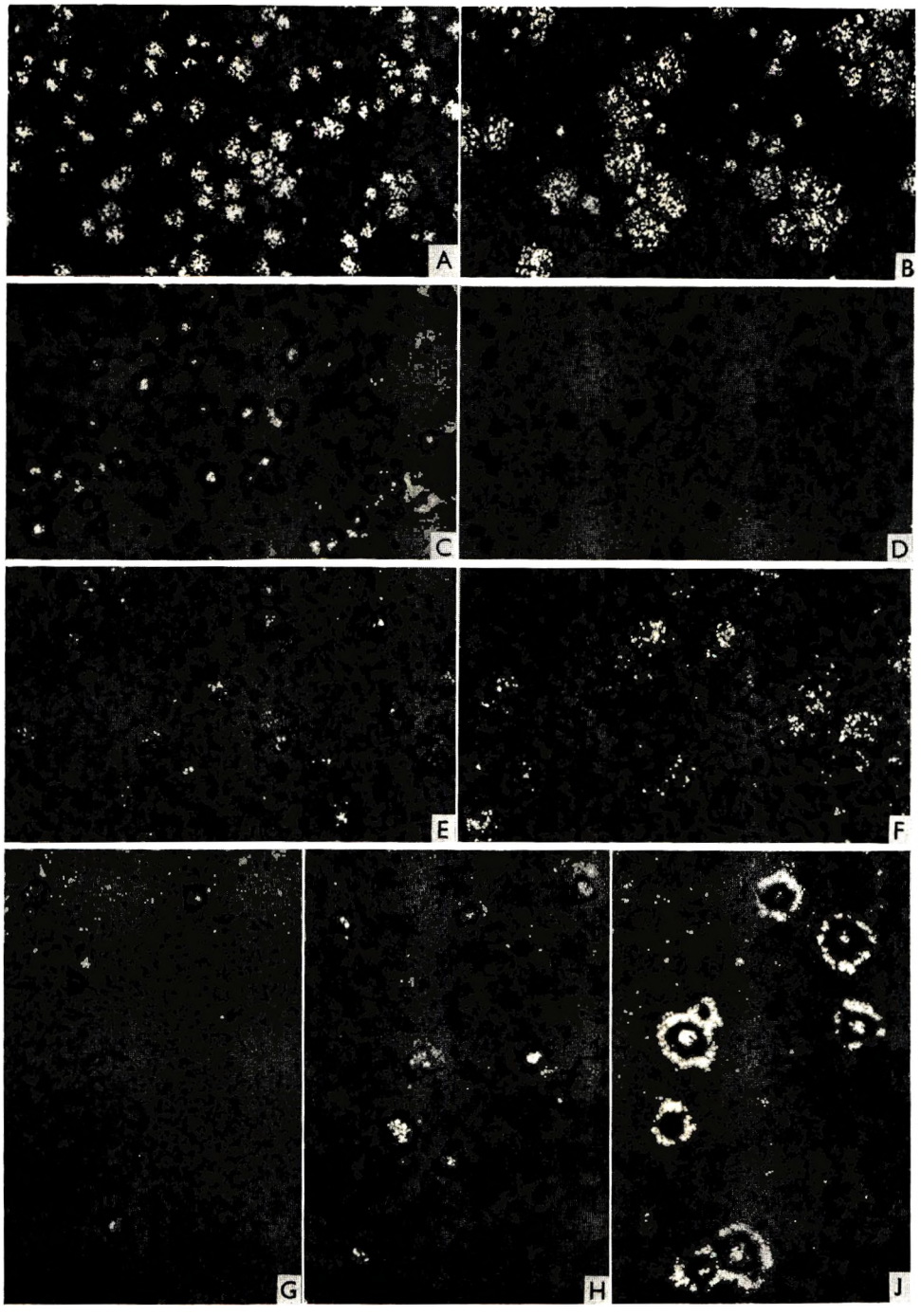
A and B. Plaques of cowpox at 2 days and at 3 days.

C and D. Plaques of variola major at 4 days and at 3 days.

E. Plaques of VC14 at 3 days.

F. Plaques of VC7 at 4 days.

G, H and J. Plaques of VC12 at 3, 4 and 5 days.



H. S. BEDSON AND K. R. DUMBELL

Facing p. 158)

J. Hyg., Camb. (1964), **62**, 159
Printed in Great Britain

An outbreak of aseptic meningitis associated with Coxsackie B5 and A9 viruses in Northern Japan, 1961. Virological and serological studies

By YORIO HINUMA, YOSHIMICHI MURAI, MINORU FUKUDA,* YOSHIO NUMAZAKI† and NAKAO ISHIDA

Department of Bacteriology, Tohoku University School of Medicine, Sendai, Japan

and TOORU NAKAO

Paediatric Clinic, Aomori Prefectural Central Hospital, Aomori, Japan

(*Received* 22 *November* 1963)

In the summer of 1961, an outbreak of aseptic meningitis took place in Northern Japan. A total of 655 cases, mostly infants or children, was reported by three local paediatricians and by one of the authors (T.N.). It is probable that the total number of cases in the entire district was more than 2000. The highest incidence was observed in Aomori Prefecture, which is in the northern end of Honshu (Japan proper), and a peak incidence occurred between June and August.

From many of the specimens of spinal fluids and faeces, Coxsackie B5 virus and, to a lesser extent, Coxsackie A9 virus were isolated. Serological tests with paired sera also suggested infections with these viruses.

The present paper is concerned with the aetiological studies on 295 cases and with some properties of the viruses isolated. Clinical and epidemiological aspects of the epidemic will be reported in detail in a paper by Nakao *et al.* (1964).

MATERIALS AND METHODS

Patients

Of the 295 cases studied, 234 were examined clinically by one of the authors (T.N.) at the Paediatric Clinic, Aomori Prefectural Central Hospital, Aomori City.

Specimens

Spinal fluids were collected from 270 cases, faeces from 147, and paired sera from 184 cases. The spinal fluids and faeces were obtained upon admission to the hospital. The specimens of blood were taken on admission and more than 7 days later to provide acute and convalescent phase sera. These sera were collected from May to October 1961 and stored at $-25°$ C. until use.

Isolation and identification of viruses

For the isolation of viruses, the stationary cultures of cynomolgus monkey kidney cells and/or HeLa (S3) cells were used. Unweaned mice were not employed

* Died on 23 October 1963.

† Present address: Virology Laboratories, Children's Hospital, Buffalo, U.S.A.

in the present studies. One-tenth millilitre of clarified 10% suspension of faeces or 0·2 ml. of spinal fluid was inoculated into two replicate tubes of cell culture which already contained 1·0 ml. of a maintenance medium. For the identification of viruses, the conventional neutralization test was performed. In the early stages of this study, reference sera against Coxsackie A 9, Coxsackie B 1–5, Echo 1–19 and Polio 1–3, all of which were kindly supplied by the National Foundation of the United States, were used. Later, rabbit antisera prepared in this laboratory against B 5 and A 9 viruses by immunization with the current strains, AM-69-L-61 and AM-85-L-61 respectively, were used for the typing of the viruses.

Antibody titration

Neutralizing antibody in paired sera from the patients was titrated against the current strain (AM-69-L-61) of Coxsackie B 5 virus, Poliovirus type 1 (Mahoney), type 2 (MEF 1) and type 3 (Saukett) in HeLa cell culture. For titration of neutralizing antibody against Coxsackie A 9 virus, the current strain (AM-85-L-61) was used in the monkey kidney cell cultures. The virus dose used was approximately 100 TCID 50.

RESULTS

Virus isolation

As shown in Table 1, Coxsackie B 5 virus was isolated from the spinal fluids of 60 patients out of 270 examined, and Coxsackie A 9 from 1 patient. Cytopathogenic agents were also isolated from the faeces of 82 cases out of 147; Coxsackie B 5 from 51 cases, Coxsackie A 9 from 13, type 3 poliovirus from 3, and unidentified viruses from 15 cases. The large number of isolations of Coxsackie B 5 virus strongly suggested that this was the main causative agent of the epidemic.

That Coxsackie A 9 virus was isolated from the spinal fluid of one case (case no. AM-85) in Aomori and also from the faeces of 13 cases, including case no. AM-85, in the same region, may indicate that Coxsackie A 9 was also partly the cause of the epidemic in this region.

Of the poliovirus excreted by three patients in Aomori (see also Table 8), one (that of no. AM-108) is possibly a wild strain because the patient had not received the live poliovaccine. The virus isolated from two other patients (AM-5 and AM-113), however, might have originated from the vaccine strains since these patients had received the live oral vaccine (Sabin's triple) within 2 weeks before onset of the aseptic meningitis.

Properties of the viruses isolated

Coxsackie B 5 virus

It is evident from Table 2 that this virus was more readily isolated in HeLa cells than in monkey kidney cells. Out of a total of 21 isolations from the spinal fluid, 10 grew only in HeLa cells and 1 grew only in monkey kidney cells. The remaining 10 were isolated in both cells.

The results of assay of viruses in the spinal fluids and faeces seemed to be concordant with the above results, i.e. the virus titres obtained in HeLa cells were generally higher than those in monkey cells (Table 3).

Table 1. *Virus isolation from the patients with aseptic meningitis*

Region	No. of cases	Spinal fluids: No. of specimens tested	Spinal fluids: No. of virus isolated: Coxsackie B5	Spinal fluids: No. of virus isolated: Coxsackie A9	Spinal fluids: No. of virus isolated: Total	Faeces: No. of specimens tested	Faeces: No. of virus isolated: Coxsackie B5	Faeces: No. of virus isolated: Coxsackie A9	Faeces: No. of virus isolated: Polio 3	Faeces: No. of virus isolated: Un-identified	Faeces: No. of virus isolated: Total
Aomori City	234	217	52	1	53	115	43	13	3	15	74
Hirosaki	17	14	5	0	5	17	5	0	0	0	5
Hachinohe	10	6	0	0	0	8	0	0	0	0	0
Mutsu	18	18	0	0	0	0	0	0	0	0	0
Kuzumaki	16	15	3	0	3	7	3	0	0	0	3
Total	295	270	60	1	61	147	51	13	3	15	82

Table 2. *Comparative sensitivity of HeLa cells and monkey kidney cells in isolation of Coxsackie B5 virus from the clinical specimens**

Specimen	Number of virus isolated in following culture: HeLa only	MK only	HeLa and MK	Total
Spinal fluids	10	1	10	21
Faeces	1	0	19	20
Total	11	1	29	41

* Individual specimens were inoculated into HeLa cell and monkey kidney (MK) cell cultures separately.

Table 3. *Assay of amount of Coxsackie B5 virus in clinical specimens by using HeLa cell and monkey kidney cell cultures*

		Amount of virus			
		Tube culture method		Plaque method	
Specimen	Case no.	HeLa cell (TCID 50/ml. or g. (−log))	Monkey kidney cell (TCID 50/ml. or g. (−log))	HeLa cell (p.f.u./ml. or g.)	
Spinal fluids	AM-69	< 1·8	< 1·8	10	(0)*
	AM-74	1·8	< 1·8	65	(0)
	AM-84	1·8	< 1·8	20	(0)
Faeces	AM-95	6·2	3·8	10×10^5	$(1{\cdot}5 \times 10^4)$
	AM-96	5·8	3·5	$8{\cdot}3 \times 10^5$	$(2{\cdot}0 \times 10^4)$
	AM-101	4·9	2·8	$2{\cdot}8 \times 10^5$	$(2{\cdot}0 \times 10^4)$

* The number in parentheses represents the number of large plaques.

To examine plaque characteristics of the virus in these clinical specimens, undiluted spinal fluids and appropriately diluted faecal suspensions, from the same specimens as were used for virus isolation, were inoculated into HeLa cell monolayers. The plaque formation was done principally according to the procedure described by Ketler, Hinuma & Hummeler (1961). Two morphologically different types of plaques were formed from faecal specimens; one was a minute, irregular but well-defined plaque with sharp boundary and another a large, round plaque with diffuse boundary. When plaques were observed on the 8th day after inoculation, the minute type and the large type were 0·5–1·0 mm. and 3–16 mm. in diameter respectively. The minute plaque was predominant in number; the ratio of minute plaques to large in three specimens examined was 65, 40 and 13 as shown in Table 3. From spinal fluids, only minute plaques were formed. Further studies on the plaque variants are now in progress. Strain AM-69-L-61, which was isolated from the spinal fluid of a 2-year-old girl (case no. AM-69) with aseptic meningitis was selected as a representative strain among 111 new strains of Coxsackie B5 virus, and was examined for its characteristics after three to four passages in HeLa cell cultures. This strain gave cytopathic effect on HeLa, FL and monkey kidney cells in monolayer cultures, yielding 10^5–10^7 TCID 50/ml. when titrated in HeLa cell cultures. Antigenicity of AM-69-L-61 virus and two other B5 strains isolated in Japan in 1960 was compared with that of the prototype Faulkner strain, by the cross-neutralization technique. Results are shown in Table 4. AM-69-L-61 strain did not completely cross with the Faulkner strain. On the other hand, the AM strain seemed to be very close to the other Japanese strains, although examination was done only from one side. Further antigenic analysis with both Faulkner and AM-69-L-61 strains is now being carried out. AM-69-L61 virus produced typical spastic paralysis in day-old mice when inoculated intraperitoneally with $10^{5{\cdot}3}$ TCID 50 of virus, and the mice died 4–10 days after inoculation. Marked colour changes in the interscapular fat pad were evident upon autopsy as described by

Dalldorf, Melnick & Curnen (1959). Five other Coxsackie B 5 strains so far examined in unweaned mice showed similar pathogenicity.

Table 4. *Antigenic variation of Coxsackie B 5 viruses*

Strain (year isolated)	Neutralizing-antibody titre*	
	Anti-Faulkner†	Anti-AM-69-L‡
Faulkner§ (1952)	320	80
AM-69-L (1961)	40	160
STS-604-L‖ (1960)	80	160
SD-58-K¶ (1960)	80	320

* Reciprocals of serum dilution which completely inhibited cytopathic effect of 100 TCID 50 of virus on HeLa cell culture.

† Anti-Faulkner rabbit serum obtained from the National Foundation, U.S.A.

‡ Anti-AM-69-L rabbit serum prepared in this laboratory by immunization with HeLa cell-grown virus which was pre-treated with fluorocarbon twice.

§ The strain was kindly supplied by Dr A. L. Barron, University of Buffalo. This had been passaged numerous times on suckling mice and then monkey kidney cells. Upon receipt, the virus was passaged twice on HeLa cells in this laboratory.

‖ The strain was kindly supplied by Dr R. Kono, Kyoto University. This had been isolated and passaged on FL cells. This was passed twice on HeLa cells in this laboratory.

¶ The strain was isolated from the faeces of a patient with aseptic meningitis in Sendai and passaged on FL cells in this laboratory. The virus was used after passage twice on HeLa cells.

Coxsackie A 9 virus

All the Coxsackie A 9 viruses were isolated only in monkey kidney cell cultures. The properties of strain AM-85-L-61 isolated from the spinal fluid of a 5-year-old boy with aseptic meningitis were examined. This virus was cytopathogenic to monkey kidney cells but not to HeLa and FL cells. Virus yield in monolayer culture of monkey kidney cells was usually 10^8–10^9 TCID 50/ml. Plaques formed on monkey kidney cell cultures by the strain, which had been passaged twice in monkey kidney cells, were characteristically large and round with diffuse boundaries, and the growth of the strain was less rapid than the Mahoney strain of poliovirus. Morphologically the plaques were not distinguishable from those produced by the PB strain of a prototype Coxsackie A 9 virus which was obtained by the courtesy of Dr A. L. Barron of the University of Buffalo. The plaques of AM-85-L-61 were also very closely related to those of the Grigg strain of Coxsackie A 9 illustrated in the paper by Melnick (1958). When the antigenic compositions of AM-85-L-61 and PB strains were examined by cross-neutralization test, no significant differences were seen. Day-old mice inoculated intraperitoneally with $10^{7\cdot7}$ TCID 50 of the AM-85-L-61 developed a prostrating paralysis and death followed within 2–5 days. A similar effect was observed after inoculation of 5 other freshly isolated strains of Coxsackie A 9.

Serological studies

Neutralizing-antibody titration against Coxsackie B5 virus was performed with the paired sera obtained from 184 patients with aseptic meningitis (Table 5). A fourfold or greater rise in antibody titre was demonstrated in 76 % of the patients from the spinal fluid or faeces of whom B5 virus was recovered. An additional 15 % of Coxsackie B5 virus-positive patients demonstrated the presence of antibody in a titre of 4 or greater without a rise in titre between the paired sera However, there were 4 patients without detectable antibody in the paired sera, from whom Coxsackie B5 virus was recovered from the spinal fluid. Of 100 patients with negative isolation of virus, 39 % showed a fourfold rise in antibody titre, 29 % showed antibodies present in a titre of 4 or greater without a rise in titre, and 32 % showed no detectable antibody titres.

Table 5. *Neutralizing antibody against Coxsackie B5 virus in the paired sera of* 184 *patients with aseptic meningitis*

Virus recovery		No. of patients	Antibody absent*		Antibody present			
					No rise†		Rise‡	
			No. of subjects	%	No.	%	No.	%
Positive B5 virus	Spinal fluids	52	4	8	6	12	42	80
	Faeces	32	3	9	7	22	22	69
	Total	84	7	8	13	15	64	76
Negative		100	32	32	29	29	39	39

* Less than 4. † 4 or greater with no rise. ‡ Fourfold or greater rise.

Table 6. *Neutralizing antibody against Coxsackie A9 virus in the paired sera of* 66 *patients with aseptic meningitis*

Virus recovery		No. of patients	Antibody absent		Antibody present			
					No rise		Rise	
			No. of subjects	%	No.	%	No.	%
Positive A9 virus	Spinal fluids	1	0	.	0	.	1	.
	Faeces	11	8*	73	1	9	2	18
Negative		54†	23	42	17	32	14	26

* One (case no. AM-91) of 8 was examined on a single serum obtained at the 9th day of disease.

† In the 54 patients, no viruses were isolated and rise of Coxsackie B5 antibody was not proved.

Neutralizing-antibody titration against Coxsackie A9 virus was performed with paired sera from 66 patients. The results are shown in Table 6. One patient (case no. AM-85) from whom Coxsackie A9 virus was isolated from both spinal fluid and faeces showed significant antibody rise. Of the other 11 patients who excreted

Coxsackie A 9 virus in the faeces, only 2 showed a significant rise of antibody titre, 1 showed antibody with no rise of titre and 8 showed no detectable antibody in paired sera.

Table 7. *Antibodies against Coxsackie A 9 and B 5 viruses in the paired sera of patients excreting Coxsackie A 9 virus*

		Antibody titre*						
		Coxsackie A 9				Coxsackie B 5		
	Age	NT†		CFT‡		NT		
Case no.	(year-month)	A§	C‖	A	C	A	C	Serodiagnosis
AM-85	5-7	0¶	64	0	4	16	16	Coxsackie A 9
AM-95-1	5-2	16	256	0	0	64	64	Coxsackie A 9
AM-81	3-3	64	256	0	0	16	64	Coxsackie A 9 or B 5
AM-79	9-3	64	64	0	4	64	64	Coxsackie A 9
AM-82-1	0-10	0	0	0	0	ND**	0	Unknown
AM-83-1	2-0	0	0	0	0	16	64	Coxsackie B 5
AM-83-2	2-2	0	0	0	0	64	64	Unknown
AM-84-1	0-9	0	0	0	0	16	256	Coxsackie B 5
AM-86-1	0-8	0	0	0	0	16	64	Coxsackie B 5
AM-97	5-7	0	0	ND	0	256	256	Unknown
AM-104	2-7	0	0	ND	ND	64	64	Unknown
AM-91	5-11	ND	0	ND	ND	ND	> 4	Unknown
AM-99	1-0	0	0	ND	ND	ND	> 4	Unknown

* Reciprocals of serum dilution.
† Neutralization test.
‡ Complement-fixation test.
§ Acute serum.
‖ Convalescent serum.
¶ Less than 4.
** Not done.

Complement-fixation tests were performed on paired sera of the Coxsackie A 9 virus-positive patients, using the antigen prepared from the monkey kidney tissue culture fluid infected with AM-85-L-61 virus. Negative results were obtained which were comparable with the results obtained in neutralizing-antibody determination as shown in Table 7. These results suggested that a large proportion of persons with alimentary tract infection with Coxsackie A 9 virus did not show a good antibody response. They also suggested that the aseptic meningitis of these patients might not be caused by Coxsackie A 9 virus but by other agents, even though Coxsackie A 9 virus was recovered from the faeces. Therefore, neutralizing antibody against Coxsackie B 5 virus in these paired sera was determined, because Coxsackie B 5 virus was considered to be the main causative agent for the epidemic. Results are shown in Table 7. Four patients showed a fourfold or greater rise in Coxsackie B 5 antibody titre, although one of them also demonstrated a significant rise of antibody against Coxsackie A 9. On the other hand, 54 patients in whom no viruses were recovered and a rise of Coxsackie B 5 antibody was not detected were selected and the paired sera were tested for Coxsackie A 9 antibody. Among them, 14 showed a fourfold rise in antibody against Coxsackie A 9 (Table 6). Thus the serodiagnostic results suggested that a portion of the population of patients with aseptic meningitis were caused by Coxsackie A 9 virus.

Neutralizing-antibody titration against polioviruses types 1, 2 and 3 was performed with the paired sera obtained from 12 selected patients. Among them, 3 were positive in isolation of type 3 poliovirus but the remainder had no evidence of infection with either Coxsackie B5 or Coxsackie A9. As shown by Table 8, a significant antibody rise was shown in 2 patients for type 2 poliovirus, and in one for type 3 poliovirus. In one case among the rest, simultaneous antibody rise was shown against types 2 and 3 poliovirus. The remaining 6 cases, not cited in Table 8, did not show a rise of antibody against any type of poliovirus. From these results, polioviruses type 2 and type 3 were also assumed to be the causative virus for a small portion of the aseptic meningitis, at the time of this epidemic.

Table 8. *Neutralizing antibody against polioviruses in the paired sera of patients with aseptic meningitis*

Case no.	Live polio vaccination*	Virus recovered from faeces	Neutralizing-antibody titre against poliovirus			Serodiagnosis
			Type 1	Type 2	Type 3	
AM-5	+	Polio 3	A 256	256	64	Unknown
			C 256	256	64	
AM-108	–	Polio 3	A	Serum not available		Unknown
			C 0	0	64	
AM-109	–	—	A 0	0	0	Polio type 2
			C 0	16	0	
AM-113	+	Polio 3	A 0	0	0	Polio type 3†
			C 0	0	16	
AM-344	–	—	A 0	4	0	Polio type 2
			C 0	64	0	
AM-366	–	—	A 0	64	4	Polio type 2 or 3
			C 0	1024	16	

* Both AM-5 and AM-113 received Sabin's oral triple live vaccine within 2 weeks before onset of the aseptic meningitis.

† Probably vaccination effect.

DISCUSSION

On the basis of the results of virus isolation, especially from spinal fluids of the patients, and of serological tests, causative agents of the aseptic meningitis occurring in Northern Japan in the summer of 1961 were determined to be Coxsackie B5 virus, and Coxsackie A9 virus to a lesser extent. The incidence of the illness attributable to polioviruses seemed to be very low. Yamada and his colleagues (personal communication) reported that more than 300 Coxsackie B5 virus strains were isolated from faeces of healthy children and patients with either acute febrile illness or aseptic meningitis in Hokkaido during the summer of the same year. This evidence suggests that there was an extensive seeding of Coxsackie B5 virus in the population of both northern Honshu and Hokkaido. On the other hand, there were no reports suggesting the spreading of Coxsackie B5 in other areas of Japan, except a few isolations of the virus from healthy children. It should be mentioned that an outbreak of aseptic meningitis on a comparable

scale to that in Northern Japan in 1961 had occurred in Western Japan during the summer of 1960 and the causative agent was determined to be Coxsackie B 5 virus, as reported by Kono *et al.* (1960). In that year, we found 9 patients with aseptic meningitis associated with Coxsackie B 5 virus in Aomori during August to October, but the spreading pattern of the infection was not likely to be epidemic. However, it was apparent that Coxsackie B 5 virus had begun to spread in this region at that time. The sero-immunity against Coxsackie B 5 virus in children in Aomori had been very poor before the 1961 epidemic as shown in Fig. 1. From these facts, it was considered reasonable to assume that Coxsackie B 5 virus began to invade children in Aomori in the late summer to early autumn of 1960, and in the following summer the virus spread out explosively in this region. In addition, attempts to isolate Coxsackie B 5 virus were not successful from clinical specimens of aseptic meningitis obtained in the years before and after 1960 and 1961 in Northern Japan.

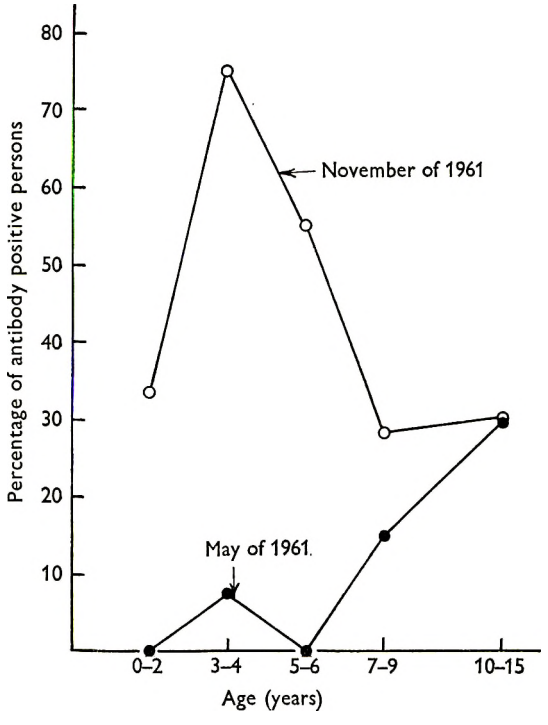


Fig. 1. Age-specific antibody patterns to Coxsackie B 5 virus of the children in Aomori, before and after epidemic in summer of 1961. The children bled on the two occasions were not the same. Positive antibody was determined as a titre of 4 or more against 100 TCID 50 of the AM-69-L-61 virus.

Coxsackie B 5 has caused fairly large epidemics of aseptic meningitis in the U.S.A. in 1956 and 1957 (Syverton *et al.* 1957; Rubin *et al.* 1958; Curnen *et al.* 1958; Gordon, Lennette & Sandrock, 1959); in Canada in 1958 (Walker, McNaughton & McLean, 1959; Cooper, Lesiak, Belbin & Labzoffsky, 1961) and in Japan in 1960 (Kono *et al.* 1960). The accumulated evidence thus appears to show that

Coxsackie B5 virus is one of the important enteroviruses which can induce extensive epidemic aseptic meningitis.

The fact that 17 patients were diagnosed as Coxsackie A9 aseptic meningitis by a serological test should not be ignored in discussing the causative agents in the epidemic described here. Although aseptic meningitis attributable to Coxsackie A9 has been described in many reports, there has been no large epidemic like that due to Coxsackie B5. However, it was interesting that Lerner, Klein, Levin & Finland (1960) reported 15 cases of Coxsackie A9 infection in Boston during July to October 1962 and Lerner, Klein & Finland (1960) also reported a small outbreak which occurred in a laboratory. It seems worth noting that the A9 virus has sometimes produced severe or fatal illness, as in the latter outbreak. The report of Hughes, Webb, Chang & Hart (1963) concerns an outbreak caused by A9 virus in Hong Kong during June to August, 1961. They isolated the virus from spinal fluids of 9 patients. The Coxsackie A9 virus described here was the first isolation in Japan and the strain AM-85-L-61 was one of relatively few strains isolated from spinal fluids so far reported (Davis & Melnick, 1958; Sabin, Krumbiegel & Wigand, 1958; Godtfredsen, 1959; Lerner, Klein & Finland, 1960; Cooper *et al.* 1961; Hughes *et al.* 1963). In addition, we have isolated Coxsackie A9 virus from the spinal fluids of 2 cases with aseptic meningitis in Aomori, 1962. The details of this study will be reported elsewhere.

During the course of the present studies, we have encountered several interesting virological and serological facts. One of them was that Coxsackie B5 virus antigen was detected by immunofluorescence in the urinary cells excreted from the patients with aseptic meningitis, as reported by Hinuma, Miyamoto, Murai & Ishida (1962). Demonstration of Coxsackie B5 plaque variants isolated from the alimentary tract in HeLa cells was also interesting, in a comparison with plaque variants of echovirus 6 in faeces observed by Suto, Karzon & Bussel (1962). As for the effect of subsequent passage upon the plaque variants, the answer should await further studies.

In general, viral infection produces the homologous antibody in the course of the illness. However, the Coxsackie A9 virus infection observed in this study was unusual. Eight of 11 patients who excreted Coxsackie A9 virus did not show detectable neutralizing antibody against 100 TCID 50 of the virus. The complement-fixing antibody was also lacking. Johnsson, Böttiger & Löfdahl (1958) reported that presence of neutralizing antibody was not demonstrated, by the conventional tube test, in the convalescent serum of a patient from whose faeces echovirus 4 was recovered. In their case, however, the complement-fixing antibody was detectable. Since both neutralizing and complement-fixing antibodies were not detectable in the serum of the Coxsackie A9 positive patient, the Coxsackie A9 infections appear to be different in their pathogenesis from the echovirus 4 infections. Why a large proportion of persons having alimentary tract infection with Coxsackie A9 did not produce detectable antibodies is unsolved as yet.

SUMMARY

Virological and serological studies on an outbreak of aseptic meningitis in association with Coxsackie B 5 virus, and Coxsackie A 9 virus to a lesser extent, are reported. Coxsackie B 5 virus was isolated from spinal fluids of 60 patients and Coxsackie A 9 virus from one spinal fluid. Fifty Coxsackie B 5 and 13 Coxsackie A 9 viruses were recovered from patients' faeces. The evidence that the epidemic was caused by these viruses was confirmed by determination of neutralizing antibody in paired sera.

The plaque variants of Coxsackie B 5 virus isolated from the faecal specimens and the poor antibody response in Coxsackie A 9 infection were reported as points of virological and serological interest.

REFERENCES

COOPER, M. R., LESIAK, J. M., BELBIN, D. & LABZOFFSKY, N. A. (1961). Isolation of enteric viruses during the poliomyelitis season in Ontario, 1956–1959. *Canad. med. Ass. J.* **84**, 200–5.

CURNEN, E. C., LONDON, A. H., TANABE, E., GELEZEN, W. P. & KOOMEN, J. (1958). An epidemic of aseptic meningitis attributable to Coxsackie B 5 virus. *Amer. J. Dis. Child.* **96**, 571–3.

DALLDORF, G., MELNICK, J. L. & CURNEN, E. C. (1959). The Coxsackie virus group. In *Viral and Rickettsial Infections of Man*, pp. 519–46, 3rd ed. Ed. T. M. Rivers and F. L. Horsfall. London: Pitman Medical Publishing Co. Ltd.

DAVIS, D. C. & MELNICK, J. L. (1958). Poliomyelitis and aseptic meningitis, a 2-year field and laboratory study in Connecticut. *J. Lab. clin. Med.* **51**, 97–117.

GODTFREDSEN, A. (1959). Strains of Coxsackie-A 9 and ECHO-9 recovered from cerebrospinal fluids. *Acta path. microbiol. scand.* **46**, 217–22.

GORDON, R. B., LENNETTE, E. H. & SANDROCK, R. S. (1959). The varied clinical manifestations of coxsackie virus infections. *Arch. intern. Med.* **103**, 63–75.

HINUMA, Y., MIYAMOTO, T., MURAI, Y. & ISHIDA, N. (1962). Detection of Coxsackie virus antigen in urinary cells by immunofluorescence. *Lancet*, ii, 179–80.

HUGHES, D. T. D., WEBB, J. F., CHANG, W. K. & HART, R. J. C. (1963). Aseptic meningitis in Hong Kong. *Brit. med. J.* ii, 649–53.

JOHNSSON, T., BÖTTIGER, M. & LÖFDAHL, A. (1958). An outbreak of aseptic meningitis with rubella-like rash probably caused by ECHO virus type 4. *Arch. ges. Virusforsch.* **8**, 306–17.

KETLER, A., HINUMA, Y. & HUMMELER, K. (1961). Dissociation of infective poliomyelitis virus from neutralizing antibody by fluorocarbon. *J. Immunol.* **86**, 22–7.

KONO, R., ASHIHARA, Y., HAMADA, C., KAWAKAMI, K., AKAGI, N., FURUKAWA, M. & MIYAO, M. (1960). 4. Studies on the epidemiology of Coxsackie B-5 virus meningitis. *Ann. Rep. Inst. Virus Res. Kyoto Univ.* **3**, 99–113.

LERNER, A. M., KLEIN, J. O. & FINLAND, M. (1960). A laboratory outbreak of infections with Coxsackie virus group A type 1. *New Engl. J. Med.* **263**, 1302–4.

LERNER, A. M., KLEIN, J. O., LEVIN, H. S. & FINLAND, M. (1960). Infections due to Coxsackie virus group A, type 9, in Boston, 1959, with special reference to exanthems and pneumonia. *New Engl. J. Med.* **263**, 1265–72.

MELNICK, J. L. (1958). Advances in the study of the enteroviruses. *Progr. med. Virol.* **1**, 59–99.

NAKAO, T., NITTA, T., MIURA, R., OGATA, K., TAKAGI, M., KUME, T., NOBUTA, K. & HINUMA, Y. (1964). *Tohoku J. exp. Med.* (in the Press).

RUBIN, H., LEHAN, P. H., DOTO, I. L., CHIN, T. D. Y., HEEREN, R. H., JOHNSON, O., WENNER, H. A. & FURCOLOW, M. L. (1958). Epidemic infection of Coxsackie virus group B, type 5. *New Engl. J. Med.* **258**, 255–63.

SABIN, A. B., KRUMBIEGEL E. R. & WIGAND, R. (1958). ECHO type 9 virus disease. *Amer. J. Dis. Child.* **96**, 197–219.

SUTO, T., KARZON, D. T. & BUSSEL, R. H. (1962). Behavior of ECHO-6 plaque variants following isolation from human alimentary tract. *Bact. Proc.* (1962), 135.

SYVERTON, J. T., MCLEAN, D. M., MARTINS DA SILVA, M., DOANY, H. B., COONEY, M., KLEINMAN, H. & BAUER, H. (1957). Outbreak of aseptic meningitis caused by Coxsackie B 5 virus. *J. Amer. med. Ass.* **164**, 2015–19.

WALKER, S. J., MCNAUGHTON, G. A. & MCLEAN, D. M. (1959). Coxsackie B 5 virus infections in children: Toronto, 1958. *Canad. J. publ. Hlth*, **50**, 461–7.

J. Hyg., Camb. (1964), **62**, 171
Printed in Great Britain

The feeding of oral poliovirus vaccine to a closed community excreting faecal viruses

By P. S. GARDNER and CHRISTINE E. COOPER

Virus and Tumour Research Section, Department of Microbiology and Department of Child Health, Royal Victoria Infirmary and University of Newcastle upon Tyne

(*Received* 23 *November* 1963)

INTRODUCTION

The long-continued faecal excretion of adenoviruses in a closed community has been recently reported (Gardner, Wright & Hale, 1961) and it was considered to be of interest to re-examine the same community a year and a half later to see if adenoviruses were still being excreted. This communication presents several new features: the finding of a changing endemic virus population; the studying of the effect of oral poliovirus vaccination on these viruses; the effect of these viruses on oral poliovirus vaccine.

These have been studied in individuals (Hale, Lee & Gardner, 1961) and the effect of enteroviruses on poliovirus vaccination in large communities has been well recorded. Sabin *et al.* (1960) found that, in Mexico, 50% of the population excreted non-polio enteric viruses both before and after vaccination while Dömök, Molnan & Jancso (1961) found that there was a rapid decrease in Coxsackie and echovirus excretion after the first oral vaccination.

Another aspect of this investigation has been to consider the general health of this community throughout the period in relation to the various adenoviruses and enteroviruses found in the stools.

The clinical significance of echovirus excretion is still incompletely understood but outbreaks of aseptic meningitis, fever, rash, respiratory infection and abdominal symptoms, especially in summer and autumn, may be associated with echovirus infections (Wright *et al.* 1961; Pelon, 1961; Ramos-Alvarez & Sabin, 1958).

INVESTIGATION

The residential home was in the grounds of a country house, in the charge of a matron. The staff consisted of qualified nurses and student nurses, most of whom were non-resident. The non-resident domestic staff were recruited locally. The length of time the children stayed in the home varied from a few weeks to many months, the latter being more usual. There were about 18 children in the home and they lived in two groups; the first group was the babies under a year old who lived in their own nursery; the second group was aged from 1 to 6 years and mixed with great freedom together. The two groups were kept separate though occasionally toddlers wandered into the nursery, and sometimes one of the older babies visited the toddlers for tea.

Faecal specimens from the children in the first 2 months of the investigation were sent twice a week and thereafter at weekly intervals. Ten per cent suspensions of faeces were made in Hanks basic salt solution containing antibiotics and 0·2 ml. were inoculated into HeLa, Hep 2 and monkey kidney tissue cells. Enteroviruses were identified by neutralization tests and adenoviruses by complement fixation as well as by neutralization (Hale *et al.* 1961; Gardner *et al.* 1961). Where there was a possibility that two enteroviruses might have been present and only one isolated, the investigation of the specimen was repeated and antiserum was incorporated to neutralize the virus already isolated in order to reveal any other virus which might have been present (Hale *et al.* 1961). This type of investigation was performed on the majority of specimens when polioviruses were being freely excreted and later on when there was evidence of reappearance of echovirus 11.

As in the previous investigation (Gardner *et al.* 1961), no attempt was made to subject the children to periodic bleeding as this would have led to the loss of confidence between children and staff which we felt was of prime importance. Since the primary interest of this investigation was the spread and the interaction of viruses in this community, the evidence could be found in faecal excretion.

All children in this home were given triple oral poliovirus vaccine at the beginning of September 1962, a second dose was given half-way through November and the third dose in mid-December. No further poliovirus vaccine was given during the remaining time of the study.

RESULTS

The endemic virus population

In the period before the feeding of the nineteen children in the home with poliovirus vaccine, that is, in the last 2 weeks of August 1962, fourteen were excreting echovirus 11 and one Coxsackie B3 virus. As soon as oral poliovirus vaccine was given, echovirus 11 completely disappeared and was replaced by the avirulent vaccine strains of poliovirus. All children in the nursery were vaccinated, but some were discharged too soon for follow-up studies. However, out of the twelve children given oral poliovirus vaccine and followed up, only one child who was 8 weeks old failed to show poliovirus excretion. He had five stools examined before vaccination and many more after; none contained virus which was detectable by tissue culture techniques.

Early in October 1962, echovirus 6 appeared for the first time and spread rapidly in the home. During October there were eighteen children in the home, of whom fifteen were excreting echovirus 6. Fourteen children arrived in the home between the first and second oral vaccinations; only two became infected with poliovirus type 2 vaccine strain which contrasted sharply with the ten children who acquired echovirus 6 infection during this period. The likelihood of infection did not vary with the age of the child and a child less than 1 year of age acquired an echovirus infection as easily as a toddler or older child.

At the end of December adenoviruses first appeared. As previously observed (Gardner *et al.* 1961) these were mainly of the non-epidemic types, i.e. 1, 2, 5 and 6.

They spread around the nursery, but did not in any way curtail the excretion of echovirus 6 which persisted well into May 1963. Adenoviruses were consistently present in the children under 1 year of age, nine out of ten being infected in comparison with three out of twelve of those over 1 year old; this was in accordance with previous observations (Gardner *et al.* 1961). Echovirus 11 reappeared at the end of February 1963 but was not widespread and did not re-infect the few remaining children who had originally been excreting echovirus 11.

There seemed to be no mutual interference between the two echoviruses and the adenoviruses, and many combinations of these viruses were found in the same stools. It appeared, too, that echovirus 6 and 11 did not exclude each other. When echovirus 6 became established in a child there was a prolonged excretion and one child so far has excreted this virus for 6 months and many other children have excreted for slightly shorter periods.

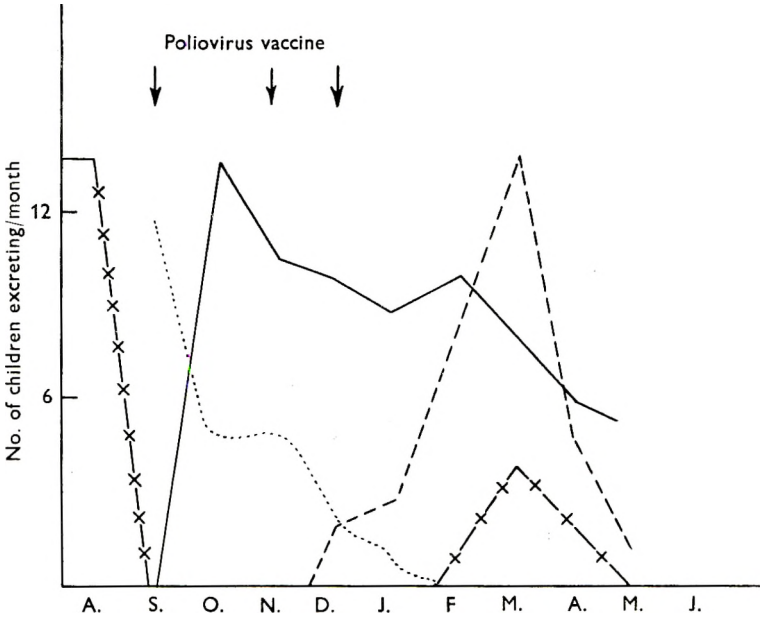


Fig. 1. Virus excretion in the residential home, August 1962–June 1963. –×–×, Echovirus 11; ——, echovirus 6; . . . . ., poliovirus from vaccine; – – – – –, non-epidemic adenovirus.

Figure 1 shows a diagrammatic representation of virus excretion in this nursery over the period of the investigation.

Clinical picture in relation to virus excretion

The children in the home were, on the whole, very well during the period under study and though there occurred an occasional coryzal and diarrhoeal episode, it bore no relationship whatsoever to the excretion of echoviruses or adenoviruses with the possible exception of one child. He developed a rash and fever which coincided exactly with the appearance of echovirus 6 in his stools which had

previously been negative. This child, too, suffered from fibrocystic disease which might account for his increased susceptibility.

There was an outbreak of chickenpox in February and March during which time a child who developed measles was admitted. It was decided to give all children over the age of four months gamma-globulin, in order to prevent the spread of measles throughout the home. The result of this was highly successful and no further cases of measles occurred. Eleven children received preventive doses of gamma-globulin but the gamma-globulin did not halt the excretion of virus already present, neither did it prevent a number of children acquiring echovirus 6 and adenovirus; one child even became infected with Coxsackie B2 virus during this period, presumably introduced from outside.

Acquisition of echovirus 6 and poliovirus by new arrivals

It was difficult to estimate how soon children, newly arrived in the home, acquired echovirus 6 infection because in some cases there was a delay of one or two weeks before the first specimen was sent to the laboratory. It was also impossible to say whether a child had brought the echovirus 6 in from outside when this virus was present in the first stool examined; this, however, was unlikely as echovirus 6 infection was not present in the general population, as judged from our concurrent surveys of normal children. If it is assumed that the echovirus 6 infections were acquired in the home, then over 80 % were acquired within the first fortnight. At that time half the children were under 1 year of age and half were over a year, age making no difference to infection by echovirus 6.

Stools from a number of children were sent to the laboratory very shortly after admission. In these children it was found that echovirus 6 was acquired in a period as short as 1–5 days. The speed of acquiring infection was not related to age and infants in cots were infected in just as short a time as older children who were running about and mixing freely with their own age group. On the other hand, vaccine strains of poliovirus showed little spread around the nursery and only two children acquired these strains between the first and second vaccinations; both these infections were with the dominant type 2 poliovirus strain (Hale *et al.* 1961).

The interaction of poliovirus vaccine and the endemic viruses

Twelve children were followed up for a sufficient length of time after their first poliovirus vaccination to determine the extent of virus excretion. Table 1 shows the effect that poliovirus had on echovirus 11 which was present in the nursery before poliovirus vaccination; a child was also excreting Coxsackie B3 at the time. The child who failed to excrete poliovirus showed no evidence of being previously infected with another virus to account for this.

Table 1 shows the effect of feeding poliovirus vaccine in November and December to the new arrivals in the home; it also summarizes the effect of the second and third doses of vaccine in the presence of echovirus 6 and adenovirus excretion.

An attempt was made to estimate the length of poliovirus excretion in the home. It was found that the eleven children excreting poliovirus after feeding at the first occasion had an average excretion time of 30 days. When the children who had

primary poliovirus vaccination after the appearance of echovirus 6 and adenovirus in the home were examined for length of virus excretion, the average excretion time of poliovirus was 10 days. This included two children who had not had an echovirus 6 infection and excreted for a much longer time than this average.

Table 1. *The effect of oral poliovirus vaccine on the excretion of echovirus and adenovirus*

Feed	First	Second		Third		
Month	September	November		December		
Dose	1st	1st	2nd	1st	2nd	3rd
Given oral poliovirus vaccine	12*	9	4	3	6	3
Excreting poliovirus more than once	11	4	1	1	2	0
Previously excreting echovirus 11	8	.	.	.	.	.
Previously excreting Coxsackie virus B3	1	.	.	.	.	.
Excreting echovirus 11 or Coxsackie virus B3 after vaccination	0	.	.	.	.	.
Excreting echovirus 6 both before and after vaccination	.	5	4	3	.	.
Excreting echovirus 6 or adenovirus both before and after vaccination	.	.	.	.	5	3
Previously excreted echovirus 6, and excreted poliovirus more than once	.	1	.	1	.	.
Excreted poliovirus after vaccination, with no previous echovirus 6 excretion	.	0	.	.	.	.

* More than 12 were given the first feed of poliovirus vaccine, but only 12 were followed up.

DISCUSSION

The residential home has presented a complex picture; two echoviruses, 11 and 6, and a variety of non-epidemic adenoviruses have spread freely amongst the children. Though echovirus 11 may be found without any clinical illness, it is often associated with a variety of respiratory infections, enteritis, febrile illnesses with rash and very occasional paralysis and aseptic meningitis (Philipson, 1958; Ramos-Alvarez & Sabin, 1958; Sommerville, 1958; Elvin-Lewis & Melnick, 1959). On the other hand, echovirus 6 has rarely been described without clinical symptoms. There have been many descriptions of outbreaks of viral meningitis, enteritis and encephalitis due to this virus (Sandford & Sulkin, 1959; Karzon, Barron, Winkelstein & Cohen, 1956; Davis & Melnick, 1956). A surprising feature in this nursery is that in spite of the rapid infection of new arrivals, particularly by echovirus 6, in only one instance could an illness be associated with a virus, in this case echovirus 6. In this nursery the echoviruses and adenoviruses had little clinical

significance, which was at variance with the larger similar investigation of the American Junior Village when febrile illnesses were associated with new virus infections (Bell *et al.* 1961).

The next unexpected finding was that as soon as oral poliovirus vaccine was fed to children, echovirus 11 was completely replaced. This may be an example of interference, and in our experience here (Pal, McQuillin & Gardner, 1963) it was found that echovirus 11 was exceedingly slow growing and it is possible that the more rapidly growing poliovirus completely replaced echovirus 11 in the children's bowels.

Hale *et al.* (1961), showed that children who were susceptible to a particular type of poliovirus would excrete that virus. As it was not feasible in this home to employ serological techniques, the assumption was made that the children of these age groups were unlikely to be immune to all three types of poliovirus and should, therefore, excrete at least one of the types on at least one occasion. The younger the age group, the more likely they would be to excrete all three types. It was, therefore, decided to use faecal excretion of poliovirus as an index of the susceptibility of these children to poliovirus and if such excretion were absent it might be related to interference by echovirus 6 or adenovirus.

The age distribution of the twelve children given poliovirus vaccine for the first time on the first occasion was the same as the age distribution of the twelve children fed for the first time on the second and third occasions when echovirus 6 and adenovirus were present. In the first group of twelve children all except one excreted poliovirus showing that they were all susceptible. This one failure, aged 8 weeks, could not be shown to be due to a virus detectable by tissue culture techniques. Only five of the twelve children who received vaccine for the first time in November and December excreted poliovirus, and of the nine children excreting echovirus 6 before poliovirus vaccination, only two eventually excreted poliovirus (see Table 1). The difference in poliovirus excretion by the two groups is significant ($\chi^2 = 4{\cdot}7$, $0{\cdot}05 > P > 0{\cdot}02$). It was also noted that some of those children who were excreting poliovirus during the echovirus 6 outbreak had the length of poliovirus excretion curtailed as they acquired their echovirus 6 infection. In no instance did poliovirus vaccine stop either echovirus 6 or adenovirus excretion and these viruses, if present before vaccination, were still present after.

Those children who were fed poliovirus vaccine the second time should still show some poliovirus excretion (Hale *et al.* 1961) and Table 1 shows that out of ten children only three excreted poliovirus while nine excreted echovirus 6 and adenovirus before and after vaccine feeding. These children are unlikely to be immune to all three types of poliovirus after only one feed and so this again demonstrates some interference. Table 1 shows that there is no excretion of poliovirus in those three children who were in residence long enough to receive all three feeds, and this is almost certainly due to immunity though the possibility of interference from echovirus 6 and adenovirus could not be completely eliminated. The picture that has been presented clearly demonstrates that residential nurseries and homes are not the ideal place to attempt oral poliovirus vaccination because virus interference may be an all important factor in unsuccessful immunization.

It is worth noting, too, that though mixtures of echovirus 6 and 11 with adenovirus occurred, on no occasion was poliovirus isolated from such a specimen, and in only the two instances noted did some poliovirus excretion occur after echovirus 6 excretion. Related to this problem of virus replacement was the ease with which echovirus 6 spread rapidly through the whole home, and in some cases it was apparent that children under 1 year of age and confined to their cots acquired an echovirus 6 infection in as little as 2 or 3 days. On the other hand, poliovirus vaccine strains did not spread rapidly round the home and this has been the experience of others (Hoskins *et al.* 1962). The obvious difference in infectivity of these strains might help to account for the interference picture produced by echovirus 6. As discussed previously (Gardner *et al.* 1961), the possibility of interferon production by poliovirus in the bowel might lead to suppression of other viruses and factors such as these may play a part in this very complex pattern.

Lastly, it should be noted that the giving of gamma-globulin had no effect whatsoever on the excretion of echovirus and adenoviruses, nor had it any preventive action on the acquisition of these viruses.

SUMMARY

The residential home was investigated to study the endemic virus population. During this study, oral poliovirus vaccine was given to the children and the effect of vaccination on endemic enteroviruses and adenoviruses was investigated. In the light of these findings, the problem of virus interference is discussed. The health of the children is related to their virus infections.

We are indebted to Prof. S. D. M. Court and Prof. C. A. Green for the active interest they have taken in this investigation; to Miss J. McQuillin, B.Sc., F.I.M.L.T., for her technical assistance; also to the matron of the home and her staff for their co-operation in supplying the specimens, and to Mr L. J. Whitlock of the Royal Victoria Infirmary and his staff for arranging their transport to the laboratory.

REFERENCES

Bell, J. A., Huebner, R. J., Rosen, L., Rowe, W. P., Cole, R. M., Mastrota, F. M., Floyd, T. M., Chanock, R. M. & Shevedoff, R. A. (1961). Illness and microbial experiences of nursery children at Junior Village. *Amer. J. Hyg.* **74**, 276.

Davis, D. C. & Melnick, J. L. (1956). Association of ECHO virus type 6 with aseptic meningitis. *Proc. Soc. exp. Biol., N.Y.*, **92**, 839.

Dömök, I., Molnan, E. & Jancso, A. (1961). Virus excretion after mass vaccination with attenuated polioviruses in Hungary. *Brit. med. J.* i, 1410.

Elvin-Lewis, M. & Melnick, J. L. (1959). ECHO 11 virus associated with aseptic meningitis. *Proc. Soc. exp. Biol., N.Y.*, **102**, 647.

Gardner, P. S., Wright, A. E. & Hale, J. H. (1961). Faecal excretion of adenovirus in a closed community. *Brit. med. J.* ii, 424.

Hale, J. H., Lee, L. H. & Gardner, P. S. (1961). Interference patterns encountered when using attenuated poliovirus vaccines. *Brit. med. J.* ii, 728.

Hoskins, J. M., Hobson, D., Udall, V., Madland, T., Goffe, A. P., Stuart-Harris, C. H. & Herzog, E. G. (1962). Small-scale trial with Sabin attenuated Type I poliovirus vaccine in a semiclosed community. *Brit. med. J.* i, 747.

Karzon, D. T., Barron, A. L., Winkelstein, W. & Cohen, S. (1956). Isolation of ECHO virus type 6 during outbreak of seasonal aseptic meningitis. *J. Amer. med. Ass.* **162**, 1298.

PAL, S. T., MCQUILLIN, J. & GARDNER, P. S. (1963). A comparative study of susceptibility of primary monkey kidney cells, Hep 2 cells and HeLa cells to a variety of faecal viruses. *J. Hyg., Camb.*, **61**, 493.

PELON, W. (1961). Classification of the '2060' viruses as ECHO 28 and further studies on its properties. *Amer. J. Hyg.* **73**, 36.

PHILIPSON, L. (1958). Aetiology of non-diphtheritic croup. II. Virologic investigation. *Acta Paediat.* **47**, 611.

RAMOS-ALVAREZ, M. & SABIN, A. B. (1958). Enteropathogenic viruses and bacteria. *J. Amer. med. Ass.* **167**, 147.

SABIN, A. B., RAMOS-ALVAREZ, M., ALVAREZ-AMEZQUITA, J., PELON, W., MICHAELIS, R. H., SHIGLAND, I., KOCK, M. A., BARNES, J. M. & RHIM, J. S. (1960). Live, orally given poliovirus vaccine. Effects of rapid mass immunization on population under conditions of massive enteric infection with other viruses. *J. Amer. med. Ass.* **173**, 1521.

SANDFORD, J. P. & SULKIN, S. E. (1959). The clinical spectrum of ECHO-virus infection. *New Engl. J. med.* **261**, 1113.

SOMMERVILLE, R. G. (1958). Enteroviruses and diarrhoea in young persons. *Lancet*, ii, 1347.

WRIGHT, A. E., GARDNER, P. S., HALE, J. H., CREERY, R. D. G., MOODY, D., SMITH, A., BELL, D. J., SMITH, D. F. & BELL, G. M. (1961). An account of two outbreaks of ECHO 9 virus infection on Tyneside. *Newc. med. J.* **27**, 13.

J. Hyg., Camb. (1964), **62**, 179
Printed in Great Britain

'False positive' complement fixation with psittacosis–trachoma antigens due to antibodies in complement preparations*†

By ALEXANDER L. TERZIN‡

From the Department of Microbiology, U.S. Naval Medical Research Institute, Bethesda, Md., and Medical Faculty, Sarajevo (Yugoslavia)

(*Received* 3 *December* 1963)

In the course of serological studies with psittacosis and trachoma during the last few years, first in Sarajevo and later in Bethesda, difficulties have been experienced attributable to the presence of antibodies to the psittacosis-group agents in guinea-pig sera used as source of complement. In view of the increasing interest in the serology of trachoma and psittacosis group agents, it is worth while to describe briefly the observations made and to point out an important source of potential error.

METHODS

Complement fixation (C.F.) and C.F.-inhibition tests have been performed as described by Hoyle (1948), and by an adaptation of the same technique (Terzin, 1958), respectively. The 'units' of antigen and antibody were determined by checkerboard titration, as described elsewhere (Terzin, 1960).

Samples of psittacosis-positive and psittacosis-negative sera of rabbits, as well as guinea-pig sera primarily intended for use as complement, were inactivated for 30 min. at 56° C. before testing for presence of antibodies.

Samples of guinea-pig sera tested for their complement titres were stored lyophilized at 4° C., or frozen at −60° C. and rehydrated or melted, respectively, immediately before titration. Samples of at least ten guinea-pig sera intended for use as complement were absorbed with red blood cells of sheep as recommended by Mayer (Kabat & Mayer, 1961). Samples of absorbed and unabsorbed guinea-pig sera, when inactivated and titrated for the presence of Bedsonia antibodies, showed no detectable difference in C.F. titres or anticomplementary activity. Consequently, the following findings observed in samples of unabsorbed guinea-pig sera apply also to the absorbed specimens.

RESULTS

Comparison of different batches of complement

A total of fifteen batches of complement, originating from five different sources, were inactivated and tested by C.F. test against influenza, mumps and typhus or

* This investigation was conducted under a Fight for Sight Postdoctoral Research Fellowship of the National Council to Combat Blindness, Inc., New York.

† The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

‡ Present address: Medical Faculty, Novi Sad, Yugoslavia.

Q-fever antigens. Twelve of these showed completely negative results in dilutions 1/2 or higher, while three samples revealed anticomplementary activity as shown in Table 1. The same fifteen batches of guinea-pig sera were tested also against Bedsonia antigen with known antibody-free guinea-pig complement. The C.F., C.F.-inhibiting and anti-complementary titres, found in tests repeated several times, are shown in Table 1. Ten batches showed measurable C.F. or C.F.-inhibiting titres, three of them as high as 1/16 or 1/32. Two C.F. negative samples showed C.F.-inhibition titres of 1/4 and 1/16, respectively.

Table 1. *Anti-Bedsonia titres found in fifteen batches of guinea-pig sera intended for use as complement**

	Commercial sources							Bethesda laboratory				Sarajevo laboratory			
	a		*b*			*c*									
Batches	1	2	1	2	3	1	2	1	2	3	4	1	2	3	4
C.F.	8	16	< 2	4	4	2	16	< 2	< 2	4	4	< 2	4	4	32‡
C.F. inhibition	< 2	< 2	< 2	< 2	< 2	< 2	< 2	4	4	< 2	< 2	16	< 2	< 2	4
A.C.†	4	2	< 2	< 2	< 2	< 2	4	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

* Each was titrated with 2 and 9 units of homologous antigen.
† Anticomplementary activity (saline control).
‡ Specimen showing prozone in the C.F. test.

When C.F.-positive guinea-pig sera, with anti-psittacosis titres as high as 1/16 and 1/32, were used as sources of complement in Bedsonia anti-Bedsonia C.F.-systems, they caused apparently nonspecific positive reactions. When the guinea-pig serum with a C.F.-inhibiting titre of 1/16 was used as a source of complement, it caused a two- to fourfold decrease in the C.F. titres of Bedsonia positive sera.

As seen from Table 1, both the highest C.F. and C.F.-inhibiting titres were obtained with sera of guinea-pigs from Sarajevo. These titres could be attributed to an otherwise inapparent, accidental cross-infection of the complement-donating animals with ornithosis strain P-4.

Effect of the anti-Bedsonia C.F. *titre of a complement preparation on antigen and antibody titres*

Table 2 shows the increment of antigen titres, and of rabbit antibody titres in C.F. tests when different samples of Bedsonia-positive guinea-pig sera (C′2, C′3, C′4, C′5 and C′6) were used as sources of complement. These are compared with the respective titre increments obtained in similar tests performed with a Bedsonia-negative guinea-pig serum as source of complement (C′1).

Since each of the six C′ samples showed a complement titre 1/60, all six complements were used in 1/30 dilution. Consequently, each of the test tubes set up with C′2–C′6 contained additional Bedsonia antibody units which amounted to about 1/30 of the antibody units contained per volume of the respective undiluted guinea-pig serum used as complement. Such additional amounts of antibody originating from the sera of Bedsonia-positive complement donors, when added to

the antibody amounts contained in the sera of humans or rabbits actually titrated against the Bedsonia antigen, account for the change of titres observed in C.F. tests, set up with the same antigen-antiserum system versus different complements (e.g. with C′1 or with C′2–C′6, respectively).

Table 2. *Tests with six complement samples showing different anti-Bedsonia titres*

Complement samples ...		C′1	C′2	C′3	C′4	C′5	C′6
Anti-Bedsonia titres (C.F.) ...		< 1/2	1/2	1/4	1/8	1/16	1/32
C.F. units per 1 vol. of a 1/30 dilution of C′		⩽0·033	0·066	0·133	0·266	0·533	1·066
One volume of the respective C′ 1/30 causes in C.F. test	Titre increase of Bedsonia antigen*	× 1	× 1	× 1	× 1	× 1–2	× 2–4
	Titre increase of Bedsonia positive serum†	× 1	× 1	× 1–2	× 2	× 2–4	× ∞
	Apparent Bedsonia titre in a negative serum	< 1/1	< 1/1	< 1/1	< 1/1–1/2	1/1–1/4	N.E.‡

* Titrated with *ca.* 3 units of homologous test serum.
† Titrated with *ca.* 3 units of homologous antigen.
‡ No end-point (all tubes show positive C.F.).

Although, in general, the results presented in Table 2 hold true for all the observations made in our experiments, in some instances the results observed showed a departure from the details given in that table.

In some instances the increased antigen titres did not appear in the series containing the one or more additional antibody units per tube, but rather in those containing 0·53 or 0·26 antibody units per volume of complement. It is known that sera of some species of animals, including guinea-pigs (Terzin, 1960) as well as certain preparations of the Bedsonia group antigen (Terzin, Matuka, Fornazarić & Hlača, 1961) show prozones in the area of antibody excess when checkerboard titrated by C.F.-test. In such cases, a small increment of antibody near a critical point may result in an increase of antigen titre greater than that seen with a larger increment of antibody (see Fig. 1).

As one would expect, if in serum-titration tests one uses complement dilutions containing one or more units of homologous antibody per volume (C′6 in Table 2), the C.F. test will show positive results in all tubes, whether the serum serially diluted was Bedsonia positive or negative, and regardless of how far the dilutions were carried (no end point). However, with Bedsonia-negative serum samples even if enriched by 0·26 or 0·53 antibody unit per test tube (as in tests set up with the C′4 or C′5 samples of complement) one would not expect a positive C.F. reaction in dilutions higher than 1/1. In contrast to that, as shown in Table 2, the Bedsonia negative sera enriched with samples of C′4 and C′5 (each containing less

than 1 full unit of homologous antibody) did show C.F.-titres of 1/2 or 1/4, respectively.

Due to possible errors of the twofold serial dilution technique, small amounts of originally undetected antibody contained in the 'Bedsonia-negative' serum, associated with added small amounts of antibody contained in the complement, may account for the antibody titre higher than expected in specimens of 'Bedsonia-negative' sera.

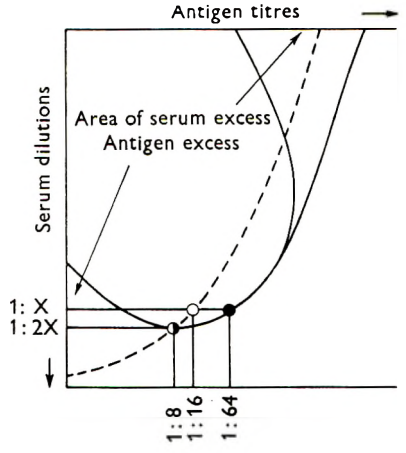


Fig. 1. Possible C.F.-titre relations of guinea-pig and rabbit serum against Bedsonia antigen. – – – –, rabbit serum; ——, guinea-pig serum.

DISCUSSION

As indicated earlier (Terzin, 1960), it is possible to find a different number of antigen units per volume of the same antigen, due to a difference in the combining capacity of the various antisera used in the respective titrations. Consequently, samples of the same antigen may show twofold or more variation in the number of antigen 'units' found per volume unit, depending on the type of serum against which the 'units' were determined (see Fig. 1). Also a twofold greater dilution of different sera (1:2 × in the graph) may reveal a two- or eightfold decrease of the antigen titre, depending on the respective type of the serum and antigen used in the checkerboard titration.

Any of these facts may account for the apparent disproportion between the small amount of guinea-pig antibody present in certain complement preparations used (C'3, C'4 or C'5) and the great effect they showed upon the respective C.F. titres presented in Table 2.

Figure 1 shows possible checkerboard titration patterns of a guinea-pig and a rabbit serum titrated against samples of the same Bedsonia antigen (Terzin, 1960; Terzin *et al.* 1961).

From several mammalian hosts, including at least two species of rodents, agents closely related to psittacosis have been recovered (Meyer, 1959), all sharing the common soluble antigen characterizing the strains of the Bedsonia group. Recently, an agent belonging to the Bedsonia group was isolated and identified (Murray,

1964), which caused subclinical or manifest conjunctivitis in guinea-pigs. The agent seems to be widespread in guinea-pig colonies and cross-infection with the agent stimulates the production of circulating antibodies indistinguishable from Bedsonia antibodies. In view of these facts it seems natural to expect a high incidence of Bedsonia-positive guinea-pigs in colonies of animals intended as a source of complement for routine use in laboratories. Recently, we identified a common Bedsonia-group antigen (CBA) contained in the yolk of normal hens' eggs. When titrating the same yolk preparation with constant amounts of an anti-psittacosis serum in parallel tests, set up with samples of normal and Bedsonia-positive complement, we obtained CBA titres 1/4 and 1/64, respectively.

In view of the observations discussed in this report, whenever C.F. tests with systems of the psittacosis–trachoma group agents are performed, it seems to be worth while to test carefully for possible presence of Bedsonia antibodies in the sera of guinea-pigs to be used as source of complement.

As has been shown, under certain circumstances the presence of such small amounts as 0·53 or 0·26 C.F. units per volume of Bedsonia antibodies may cause apparent increase of the Bedsonia titres. When used as source of complement, a guinea-pig serum with a 1/32 Bedsonia titre may reveal a completely 'false positive' C.F. test. Of the seven commercial preparations of complement (each labelled with a different lot number), produced by firms *a*, *b* and *c*, respectively (as shown in Table 1), five were found positive when tested for Bedsonia antibodies. It seems to be indicated therefore, to test the sera of complement-donor guinea-pigs individually or in small pools (not exceeding four individuals per pool if testing them starting with dilution 1/1) with several dilutions of the Bedsonia group antigen. Larger pools to be used as source of complement may be conveniently prepared from the samples found negative.

SUMMARY

Complement-fixation tests with psittacosis-trachoma group antigens, if set up with complement prepared from guinea-pigs cross-infected with any of the Bedsonia agents, may give completely false positive results. The use of C.F. positive or C.F. inhibition positive samples of guinea-pig sera as a source of complement can induce also a significant increase or decrease, respectively, of the actual C.F. titres in Bedsonia-positive serum samples tested. Observations made both in routine serology and in experimental studies show the necessity of testing carefully, for possible presence of Bedsonia titres, individual sera of guinea-pigs intended for use as source of complement in C.F. tests performed with Bedsonia group antigens.

I have pleasure in thanking Dr F. B. Gordon and Dr E. Weiss for the valuable suggestions made and HM3 C. O. Wiese for the technical assistance.

REFERENCES

HOYLE, L. (1948). Technique of complement fixation test. *Mon. Bull. Minist. Hlth Lab. Serv.* **7**, 114.

KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry*, 2nd ed. Springfield, Illinois: C. C. Thomas.

MEYER, K. F. (1959). Psittacosis-lymphogranuloma venereum group. In *Viral and Rickettsial Diseases of Man*, 3rd ed. Ed. T. M. Rivers and F. L. Horsfall. London: Pitman Medical Publishing Co. Ltd.

MURRAY, E. S. (1964). Guinea pig inclusion conjunctivitis virus. I. Isolation and identification as a member of the psittacosis-lymphogranuloma-trachoma group. *J. infect. Dis* **114**, 1.

TERZIN, A. L. (1958). The complement fixation inhibition test as a serological tool in the virus laboratory. *J. Hyg. Epidemiol. (Praha)*, **2**, 36.

TERZIN, A. L. (1960). Different types of serologic reactivity to Bedsonia (psittacosis group) antigen in various hosts. *J. Immunol.* **85**, 90.

TERZIN, A. L., MATUKA, S., FORNAZARIČ, M. R. & HLAČA, D. M. (1961). Preparation of group-specific Bedsonia antigens for use in complement-fixation reactions. *Acta virol., Prague*, **5**, 78.

J. Hyg., Camb. (1964), **62**, 185
Printed in Great Britain

Serological studies on group- and species-specific antigens of trachoma and inclusion conjunctivitis (TRIC) agents*†

By A. L. TERZIN,‡ N. A. VEDROS and J. B. JOHNSTON
U.S. Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

(*Received* 3 *December* 1963)

INTRODUCTION

It was shown by Bedson (1936) that psittacosis virus possesses both heat labile and heat stable C.F. antigens. The heat stable antigen was found to be shared by other viruses of the same group (Bedson, Barwell, King & Bishop, 1949; Monsur & Barwell, 1951; Hilleman, 1955; Ross & Gogolak, 1957).

A whole series of procedures have been described for the preparation of ether soluble, alkali soluble and other materials revealing in C.F. reactions group antigen activity of indistinguishable specificity. Both the group- and the species-specific antigenic activity of elementary body suspensions, first observed by Bedson in 1936 in his studies on psittacosis, seem to be confirmed also by recent studies on trachoma. Collier & Sowa (1958) tested, by complement fixation, sera from LGV patients and Frei-negative trachoma patients against the psittacosis–lymphogranuloma group antigen and against three antigens prepared from various trachoma strains. Their 'results revealed a very close relationship between all four antigens'.

Woolridge, Jackson & Grayston (1960) reported that group antigens prepared from trachoma, psittacosis or LGV, all reacted similarly, and they concluded 'that trachoma virus contained the group antigen of the P-LV group and in addition a specific antigen'.

Murray (1962) reported on tests with group antigens prepared from over 40 different trachoma strains as well as from about 20 strains of psittacosis, LGV, meningopneumonitis, feline pneumonitis and bovine abortion. These 60 group antigen preparations were indistinguishable from each other when tested by C.F. with the sera of patients diagnosed as trachoma, psittacosis, or LGV.

Reeve & Taverne (1962) tested boiled antigens prepared from different strains of trachoma and inclusion conjunctivitis with immune sera against different strains of trachoma, inclusion conjunctivitis and with LGV convalescent serum. The results showed that 'the viruses of trachoma and inclusion blennorrhoea are anti-

* This investigation was conducted under a Fight for Sight Postdoctoral Research Fellowship of The National Council to Combat Blindness, Inc., New York.

† The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

‡ Present address: Medical Faculty, Novi Sad, Yugoslavia.

genically similar to other members of the group'. Terzin, Fornazarić & Birtašević (1963) prepared group-reactive soluble antigens from trachoma and psittacosis infected yolk membranes. About 100 serum specimens were tested with these antigens; these included normal sera, sera of men and pigeons recovered from ornithosis infections, sera of rabbits and guinea-pigs immunized with ornithosis virus and sera of Bedsonia-positive sheep, cattle, and fowl. All sera showed comparable C.F. titres with group antigens, prepared from trachoma and psittacosis organisms. Both the specificity and the sensitivity (as illustrated by results of checkerboard titration) of the two antigens was found to be indistinguishable by C.F.-test. In the sera of trachoma patients which were free of group antibodies, they were able to demonstrate the presence of species-specific C.F. antibodies, as detected by semipurified elementary body suspensions of trachoma.

The bibliographical data just reviewed briefly, supplemented with the results presented in this report, seem to fit a definite pattern of notions which will be discussed at the end of this paper.

MATERIALS AND METHODS

Diluents

Ca-Mg buffered saline (*pH* 7·2). This was used as diluent for ingredients of the complement fixation test and was that described by Mayer, Osler, Bier & Heidelberger (1946).

Sucrose phosphate saline (*SPS*). Normal saline solution containing 0·2 M sucrose and 0·02 M potassium phosphate buffer pH 7·2. Sterilized by autoclaving at 15 lb. for 20 min.

Sucrose phosphate albumin (*SPA*). SPS-solution with 0·6 % crystalline bovine albumin. The albumin was first dissolved in distilled water to make a 6 % concentration, sterilized by filtration and added to the autoclaved SPS solution in proportions to make a final concentration of 0·6 %.

Bedsonia strains

Inclusion conjunctivitis strain MRC-1/G (formerly LB-1), trachoma strains TE-55 and Cal-1 (formerly Bourassa), psittacosis strain 6BC and mouse pneumonitis strain Nigg (MoPn) were all maintained in yolk sac passages in this laboratory. Also, lines of MRC-1/G and TE-55 strains maintained in cultures of McCoy cells were used.

Complement fixation (C.F.) *tests*

The technique used in performing the C.F. tests was that described earlier (Terzin *et al.* 1963).

Antigens

Suspensions of purified elementary bodies (E.B. suspension) were prepared from agents grown both in yolk membranes of embryonated eggs and in cultures of McCoy cells.

Yolk grown E.B. suspensions. Heavily infected yolk membranes (with strains MRC-1/G, TE-55, Cal-1 or psittacosis 6BC) were homogenized three times for

10 sec. in a Waring blender. In the subsequent description, the volume of the homogenate is designated as X ml. Equal volumes of homogenate and precooled SPS were mixed and centrifuged at 35,000g for 20 min. The supernatant fluid with a thick fatty layer was discarded and the sediment resuspended in $2X$ ml. of SPS. Armour Tryptar (trypsin) diluted in SPS to 500 units/ml. was added in a volume of $2X$ ml. and the resulting $4X$ ml. suspension (with a final trypsin concentration of 250 units/ml.) was kept at room temperature for 1 hr. under constant stirring.

The trypsinized suspension was centrifuged for 15 min. at 1500 r.p.m. in a horizontal centrifuge, the sediment was discarded and the supernatant centrifuged for 20 min. at about 27,000g. The separated pellet was resuspended in X ml. of SPA diluent and mixed with celite in the proportion of 1 g. of celite per 10 g. of yolk sac. After stirring for 5 min. at room temperature, the mixture was spun for 15 min. at 1500 r.p.m. The supernatant was decanted and designated as Supernatant I and the sediment was mixed with $X/2$ ml. of SPA for 3 min. at room temperature. By centrifugation for 15 min. at 1500 r.p.m., the sediment was separated and discarded, and the supernatant pooled with 'Supernatant I'. A total volume of approximately $1{\cdot}5X$ ml. of the pooled supernatants was centrifuged for 20 min. at 27,000 g to obtain a supernatant which was discarded and a dark yellow pellet of homogeneous appearance. At this stage of the preparation some of the sediments were kept overnight at 4° C., in well-protected containers. The pellet was resuspended and washed three times in SPA diluted 1/4 with saline (using centrifugation in a Spinco centrifuge at 27,000g for 25 min.). The washed pellet was resuspended and well homogenized in $X/20$ ml. of SPA, spun for 5 min. at 1500 r.p.m. in a horizontal centrifuge to remove clumps and the supernatant decanted was designated as 'E.B. susp.' of the respective strain. The LD 50 for chick embryos of the different E.B. suspensions was found to be about $10^{-6} \pm 1$ log unit.

Tissue culture grown E.B. suspensions. Strains of TE-55 and MRC-1/G were grown in McCoy cells by methods previously developed by Gordon, Quan & Dressler (1963). Harvests of cells and fluids from twelve chronically infected T-30 flask cultures, that had been maintained for weeks or months on Eagle's medium supplemented with vitamins, glutamine and 10% horse serum, were pooled and processed as follows. After disruption of the cells in a 10 KC Raytheon sonicator at full power for 5 min., the suspension (about 100 ml.) was centrifuged for 40 min. at 8000g in a Spinco rotor. The sediment was resuspended in 20 ml. of phosphate buffer (0·15 M, pH 7·2) in saline and trypsinized for 1 hr. at room temperature (final concentration of Armour Tryptar 250 units/ml.). After centrifugation at 26,000g for 30 min., the sedimented particles were resuspended either in 10 ml. phosphate buffer (pH 7·4) which contained a final concentration of 0·2 M sucrose or in 10 ml. of SPA. The suspensions without albumin were used for inoculation of rabbits, while the E.B. suspended in SPA were used as antigens in C.F. tests. The E.B. suspensions were stored for 3 months at $-70°$ C., or at 4° C. for at least 1 month, with no detectable change in potency.

Boiled E.B. susp. of mouse pneumonitis. A yolk grown purified E.B. suspension was suspended in phosphate-saline (potassium phosphate buffer pH 7·2) at a final

concentration 0·02 M instead of diluents containing sucrose and albumin. It was heated for 10 min. in a boiling water-bath.

Samples taken from the same suspension before and after boiling when titrated with psittacosis antisera showed a four- to eightfold increase of the group-specific antigen titre due to steaming of the suspensions.

Ether soluble psittacosis antigen. This was prepared from yolk sacs infected with 6BC or P-4 strains, by a procedure described elsewhere (Terzin, Matuka, Fornazarić & Hlača, 1961). In contrast to the E.B. suspension antigens preserved by sucrose-albumin, both the boiled E.B. suspension prepared from MoPn, and the ether soluble psittacosis antigen represent monospecific Bedsonia-group reagents.

Human sera

Fifty-three serum specimens from young adult trachoma convalescents (from 1 to 16 years after the onset of the disease) were collected in Yugoslavia by Dr B. V. Birtašević; three serum specimens from trachoma patients were obtained from Dr E. S. Murray; fourteen Bedsonia negative serum specimens from apparently healthy children of age varying from 3 to 6 months were obtained from Dr A. J. Vargosko; and eight specimens of sera were drawn from the personnel of this laboratory.

Animal sera

Seven serum samples were collected at different time intervals in this laboratory from two monkeys infected experimentally with trachoma.

Anti-sera were prepared by immunization of rabbits with MRC-1/G and TE-55 strains grown in tissue culture.

The preparation of inocula used for the immunization was described under 'Tissue culture grown E.B. suspensions'. Each of four rabbits received two courses of injections separated by a month; one consisted of six and the other one of four injections. The first injection in each course was given intraperitoneally and the rest intravenously. In each of the immunization courses the animals were given single doses of the suspension increasing from 0·5 to 1·5 ml. per animal. The total amount given to each of the four animals amounted to 9 ml. of the respective suspensions. Eight days after the last injection of the second course the animals were bled.

The antisera prepared by immunization with tissue culture grown purified elementary bodies gave no reaction with the crude control antigen prepared from uninoculated tissue cultures.

The anti-psittacosis sera were obtained from the Medical Faculty, Sarajevo. There they were prepared by intraperitoneal inoculation of rabbits with organs (20 % suspension of mixed brain and spleen homogenates in saline solution) of mice infected intracerebrally with the 25th mouse passage of the psittacosis strain P-4.

Absorption of sera

Three volumes of the boiled MoPn antigen were centrifuged for 40 min. at about 30,000*g*. The supernatant was separated and used successfully as a group-specific C.F. antigen in other studies. The sediment was resuspended and well homogenized

in 1 volume of the serum intended for absorption. The suspension was kept for about 3 days at 4° C., and occasionally shaken. The absorbed serum was separated at about 70,000*g* for 50 min., decanted and passed through a Millipore filter (porosity 0·45μ). Although only the sediment of the boiled E.B. suspension was used for absorption, and the serum after absorption was centrifuged for 50 min. at 70,000*g*, the absorbed serum showed an anticomplementary activity as a rule as high as 1/16. After filtering it through Millipore pads, we could reduce its anti-complementary activity to less than 1/4 (apparently due to removal of the flooding and 'soluble' serum-antigen complexes which tend to fix complement). Serum specimens from which the anti-group titres were not removed by a single absorption were absorbed for a second time. In terms of C.F.-units, we were unable to define a generally applicable agent–antibody ratio which would be satisfactory for complete absorption. Experiments repeated with several serum samples of rabbits and guinea-pigs and with different preparations of boiled MoPn antigen showed, that for the removal of all detectable group antibodies in some instances it was enough to use 1 C.F.-unit, while in other cases it was necessary to use 20 C.F.-units of the antigen per 1 C.F.-unit of group antibody. We decided to use E.B. of mouse pneumonitis agent in our absorption experiments because, as far as we know, the similarity of the group antigens contained in mouse pneumonitis and TRIC organisms has not previously been investigated.

RESULTS AND CONCLUSIONS

(1) *Correlation of* C.F. *titres revealed by sera titrated by psittacosis and mouse-pneumonitis antigens*

Of the 85 serum samples tested, 63 (74 %) were taken from cases of trachoma (mostly convalescents), diagnosed on the basis of both clinical and epidemiological evidences and a few of them confirmed also by positive smears or isolation of the agent from the conjunctival lesions. Many of the adult patients have spent periods of their life in areas where psittacosis is known to be prevalent. Regardless of the proportion in which trachoma infection can be made responsible for the development of anti-Bedsonia antibodies in the serum specimens of these individuals, the fact remains that these specimens showed the same proportion of C.F. positive reactors and revealed comparable titres with group antigens prepared both from psittacosis and mouse pneumonitis organisms. This appears to be suggestive evidence for a very similar, if not identical specificity of the group antigens of psittacosis, mouse pneumonitis and the group antibodies found in the sera of trachoma patients and trachoma infected monkeys.

These 85 serum specimens all showed negative results ($< 1/4$) when tested both for anticomplementary activity and against Q fever antigen.

Each of the 85 serum specimens was tested against the ether soluble psittacosis antigen diluted 1/60 (which represents about six homologous group antigenic units) and against the boiled E.B. suspension of MoPn antigen diluted 1/140 (representing about 7·3 homologous group antigenic units). Figure 1 shows the correlation table of the titres revealed against the two antigens. Statistical analysis of

these data revealed a positive correlation coefficient 0·896. The probability that this positive correlation of a high degree might have occurred by mere chance was found to be much less than 0·001. Also by inspection of Fig. 1, one can note that except for one specimen (which showed a titre of < 1/4 with the psittacosis and a titre of 1/8 with the MoPn antigen), all sera showed identical titres with both antigens in the limits of ± one tube difference. This variation is considered tolerable in the serial twofold dilution C.F. test, if the tests are repeated on different days. As seen from Fig. 1, out of the 85 specimens tested, 46 (or 54 %) were positive with psittacosis, and 42 (or 49 %) with the MoPn antigen. A comparison of the significance of the proportion of positives and negatives revealed by the two antigens, gave a chi-square value as low as 0·196 indicating with great probability ($0{\cdot}7 > P > 0{\cdot}5$) that the results obtained with the two antigens were indistinguishable.

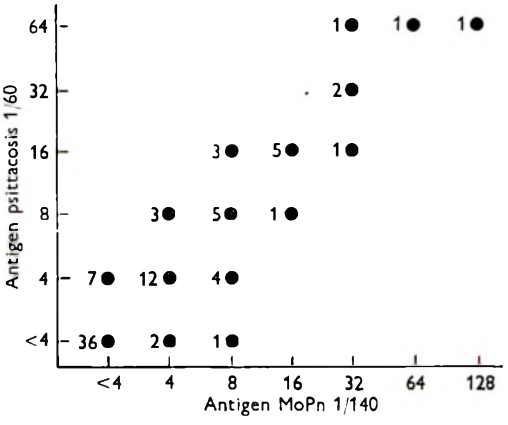


Fig. 1. Two-way frequency of reciprocals of the C.F. titres shown by 85 serum specimens with two antigens.

The data in Fig. 1 also show that the mean titre of the positive serum samples against the psittacosis antigen was 1/12·26 as compared with 1/14 of the MoPn antigen.

When analysed by *t*-test, the difference between these means is shown to be not significant. The *t* value obtained was 0·4394, at a probability level of significance of $0{\cdot}7 > P > 0{\cdot}6$, indicating with great probability that the difference observed between the two means might have been obtained just as well by re-titrating the same serum specimens twice with the same antigen.

All three statistical examinations suggest a great similarity, if not identity of the specificity of the two antigens employed, although each was prepared in a different way (ether soluble fraction of psittacosis and boiled E.B. suspension of MoPn), with the specificity of group antibodies found in sera of trachoma patients and trachoma infected monkeys.

(2) *Experiments with absorbed anti-trachoma sera*

The E.B. suspensions (with stabilized serological activity of the species-specific antigen) as well as the absorbed antisera (which were free both of the Bedsonia-

group antibodies and of anticomplementary activity) enabled us to study the specificity of the antigens (contained in TRIC organisms) not only at the group-genus level, but also at the species-specific level, and to make comparisons of the serological cross-reactivity of different TRIC strains.

Different samples of boiled elementary body suspensions prepared from mouse pneumonitis agent removed or reduced very significantly the capacity of immune sera against trachoma to react with group antigens prepared from psittacosis or other Bedsonia agents. However, the absorbed sera retained their capacity to react with elementary body suspensions prepared from trachoma or inclusion blennorrhoea strains.

Table 1. *Reciprocals of antigen titres with unabsorbed and absorbed sera*

	Antiserum (rabbit)								
	MRC-1/G			TE-55		Psittacosis			
		Absorbed			Absorbed				A.C. of antigen
Antigens	1:40	1:6	1:20	1:256	1:20	1:20	1:40	1:80	
E.B. susp. (yolk grown)									
MRC-1/G	1024	64	32	512	128	256	128	64	< 8
Cal-1	256	16	8	256	128	n.t.	n.t.	n.t.	< 8
TE-55	512	256	128	512	256	256	256	128	< 8
Psittacosis	128	< 8	< 8	64	< 4	64	32	32	< 4
E.B. susp. (TC grown)									
MRC-1/G	32	n.t.	8	n.t.	n.t.	32	n.t.	n.t.	< 4
TE-55	64	n.t.	32	n.t.	n.t.	32	n.t.	n.t.	< 8
Boiled E.B. susp. MoPn	2048	< 8	< 8	1024	< 16	1024	1024	512	< 8
Ether soluble psittacosis	256	< 8	< 4	256	< 16	256	256	32	< 4
A.C. of respective serum dilution	–	–	–	–	–	–	–	–	

A.C., Anticomplementary activity; n.t., not tested; –, negative.

The sera used in absorption experiments were prepared by immunization of rabbits with elementary body suspensions grown in tissue culture, and the elementary bodies of mouse pneumonitis used for serum absorption were grown in yolk sacs of chick embryos. It is obvious that the specificity shared by these two systems may be only that of the Bedsonia antigen shared by both organisms (trachoma and mouse pneumonitis). Consequently, the data presented in Tables 1 and 2 seem to offer strong additional evidence for the notion that the specificity of group antibodies formed against trachoma organisms is serologically indistinguishable from the specificity of the group antigen produced by mouse pneumonitis organisms.

From the data presented in Tables 1 and 2 it is easy to calculate the factors of the titre decrease which were induced by serum absorption. When titrated against constant amounts of the unabsorbed and absorbed samples of the respective sera, the E.B. suspensions prepared from MRC-1/G, Cal-1 and TE-55 organisms grown

Table 2. *Reciprocals of titres revealed by unabsorbed and absorbed sera*

	Antigens							
	MRC-1/G E.B. susp. grown		TE-55 E.B. susp. grown		Psittacosis E.B. susp. grown in yolk, 1:30	MoPn grown in yolk boiled, 1:140	Psittacosis grown in yolk ether extract, 1:100	A.C. of serum
Anti-sera (rabbit)	In TC, 1:20	In yolk, 1:20	In TC, 1:30	In yolk, 1:50				
MRC-1/G	512	64	256	256	128	128	128	< 4
MRC-1/G absorbed	128	8	32	16	< 8	< 4	< 4	< 4
TE-55	n.t.	512	n.t.	1024	n.t.	1024	512	< 16
TE-55 absorbed	n.t.	16	n.t.	32	n.t.	< 16	< 16	< 16
A.C. of respective antigen dilution	–	–	–	–	–	–	–	–

A.C., Anticomplementary activity; n.t., not tested; –, negative.

both in yolk and tissue culture showed an average of 10·25-fold drop of the C.F. titre (varying from 2- to 32-fold in individual examples shown in Table 1).

Similarly, titrations of the unabsorbed and absorbed samples of anti-MRC-1/G and anti-TE-55 sera against constant amounts of E.B. suspensions prepared from MRC-1/G and TE-55 organisms, showed on the average a 16·66-fold decrease of the C.F.-titres (varying from 4- to 32-fold decrease in individual cases shown in Table 2).

In contrast to that, the results of C.F.-tests run with ether soluble antigen and E.B. suspension prepared from psittacosis, as well as of the tests run with the MoPn antigen, all titrated against both unabsorbed and absorbed specimens of anti-MRC-1/G and anti-TE-55 sera, revealed an average 144-fold decrease of the antigen titres (varying from 32- to 512-fold in different cases) and an average 70-fold decrease of the respective serum titres (varying from 32- to 128-fold in different examples). In most titrations the amounts of the reagents which were kept constant represented 4–16 homologous C.F.-units of antiserum (Table 1) and 1–10 units of the homologous antigen (Table 2). An exception is seen in the 1/20 dilution of the yolk grown MRC-1/G E.B. suspension, which contained about 50 C.F. units of the group reactive antigen component (Table 2).

The data presented in Tables 1 and 2 seem to show clearly that the serological specificity of the group antigens of inclusion blennorrhoea and trachoma is indistinguishable from that of the mouse pneumonitis and psittacosis organism.

In contrast to that, if tested against absorbed (group-negative) sera, the species-specific antigen activity of the E.B. suspensions prepared from strains of trachoma or inclusion blennorrhoea discriminate clearly between TRIC-species and other Bedsonia agents (psittacosis, mouse pneumonitis, etc.).

The serological activity of E.B. suspensions prepared from TRIC and psittacosis agents both against unabsorbed and absorbed anti-TRIC sera, as well as against the anti-psittacosis serum, shows that the species-specific antigens of TRIC agents are different from the species antigen of the psittacosis agent (Table 1). However, a differentiation of various strains within the TRIC-species could not be demonstrated by the use of C.F.-test and purified E.B. suspensions prepared from strains of trachoma and inclusion conjunctivitis. Data shown in Tables 1 and 2 actually indicate that the species-specific antigens of the TRIC strains we compared were indistinguishable. If confirmed by more extensive studies, this finding would put strains isolated from trachoma and inclusion conjunctivitis cases in the same, serologically homogeneous, species.

(3) *Observations bearing on the species-specific antigenic activity of TRIC agents*

In contrast to the E.B. suspensions of trachoma agent suspended in SPG (Terzin *et al.* 1963), the suspensions of the E.B. preserved by SPA, after storage for 3 months at $-70°$ C. or for 60 days at 4° C., showed neither detectable drop in their species-specific titres, nor detectable increase in their group titres when tested by absorbed and unabsorbed homologous sera. The finding that sucrose-albumin preserves the species-specific C.F. antigen of trachoma-inclusion blennorrhoea

agents seems to be in agreement with the findings of Weiss & Dressler (1962) who reported that sucrose and albumin have pronounced stabilizing effect on the infectivity of purified preparations of trachoma agent.

However, these suspensions, although both stabilized and revealing pronounced species-specific activity as test-antigens for detection of trachoma or inclusion conjunctivitis antibodies, can be used only if the serum tested is free of group-specific antibodies (against absorbed or *a priori* Bedsonia-negative sera). In the presence of group antibodies the group–antigroup C.F. system would mask the distinct manifestation of the species–antispecies C.F. system.

With well-preserved specimens of normal sera of men, rabbits and guinea-pigs, the E.B. suspensions showed no signs of fixation of complement. However, with turbid, many times frozen and thawed specimens or with contaminated sera, the same suspensions tended to give nonspecific fixation of complement.

Samples of E.B. suspensions which became contaminated developed anti-complementary activity. Stock batches of E.B. suspensions should be carefully preserved, divided into small volumes and kept frozen until use. As shown in Table 1, the antigen titres obtained with anti-psittacosis serum are comparable with the respective antigen titres obtained with homologous anti-TRIC sera. Therefore, it does not seem feasible to use some 'critical' working dilutions of these E.B. suspensions which would allow a distinction between species- and group-specific serum titres. For these reasons we attempted to apply to trachoma serology the method of Jenkin, Ross & Moulder (1961) for preparing species-specific cell-wall antigens, free of the group antigen component. As starting material, we used purified suspensions of elementary bodies grown in yolk sacs. Out of seven preparations tested the first two showed promising results (species-specific reactions with absorbed sera; low or no detectable group–antigen reactivity; tests set up with boiled samples revealed both a resistance to heat of the species-specific activity and a disappearance of the initially present anticomplementary activity). However, in five additional attempts, for unknown reasons, we failed to reproduce the results which looked so promising at the beginning.

DISCUSSION

In spite of the huge amounts of money spent for treatment programmes, more than 400 million of the world's population still suffer from trachoma and its complications (Thygeson, 1962).

It is known that the clinical diagnosis of this disease requires careful examination of each patient by an eye specialist, a condition which would be very hard to procure in remote areas of Africa, Asia and South America, where trachoma is flourishing. Besides that, clinical examination may fail to diagnose cases of atypical trachoma, which nevertheless might represent sources of infection. According to Thygeson (1960) 'the obstacles in the way of detecting and treating the disease in entire populations have seemed insuperable...'.

Both the isolation of the agent from and the demonstration of inclusion bodies in scrapings and smears of the suspect conjunctiva, are the best evidences for an

actual infection of the eye with TRIC-agents. However, the low frequency of positive results obtained by these methods, as well as the expense of adequate laboratory facilities, make these methods both unreliable and unfeasible for use as diagnostic tools. In field work on a large scale, these methods cannot be used as substitutes for complete clinical examinations by the eye-specialist.

All these circumstances seem to point out quite clearly the need for a reliable, easily performed, specific serological test, which would make it possible to diagnose trachoma from blood specimens collected in the field, and transported to central laboratories.

As summarized by Thygeson (1960), 'No serological tests of diagnostic value are yet available. The sera of many, but not all, trachoma patients show group complement-fixing antibodies for the psittacosis-lymphogranuloma venereum group of viruses, but no differentiation among members of the group is yet possible.'

The purpose of this discussion is to sketch a pattern of facts and notions which seem to indicate a definite direction of effective search for serological tests of diagnostic value in trachoma.

The serological evidence available so far seems to indicate the existence of at least three taxonomic levels of the TRIC organisms:

(1) At the level of genus or group, it is known (Bedson, 1959) that a large number of organisms share a common serological specificity of their Bedsonia-group antigen or psittacosis–LGV–trachoma-group antigen (as well as similar morphology, cultural characteristics in chick embryos, propagation cycle, resistance to streptomycin, sensitivity to many antibiotics and several other features of similarity).

(2) At the species level, the TRIC agents may be distinguished from other Bedsonia organisms by similarity in host range and a species specific thermolabile antigen. In contrast to other species of Bedsonia, the TRIC agents are infectious for man, ape and monkey by ocular route, occasionally for mice intracerebrally, but not infectious for the guinea-pig by any route. The species specific antigen may distinguish TRIC strains from, e.g. the psittacosis species, but cannot distinguish trachoma from inclusion conjunctivitis strains.

The apparent existence of two separate varieties (trachoma and inclusion conjunctivitis) of the TRIC species might well be a manifestation of differences in the hosts and in some ecological factors.

(3) Within the TRIC species, as in the psittacosis–ornithosis species, it was possible to demonstrate (by active protection test of mice challenged intravenously) the existence of at least three serological types of toxins, associated with intact elementary bodies (Bell & Theobald, 1962; Chang, Wang & Grayston, 1962).

(4) The number of TRIC strains, isolated over all five continents, amounts to well above a hundred. The majority of these strains seem to be characterized by their origin, specific case history and nominal designation, rather than by stable and recognizable biological markers at a subspecies level. It is unfortunate that the E.B. of the TRIC organisms contain thermostable group antigens which are serologically indistinguishable from the group antigens in other Bedsonia

organisms; that different species of Bedsonia organisms (mammalian and avian strains of psittacosis–ornithosis, LGV, etc.) are prevalent over the globe causing both clinically manifest and subclinical infections in men (followed by long-lasting persistence of group-specific antibodies in the cross-infected population). Consequently, the mere fact that an antigen was prepared from TRIC organisms cannot justify its use for diagnosing infections caused by TRIC agents, either for studies of prevalence of the TRIC infections in population groups, or for detecting reactors to a trachoma vaccine. In order to secure a specific serological test of diagnostic value, one has to provide reagents which will interact on a monospecific TRIC-species level. Taking as example the C.F.-test, there are two theoretical alternatives:

(1) One might use well-preserved E.B. suspension (or other antigen preparations in which the species-specific component is stabilized) which would react specifically with trachoma sera which are free from group antibodies.

(2) Alternatively, one could prepare a TRIC species-specific antigen, free from the Bedsonia group antigen, which would detect specific anti-TRIC activity in sera of trachoma patients, without reacting with any group-specific antibodies that might be present.

In this report we describe the preparation of stabilized E.B. suspensions which may be used for detection of the species-specific TRIC antibodies. However, these suspensions can be used as TRIC-specific reagents only with sera free from group-reactive antibodies.

A successful separation of the TRIC-specific antigen from the Bedsonia-group antigen would provide a reagent which might be used either in C.F.-test, or in some other serological test as an invaluable serological tool, so much needed for effective trachoma research. It is unfortunate that such an antigen is not yet available.

SUMMARY

Group-reactive ether soluble psittacosis and boiled mouse pneumonitis antigens were tested in parallel, with 85 serum specimens. The results indicate that the group-specific C.F. antigens of these organisms are indistinguishable when tested against sera of trachoma patients, monkeys infected with trachoma or against sera of other individuals.

Sera of rabbits immunized with viable trachoma-inclusion conjunctivitis (TRIC) organisms, grown in tissue culture, were absorbed with boiled elementary body suspension of mouse pneumonitis agent, which removed the group reactive antibodies, and resulted in a species-specific anti-TRIC serum.

The absorbed and unabsorbed TRIC sera were titrated against purified E.B. suspensions, which were prepared both from yolk and from tissue culture grown organisms, of homologous TRIC strains and from other heterologous Bedsonia organisms.

Results of absorption experiments indicate that group reactive antigens prepared from mouse pneumonitis and psittacosis are indistinguishable by C.F. test from the group-specific component of the TRIC antigens. The species-specific antigen of the

TRIC agents was well distinguished from the species-specific antigen of the psittacosis agent. However, the C.F. test did not distinguish the strains isolated from trachoma from those isolated from cases of inclusion conjunctivitis.

The stabilizing effect of sucrose and albumin upon the species-specific C.F. antigen of purified elementary bodies of TRIC organisms was found to be pronounced.

Our attempts to produce a species-specific antigen preparation, free from group component, failed.

We have pleasure in thanking Dr F. B. Gordon for suggestions; Dr B. V. Birtašević, Mr H. R. Dressler, Dr E. S. Murray and Dr A. J. Vargosko for procuring sera of patients or organisms grown in tissue culture; Dr T. A. Strike for the help with statistical computations; HMC P. R. Hill, HM3 C. O. Wiese and Mr B. L. Ward for invaluable technical assistance.

REFERENCES

BEDSON, S. P. (1936). Observations bearing on the antigenic composition of psittacosis virus. *Brit. J. exp. Path.* **17**, 109.

BEDSON, S. P. (1959). The Harben Lectures. The psittacosis-lymphogranuloma group of infective agents. *J. R. Inst. publ. Hlth*, **22**, 67.

BEDSON, S. P., BARWELL, C. F., KING, E. J. & BISHOP, L. W. J. (1949). The laboratory diagnosis of lymphogranuloma venereum. *J. clin. Path.* **2**, 241.

BELL, S. D. & THEOBALD, B. (1962). Differentiation of trachoma strains on the basis of immunization against toxic death of mice. *Ann. N.Y. Acad. Sci.* **98**, 337.

CHANG, I., WANG, S. & GRAYSTON, J. T. (1962). Antigenic relationship of trachoma virus strains in the mouse toxicity prevention test. In *The Biology of the Trachoma Agent. Ann. N.Y. Acad. Sci.* **98**, 347.

COLLIER, L. H. & SOWA, J. (1958). Isolation of trachoma virus in embryonate eggs. *Lancet*, i, 993.

GORDON, F. B., QUAN, A. L. & DRESSLER, H. R. (1963). Observations on growth of trachoma strains in cell cultures. (Abstract.) *Bact. Proc.* p. 132.

HILLEMAN, M. R. (1955). Serological procedure for detecting psittacosis infection in birds. In *Psittacosis*, p. 74. Ed. F. R. Beaudette, New Brunswick, New Jersey: Rutgers University Press.

JENKIN, H. M., ROSS, M. R. & MOULDER, J. W. (1961). Species-specific antigens from the cell walls of the agents of meningopneumonitis and feline pneumonitis. *J. Immunol.* **86**, 123.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1946). Activating effect of magnesium and other cations on hemolytic function of complement. *J. exp. Med.* **84**, 535.

MONSUR, K. A. & BARWELL, C. F. (1951). Observations on the antigenic relationship between the virus of enzootic abortion of ewes and viruses of the psittacosis-lymphogranuloma group. *Brit. J. exp. Path.* **32**, 414.

MURRAY, E. S. (1962). Discussion in *The Biology of the Trachoma Agent. Ann. N.Y. Acad. Sci.* **98**, 325.

REEVE, P. & TAVERNE, J. (1962). Some properties of the complement-fixing antigens of the agents of trachoma and inclusion blennorrhea and the relationship of the antigens to the developmental cycle. *J. gen. Microbiol.* **27**, 501.

ROSS, M. R. & GOGOLAK, F. M. (1957). The antigenic structure of psittacosis and feline pneumonitis viruses. I. Isolation of complement-fixing antigens with group and species specificity. *Virology*, **3**, 343.

TERZIN, A. L., FORNAZARIĆ, M. R. & BIRTAŠEVIĆ, B. V. (1963). Demonstration of complement-fixation titres against the species-specific trachoma antigen in sera of trachoma patients. *J. Hyg., Camb.*, **61**, 31.

TERZIN, A. L., MATUKA, S., FORNAZARIĆ, M. R. & HLAČA, D. M. (1961). Preparation of group-specific Bedsonia antigens for use in complement-fixation reactions. *Acta Virol.* **5**, 78.

THYGESON, P. (1960). Trachoma manual and atlas. Washington, Publ. Health Serv. Publ. No. 541.

THYGESON, P. (1962). Trachoma virus: historical background and review of isolates. In *The Biology of the Trachoma Agent. Ann. N.Y. Acad. Sci.* **98**, 6.

WEISS, E. & DRESSLER, H. R. (1962). Investigation of the stability of the trachoma agent. In *The Biology of the Trachoma Agent. Ann. N.Y. Acad. Sci.* **98**, 250.

WOOLRIDGE, R. L., JACKSON, E. B. & GRAYSTON, J. T. (1960). Serological relationship of trachoma, psittacosis and lymphogranuloma venereum viruses. *Proc. Soc. exp. Biol., N.Y.*, **104**, 298.

J. Hyg., Camb. (1964), **62**, 199
Printed in Great Britain

The serological differentiation of *Mycoplasma* strains (pleuropneumonia-like organisms) from various sources

By RUTH M. LEMCKE
The Lister Institute of Preventive Medicine, London, S.W. 1

(*Received* 28 *December* 1963)

INTRODUCTION

Over a period of several years, strains of *Mycoplasma* from a variety of sources have either been isolated in this laboratory or sent here for examination. Because some of these cultures were associated with disease conditions and it had been suggested that others (e.g. those from tissue cultures) were L-forms of bacteria, it seemed desirable to establish the identity of these cultures by comparing them with *Mycoplasma* cultures of known origin and properties.

Cultural and morphological characters are inadequate for classification, especially as they are readily affected by the type of medium and the conditions of cultivation. Biochemical tests also have a limited value; several species give no reactions and the range of substrates fermented by the others is very similar (Tourtellotte & Jacobs, 1960). Serological methods seem to be the most satisfactory for distinguishing strains.

Agglutination, and sometimes agglutinin absorption have been used for this purpose (Klieneberger, 1938, 1940; Warren & Sabin, 1942; Norman, Saslaw & Kuhn, 1950; Bailey *et al.* 1961). A drawback of this method is that some PPLO suspensions are auto-agglutinable.

A serological method depending on the inhibition of growth by specific antibody was devised by Edward & Fitzgerald (1954). The method had the advantage of being less sensitive than the agglutination test to small antigenic differences between strains of the same species. It was used in conjunction with a complement-fixation test by Huijsmans-Evers & Ruys (1956) to distinguish strains from the human mouth and genital tract, and in a modified form by Bailey *et al.* (1961). However, the growth-inhibition method was found unsatisfactory by Klieneberger-Nobel (1962) who, like Huijsmans-Evers & Ruys (1956), found that some antisera had no effect, even on the homologous strain. She also found that others lost their inhibitory power on storage.

Complement fixation tests were successfully used by Edward (1950), Edward & Fitzgerald (1951), Huijsmans-Evers & Ruys (1956) and Card (1959) to differentiate PPLO from various animal and human sources, and this method was chosen for the present work.

METHODS

Organisms

In all, 82 cultures were examined, 23 of human origin, 16 from tissue cultures and one from Eaton Agent propagated in tissue cultures, 27 from laboratory rats and mice, 2 from sewage, 8 from cattle and goats, 4 from poultry or embryonated eggs and one strain of unknown origin. The designation and source of the strains examined and the worker who isolated them are given in Table 1.

In addition to these 82 strains, antigens were made from three avian cultures, M, Fowl and TU, but were compared only with the other avian strains; no antisera were prepared against them. M (*Mycoplasma iners*) and Fowl (*M. gallinarum*) were recognized as distinct species by Edward & Kanarek (1960); TU, an unnamed non-pathogenic strain isolated in the U.S.A. and assigned by Kleckner (1960) to his group C, was found by Chu & Newnham (personal communication) to be distinct from *M. iners*, *M. gallinarum*, the non-pathogenic A 36 from the U.K. and the pathogenic 'coccobacilliform bodies' or *M. gallisepticum*.

Media and cultural conditions

The liquid medium used for maintaining cultures and growing antigen for the C.F.T. consisted of a tryptic digest of horse meat broth (TDB) supplemented with pooled normal human serum inactivated at 56° C. for 30 min. (10 %, v/v), Oxoid yeast extract (0·5 %, w/v), deoxyribonucleic acid (20 μg./ml.) and penicillin (50 units/ml.). For the living cultures used to immunize rabbits, the human serum was replaced by pooled normal rabbit serum, and for the preparation of the C.F.T. antigen of *M. pneumoniae* by unheated horse serum (20 %, v/v).

Cultures were incubated at 37° C.; all were shaken continuously during incubation with the exception of B 3 and G 2, which grew well only anaerobically, and 823, 826, 837, 844 and BM which grew better as stationary cultures.

For testing glucose fermentation, cultures on ox-heart infusion agar (1·5 %, w/v) with human serum (15 %, v/v), glucose (1·0 %, w/v) and phenol red (0·004 %, w/v) were incubated at 37° C. for 6 days. As controls, plates of the same medium without glucose were inoculated simultaneously and a known glucose-fermenting strain, A 36, was also inoculated on to both media.

Double-diffusion agar precipitation

This technique was used to test antisera for the presence of antibody against constituents of the media. Difco Noble agar (1·0 %, w/v) in normal saline buffered to pH 7·2, with 0·01 M sodium azide as preservative, was poured to a depth of 4 mm. A pattern of seven wells 6 mm. in diameter was used, the centres being 12 mm. apart. Precipitation lines were allowed to form at room temperature.

Reagents for the complement fixation test

These were the same as those described by Card (1959) except that preserved guinea-pig complement and glycerinated rabbit haemolytic serum for sheep corpuscles (Wellcome Research Laboratories) were used.

Table 1. *The* 17 *serotypes established by* C.F.T. *among* 82 *strains of* Mycoplasma

Type or species	No. of strains	Host or source	Designations of strains
Common human genital (*M. hominis type* 1)	17	Man	H34*, infected abdominal wound; H68, Bartholin's abscess — E. J. Stokes (London) 4387 P*, non-gonococcal urethritis (NGU), R. G. Wittler (Washington) MJW, NGU; M. J. Whittington (London) H27, Reiter's disease (urethra); H33, 587, 731, 105, NGU (urethra); D, E, H, Q, M, O, S, NGU (external genitals); L, primary syphilis (external genitals) — R. M. Lemcke (London)
	11	Tissue cultures	ERK, MK2, CH, NCH, Tang, AE, CE, Payne; R. M. Lemcke 417, 420; L. Hayflick (Philadelphia) HEp2; M. F. Barile (Bethesda)
Eaton agent (*M. pneumoniae*)	1	Man	FH*, egg-propagated line of Eaton agent; L. Hayflick
Human oral (probably corresponding to *M. salivarium*)	1	Man	B3*, human mouth; D. H. Card (London)
G strain (*M. fermentans*)	1	Man	G2*, fusospirillary infection of human genital mucosa; M. Ruiter and H. Wentholt (Groningen)
Previously undescribed	1	Man	Navel*, fusospirillary infection of umbilicus; M. Ruiter and H. Wentholt (Groningen)
Previously undescribed	4	Tissue cultures	823*, 826, 837*, 844; L. Hayflick
	1	—	BM, Eaton Agent propagated in tissue cultures; B. P. Marmion (Melbourne)
Rat or mouse lung (probably corresponding to *M. pulmonis*)	9	Rats	Kon*, bronchiectasis; E. Klieneberger-Nobel (London) R42L, R45L, R46L, R25NP, RME (1), respiratory tract or middle ear; R42A (1), abscess — R. M. Lemcke 2098, respiratory tract; D. Stephenson (Sittingbourne) SPF47, respiratory tract; H. C. Bartlema (Rijswick)
	8	Mice	DGE, respiratory tract; D. G. ff. Edward (Beckenham) M1*, M2, 68NP, 72L; respiratory tract MB*, Peter, 73B; brain — R. M. Lemcke
Mouse 'rolling disease' (probably corresponding to *M. neurolyticum*)	1	Mouse	KSA*, mouse brain; R. M. Lemcke
Rat polyarthritis (probably corresponding to *M. arthritidis*) (*M. hominis, type* 2)	5	Rats	Jasmin*, tumour; Baxter, polyarthritis — E. Klieneberger-Nobel DW, tumour; R. M. Lemcke LX; L. Dienes (Boston) VII-2, respiratory tract; H. C. Bartlema
	2	Man	Campo*, urethra; L. Dienes O7, cervix; M. H. Hatch (Baltimore)
	1	Unknown	H606, unknown (Washington)
Partially related to *M. arthritidis*	3	Rats	R38*, rhinitis; RME (2), middle ear; R42A (2), abscess — R. M. Lemcke
Contagious bovine pleuropneumonia, *M. mycoides* var. *mycoides*	2	Cattle	pp. cattle*, contagious bovine pleuropneumonia (CBPP); unknown Gladysdale*, CBPP; J. R. Hudson (Melbourne)
	2	Goats	G1/61*, G11, contagious caprine pleuropneumonia (CCPP) in Sudan; C. Pillai (Khartoum)
Contagious caprine pleuropneumonia	1	Goat	pp. goat*, CCPP in Turkey; W. I. Beveridge and H. P. Chu (Cambridge)
Agalactia of sheep (probably corresponding to *M. agalactiae*)	1	Goat	Agalactia*, infected goat milk from Italy; E. Klieneberger-Nobel
Bovine genital (*M. bovigenitalium*)	2	Cattle	PG11*, genital tract; D. G. ff. Edward 37, mastitis; I. Davidson and P. Stuart (Weybridge)
Saprophytic (*M. laidlawii*)	2	Sewage	Laidlaw A*, Laidlaw B; P. P. Laidlaw and W. J. Elford
	1	Rat	STR; L. Dienes
	1	Tissue culture	TC7277; unknown
Non-pathogenic avian (unnamed species)	1	Chicken	A36*, tracheal exudate; H. P. Chu
	2	Embryonated eggs	AM*, embryonated egg material; R. M. Lemcke PSU4, 'pipped' chick embryo after further egg passage; W. H. Kelton (Iowa)
Nelson's 'coccobacilliform bodies' (*M. gallisepticum*)	1	Embryonated egg	T*, eggs used to passage NGU exudates; M. C. Shepard (Camp Lejeune) (identified as 'coccobacilliform body' strain by Klieneberger-Nobel (1962))

* Strains against which test antisera were prepared.

The preparation of the antisera

At first rabbits were inoculated intravenously (i.v.) with washed saline suspensions harvested from 6-day cultures. Suspensions of several strains, for example *M. hominis type* 1, were found to contain very few if any viable cells after such an incubation period. Later it was found that more potent sera could be obtained with younger, viable cultures and, with the exception of the slow-growing *M. pneumoniae*, cultures 24–72 hr. old, depending on the rapidity of growth of particular strains, were used for injection. *M. pneumoniae* cultures were incubated 11–13 days. Six injections of 1, 1, 2, 2, 4 and 6 ml. were given, i.v., on alternate days, the suspensions for the last 5 being stored at $-30°$ C. until required. Experiments in which, for comparison, 2 rabbits were injected each time with fresh unstored suspensions, showed that such brief storage did not reduce the efficacy of the immunizing antigen. As a rule, two courses of 6 injections were given, separated by an interval of 2–3 weeks. A third series of 3 injections was sometimes given where the serum titre began to drop before the final exsanguination. Blood was taken before immunization and 5–10 days after the final injection of each series.

Antisera were also prepared by subcutaneous (s.c.) injection of suspensions in Freund's complete adjuvant. Washed cells harvested from 250 or 500 ml. of 24–72 hr. cultures were given at one time in several sites. Homologous serum titres were increased 4- to 16-fold if one or more s.c. injections were followed after an interval of 2–3 weeks by a course of 6 i.v. injections, but only low titre sera were obtained if the i.v. were given before the s.c. injections.

To determine whether antibody to traces of medium constituents in the immunizing antigen could account for the low titre cross-reactions observed, 4 rabbits were given, i.v., TDB, the residue centrifuged from TDB and resuspended in saline (TDB residue), Oxoid yeast extract (10 %, w/v) and deoxyribonucleic acid (0·2 %, w/v). Except for the TDB, of which a total of 30 ml. was given, these substances were injected in amounts which were present in 1000 ml. of the complete medium, i.e. the total volume from which immunizing antigen was usually harvested.

Preparation of antigens for complement fixation test

Unheated saline suspensions preserved with merthiolate were prepared as described by Card (1959). However, antigens of G 2, 37, Laidlaw A and Laidlaw B prepared in this way were too anticomplementary for use. Attempts to reduce the anticomplementary effect by sonic treatment and ether extraction failed.

Satisfactory antigens, however, were made from 37 and Laidlaw A by incubating cultures for 15–24 days, instead of the usual 5–6 days. This method did not work with G 2 or Laidlaw B, but satisfactory antigens were obtained by substituting rabbit or horse for human serum in the growth medium and incubating for 15 days.

The complement fixation test

At first titrations were carried out using the method of Card (1959). In subsequent tests, in place of antigens of standard optical density and a concentration of

complement (C′) determined for each antigen, the concentration of each antigen giving the maximum titre with its homologous serum, or with a strongly reacting serum, in the presence of 5 HD 50 of C′ was determined and then used for all titrations with other sera. With these optimal antigen concentrations, which were invariably less than those of standard turbidity, homologous titres were sometimes higher than in the original test, whereas cross-reactions with heterologous antisera and pre-immunization sera were lower.

In determining the identity of an antigen, its titre with a particular antiserum was compared in the same test with that of the homologous antigen and with that of the corresponding pre-immunization serum. Doubling dilutions of serum starting at 1/20 were used, and the titre expressed as the reciprocal of the highest serum dilution giving at least 50% fixation of complement. Antigens reacting with an antiserum to within $\frac{1}{2}$ or $\frac{1}{4}$ of the homologous titre were considered to be indistinguishable from the homologous strain, since even homologous titres varied to this degree in tests made at different times. Homologous titres are printed in bold type in the tables, those significant of antigenic relationship in ordinary type, and cross-reactions not regarded as significant of relationship, in italic figures.

RESULTS

Establishment of serotypes

As the survey progressed, antisera were prepared against strains that clearly did not belong to the serotypes represented by the antisera already in use. Eventually antisera against 17 distinct serotypes were available (Table 1). Not all the 82 strains were tested against all the antisera, but at least one strain of a given serotype, whether already established or newly discovered, was tested against the full battery of 17 antisera. Within the groups, however, in many cases up to 2 or 3 antisera of the same serotype were tested.

The 17 antisera used for fully cross-testing the serotypes were those against H 34, FH, B 3, G 2, Navel, 823, M 1, KSA, Campo, R 38, Gladysdale, pp. goat, Agalactia, PG 11, Laidlaw A, AM and T (Table 1); all had homologous titres in the range 1280–10,240.

With the exception of R 38 and Campo, cross-reactions occurred mostly at $\frac{1}{32}$ or less of the homologous titre. A few occurred at $\frac{1}{16}$ with antisera to FH, B 3, pp. goat and Laidlaw A, all of which had the comparatively low titre of 1280; these reactions did not suggest a close antigenic relationship. Only one high-titre serum, KSA, cross-reacted with several heterologous antigens at $\frac{1}{16}$ of the homologous titre (10,240). Gel-diffusion tests (see below) suggested that this may have been due to the presence of non-specific antibody to certain medium constituents.

With R 38 and Campo, the heterologous antigen reacted at $\frac{1}{16}$–$\frac{1}{4}$ of the homologous titre, suggesting partial, though not complete relationship.

The nature of the cross-reaction

The modification of the C.F.T. reduced the heterologous titres; it did not eliminate them completely. Low-titre reactions occurred with many of the pre-

immunization sera, but after immunization the reactions with heterologous antigens usually increased two- to fourfold. The occurrence of such persistent cross-reactions suggested either that non-specific antibody to certain constituents of the medium was present in the antisera, or that different species of *Mycoplasma* possessed certain antigens in common.

Of the four medium constituents injected into rabbits, only TDB and 'TDB residue' induced a two- to fourfold rise in titre over the pre-immunization sera against several mycoplasma antigens and TDB residue. The antibody responsible was probably scanty because it produced no precipitate on gel-diffusion plates with the various medium constituents. Nor was any precipitate demonstrable by gel-diffusion between these substances and the antisera used in the C.F.T., except sera against KSA and Gladysdale, which gave diffuse bands both with TDB and the complete medium. It is probable that TDB antibody was responsible for at least part of the cross-reactions which occurred in the C.F.T., and the possibility of removing non-specific antibody by absorbing antisera with horse tissue powders is being investigated.

The observed cross-reactions may not entirely be due to non-specific antibody. The observations of Villemot & Provost (1959) and Taylor-Robinson, Somerson, Turner & Chanock (1963) suggest that different species of *Mycoplasma* have antigens in common. This possibility requires further investigation.

Strains from man

Seventeen strains from the genital tract were tested against antisera to two of them (H 34 and 4387 P), to a human oral strain, B 3, and against an antiserum made by Card (1959) to a human genital strain representative of the single broad serotype which she found among 56 genital strains. Nine of these 17 strains were from the genital tract proper, including 2 strains which had undergone 6–8 passages in chick embryo cell cultures (Csonka & Furness, 1960). The other eight were from the external genitals in patients with non-gonococcal urethritis (NGU) or primary syphilis.

All reacted identically with the four sera, to titre with the three 'genital' antisera and at $\frac{1}{16}$–$\frac{1}{32}$ of the homologous titre with the 'oral' B 3 antiserum and may be considered serologically identical with Card's 56 strains. Thus, 73 strains tested by C.F.T. in this laboratory belonged to one broad serological group.

This agrees with the finding of Nicol & Edward (1953), Huijsmans-Evers & Ruys (1956) and Bercovici, Persky, Rozansky & Razin (1962), that the majority of the human genital strains examined belong to one serotype. Edward & Freundt (1956) have named this common human genital type *M. hominis type* 1. Since four cultures of *M. hominis type* 1, PG 21, PG 23, PG 25 and PG 26 obtained from Dr D. G. ff. Edward, reacted to high titre with an antiserum against a strain representative of those isolated in this laboratory (Card & Klieneberger-Nobel, unpublished observations) it would seem that our 73 strains belong to this species.

Three other strains of human origin, FH from Eaton's agent of Primary Atypical Pneumonia (*M. pneumoniae*, Chanock *et al.* 1963), B 3 (oral) and G 2 (*M. fermentans*) were serologically distinguishable from one another and from *M. hominis*

Table 2. *Homologous and cross-*C.F. *titres of* Mycoplasma *spp. from man and rats*

Antigen of strain	Origin	C.F. titre with antiserum: H34 *M. hominis, type* 1	B3 (human oral)	G2 *M. fermentans*	FH *M. pneumoniae*	Navel	Campo (PG27) '*M. hominis, type* 2'	Jasmin *M. arthritidis*
		Homologous titre						
		10240	**1280**	**2560**	**1280**	**5120–10240**	**10240**	**2560**
H34	Man	**10240**	*80*	*80*	*20*	*80*		
B3	Man	*80*	**1280**	*80*	*40*	*80*		
G2	Man	*80*	*80*	**2560**	*20*	*80*	*20–80*	NT
FH	Man	*80*	*40*	*40*	**1280**	*80*		
Navel	Man	*80*	*80*	*40*	*20*	**5120–10240**		
Campo-line PG27	Man							
Campo-line HEM	Man							
O7	Man							
Jasmin	Rats	*40–80*	*40–80*	*40–80*	*20–40*	*40–80*	5120–10240	1280–2560
Baxter	Rats							
LX	Rats							
DW	Rats							
VII-2	Rats							
H606	Unknown							

NT = Not tested.

type 1 (Table 2). This confirms the observations of Nicol & Edward (1953), Dienes & Madoff (1953), Huijsmans-Evers & Ruys (1956), Card (1959), Chanock, Hayflick & Barile (1962) and Taylor-Robinson *et al.* (1963). These 4 serotypes were distinct from a fifth human type represented by the strain Navel (Table 2) and from the 12 other types shown in Table 1.

Navel could be serologically distinguished from all of the other 16 serotypes included in this survey; heterologous antigens all cross-reacted at less than $\frac{1}{32}$ of the homologous titre. The results were quite clear-cut, in contrast to those obtained by Taylor-Robinson *et al.* (1963) in a micro-C.F.T., using antiserum supplied by the present author. These workers obtained very high levels of cross-reaction with antigens of certain oral and genital strains and it was difficult to assess from their results how closely Navel was related to these strains.

Apart from the above report, Navel does not seem to have been compared serologically with other species of *Mycoplasma*. Ruiter & Wentholt (1955), who isolated it from a patient with umbilical dermatitis, noted that it differed from their G-strains (Ruiter & Wentholt, 1952) in its failure to ferment glucose, its non-pathogenicity for mice and its eventual adaptation to growth under aerobic conditions.

Campo, a strain of the so-called '*M. hominis type* 2', was distinct from the other 5 serotypes isolated from man, but indistinguishable from a rat strain Jasmin (Table 2). To verify that the reactions of our line of Campo (PG 27, obtained some years previously from Dr D. G. ff. Edward) were typical of the species, another line of Campo (HEM) and another 'type 2' strain, 07 (Morton, Lecce, Oskay & Coy, 1954), were obtained from Dr H. E. Morton. All these cultures were indistinguishable from 5 rat strains originating in different countries (Table 2). The pathogenicity of two of the rat strains, Jasmin and Baxter, and their similarity to the original L4 (Klieneberger-Nobel, 1960) suggests that they are rat polyarthritis PPLO, the *M. arthritidis* of Edward & Freundt (1956). Strain H 606 also belonged to this group, but whether it was of rat or human origin could not be established.

'Type 2' strains and *M. arthritidis* are reported as failing to ferment glucose (Edward, 1950, 1954; Lecce & Morton, 1954; Tourtellotte & Jacobs, 1960). None of the 'type 2' and rat strains listed in Table 2 produced acid from glucose. Further evidence for the identity of the two species is their ability to produce subcutaneous abscesses in mice (Edward, 1954).

In summary, five serological types were found among mycoplasma strains from man. One, comprising the greatest number of strains, corresponded to the species *M. hominis type* 1, the second to *M. pneumoniae*, the third probably to the oral species, *M. salivarium*, and the fourth to *M. fermentans*. The fifth serotype, comprising only one strain, Navel, has not so far been assigned to any recognized species. Because of its close antigenic relationship to *M. arthritidis*, the position of '*M. hominis type* 2' as a distinct species is in doubt.

Strains from tissue cultures

Sixteen strains of *Mycoplasma* from various mammalian cell cultures were examined. Nine were from HeLa lines, 5 maintained in four London laboratories, 1 from Canada and 3 from one laboratory in the U.S.A. Six cultures came respectively from the cell lines ERK, MK2, mouse sarcoma S180, human pleural D116P, HEp.II and the Fjelde line of human epidermoid carcinoma of skin. ERK was shown to be of human rather than rabbit origin (Coombes, Daniel, Gurner & Kelus, 1961) and both ERK and MK2 are thought to be HeLa lines. Thus, apart from S180, all the cell lines were of human origin. The type of cell culture from which the remaining strain, TC7277, was isolated is unknown: the only information available is that it was isolated in Saudi Arabia from a trachoma culture. A seventeenth strain, BM, may have originated in a tissue culture since it was found in a subculture of a strain of Eaton Agent (line PI898) that had been passaged only in primary monkey kidney tissue cultures.

Eleven of these strains, 10 obtained from cell lines of human origin and 1 from the mouse sarcoma line, S180, reacted to high titre with antisera against *M. hominis type* 1, but gave no significant reaction with the 'human oral' B3 antiserum (Table 3).

Five others, 823, 826, 837 and 844 from the three American HeLa lines and the HEp.II line, and BM from the Eaton Agent strain formed a homogeneous group, as judged by tests with sera against two of them (Table 3). This group was distinct from *M. hominis type* 1, the human oral (Table 3) and 14 other serotypes of *Mycoplasma* (Table 1).

Morphologically, 823 and related strains were highly filamentous when grown in broth. On solid media, all 5 grew better anaerobically; in liquid, growth occurred aerobically but was more profuse if cultures were not shaken. It is not clear whether this preference for a low oxygen tension is an inherent property of this group or whether it is the result of continued propagation in tissue cultures. In this laboratory human genital strains, which usually grow well aerobically, grow very poorly, if at all, on aerobic plates when first isolated from tissue cultures, indicating the need for anaerobic as well as aerobic cultivation when attempting to isolate PPLO from tissue cultures.

TC7277, the strain from an unidentified tissue culture, was not related to *M. hominis type* 1 or to the 823 serotype, but was serologically identical with *M. laidlawii*.

To summarize, most of the tissue culture strains belonged to the species *M. hominis type* 1; one was *M. laidlawii* and the remainder constituted a distinct serological group, unrelated to other known species.

A strain probably derived from embryonated eggs

Fluid from embryonated eggs through which material from a patient with a recurrent skin infection had been passaged was sent to this laboratory by Prof. A. M. Macdonald. The material had produced pock-like lesions about 2 mm. in diameter on the chorio-allantoic membrane (C.A.M.). The pock-producing agent had

Table 3. C.F. *titres of* Mycoplasma *strains from tissue cultures with antisera against* M. hominis type 1, *a human oral and two tissue culture strains*

Antigen of strain	Tissue culture of origin	C.F. titre with antiserum: H34 *M. hominis, type* 1	4387P *M. hominis, type* 1	B3 (human oral)	823	837
		Homologous titre: **10240**	**2560**	**1280**	**10240**	**5120**
ERK	Embryo rabbit kidney (probably HeLa)	2560–5160	640–2560	*40–160*	*40–80*	*40–80*
MK2	Monkey kidney (probably HeLa)					
CH	HeLa (London, laboratory 1)					
NCH	HeLa (London, laboratory 2)					
Tang	HeLa (London, laboratory 3)					
AE	HeLa (London, laboratory 4, line 1)					
CE	HeLa (London, laboratory 4, line 2)					
Payne	HeLa (Canada)					
H.Ep2	Human Epidermoid carcinoma (Fjelde line)					
420	Human pleural, D116P					
417	Mouse sarcoma, S180					
823	HeLa (U.S.A.)	*80–160*	*80*	*40–80*	2560–10240	1280–5120
837	HeLa, RB10 (U.S.A.)					
826	HeLa (U.S.A.)					
844	H.Ep II (U.S.A.)					
BM	Eaton agent propagated in tissue culture					

been serially propagated on the C.A.M., producing lesions that contained minute stainable organisms resembling PPLO (Macdonald, personal communication). The present author isolated a mycoplasma strain, AM, from the egg material.

In C.F. tests with antisera of 17 serotypes, AM reacted significantly only with an antiserum against a non-pathogenic avian strain A 36; A 36 also reacted to high titre with AM antiserum (Table 4). Miss A. G. Newnham (Cambridge) confirmed the serological relationship of AM to A 36 by means of an agglutination test. AM differed from A 36, however, in failing to produce acid from glucose.

Table 4. C.F. *titres of various avian* Mycoplasma *strains with* A 36 *and* AM *antisera*

	C.F. titre with antiserum	
Antigen of strain	A 36	AM
A 36 (non-pathogenic avian, U.K.)	**640–1280**	640–1280
AM (embryonated egg)	640–1280	**2560–5120**
PSU 4 (embryonated egg)	640	640–1280
TU (non-pathogenic avian U.S.A.; Kleckner's group C)	*40–80*	*40–80*
M (*M. iners*)		
Fowl (*M. gallinarum*)		
T (*M. gallisepticum*)		

Another strain PSU 4, isolated from a 'pipped' chick embryo after further egg passages, was also related to AM and A 36. These three strains seemed distinct from 4 other recognized avian types or species, TU, *M. iners*, *M. gallinarum* and *M. gallisepticum* (Table 4).

Chu & Newnham (personal communication) have found that A 36 is distinct from the 4 avian types, TU, *M. gallinarum*, *M. iners*, and *M. gallisepticum*, and consider that it probably corresponds to the group D of Kleckner (1960). The present work showed that it is also distinct from 15 serotypes of non-avian origin (Table 1).

The relationship of AM to A 36 suggests that it was derived from the embryonated eggs and not the skin lesions of the patient. This view is consistent with the failure of broth cultures of AM, after 7 transfers on artificial medium, to produce pock-like lesions in the C.A.M. Although it was possible that the *Mycoplasma* had produced the pock-like lesions originally observed and had lost this property during transfer on artificial media, it seems more probable that the pocks were produced by another agent from the human skin infection. The growth of the pock-producing agent on the C.A.M. probably enabled the mycoplasma already present in the eggs to proliferate in the lesions. In experimental animals, analogous situations have been found where latent mycoplasma infections have been made manifest by the inoculation of other infective agents (Klieneberger-Nobel, 1962).

Strains from laboratory rats and mice

Seventeen strains from rats were classified by means of antisera prepared against three of them: Kon, from a lung lesion of a rat with broncho-pneumonia, Jasmin (*M. arthritidis*) and R 38 from purulent rhinitis in a rat. Kon and Jasmin appeared

distinct; there was some antigenic relationship between Jasmin and R38 (Table 5), but it was possible to distinguish between them by the C.F.T.

Eight of these rat strains, R42L, R45L, R46L, R25NP, 2098 and SPF47, all from the respiratory tract, and RME (1) and R42A (1) were indistinguishable from the rat lung strain, Kon (Table 5). RME (1) was isolated from a natural infection in the middle ear and R42A (1) from a subcutaneous abscess produced by the inoculation of R38. Both these organisms may have originated in the respiratory tract; in one rat an identical strain (R42L) was isolated from the lung; in the other (Tuffery, personal communication) there were, on the lung surface, dark spots characteristic of one stage of chronic PPLO bronchopneumonia (Klieneberger & Steabben, 1937, 1940).

Table 5. C.F. *titres of* Mycoplasma *strains from rats with antisera against Kon, Jasmin and R*38

Antigen of strain	Origin of strain	C.F. titre with antiserum: Kon (Homologous titre)	Jasmin (Homologous titre)	R38 (Homologous titre)
		1280	**2560**	**2560**
Kon, R42L, R45L, R46L, R25NP, 2098, SPF47	Respiratory tract	640–1280	*40–80*	*40–80*
Jasmin	Tumour	*40–80*	1280–2560	320–640
Baxter	Polyarthritis			
DW	Tumour			
LX	(Lesion unknown)			
VII-2	Respiratory tract			
R38	Rhinitis	*80*	320	2560
R42A	Abscess produced by inoculation of R38	640	320	2560
R42A (1)	Isolate from mixed culture R42A	640	*80*	*80*
R42A (2)	Isolate from mixed culture R42A	*80*	320	2560
RME	Infection of middle ear	1280	320	2560
RME (1)	Isolate from mixed culture RME	1280	*80*	*80*
RME (2)	Isolate from mixed culture RME	*80*	320	2560

Two rat strains were indistinguishable from R38. These were R42A (2) which was recovered, together with the lung type R42A (1), from a subcutaneous abscess produced by the inoculation of R38, and RME (2), which was recovered, together with the lung type RME (1), from the middle ear. The presence of 2 distinct strains in the original cultures from the abscess and the middle ear was first shown by the C.F.T.; antigens from these cultures reacted to titre with both Kon and R38 antisera (Table 5). Re-examination of both cultures revealed 2 colony types

and antigens prepared from single colony subcultures showed that both R38 and the lung type were present in the original cultures (Table 5).

Four rat strains, Baxter, DW, LX, and VII-2 were identical with Jasmin in their reactions with antisera against Jasmin, Campo ('*M. hominis type* 2') and R38 (Tables 2 and 5). Another rat strain, STR, which was indistinguishable from *M. laidlawii* may have been present on the rat as a saprophytic contaminant.

Eight strains from mice, DGE, M1, M2, 68NP and 72L from the respiratory tract and MB, Peter and 73B from the brain, were identical with Kon. This was confirmed by titrations with M1 and MB antisera. This serotype has been shown to be associated with respiratory disease in mice (Lemcke, 1961) and probably spreads to the brain when the respiratory tract is heavily infected. Unlike *M. neurolyticum*, another type found in the mouse brain, it is not pathogenic when inoculated intracerebrally (Lemcke, 1961).

Thus, the same serotype is found in the respiratory tract of both rats and mice. Edward (1954) also reported that some PPLO from mice with catarrh had similar pathogenic and cultural properties to L3, the original rat lung type described by Klieneberger (1938). The association of the serotype represented by Kon with rat bronchopneumonia and respiratory disease in mice suggests that it corresponds to the species *M. pulmonis* (Edward & Freundt, 1956).

Another strain, KSA, from the brain of a mouse differed both in colonial morphology and antigenically from Kon and its related strains. It was also distinct from *M. arthritidis*, R38, and 13 other serotypes (Table 1). On primary isolation it produced the symptoms of 'rolling disease', as described by Findlay, Klieneberger, MacCallum & MacKenzie (1938), when inoculated with an agar adjuvant intracerebrally into mice (Lemcke, 1961). A later subculture was less virulent, only one out of nine mice exhibiting the characteristic 'rolling'. The rest survived, developing a pronounced hydrocephalus; one month after inoculation, their serum titre with KSA antigen was 40–80, compared with < 10 in mice inoculated only with agar. The properties of KSA suggest that it is the same as the L5 strain of Findlay *et al.* (1938), the species *M. neurolyticum* of Edward & Freundt (1956).

In summary, all the strains from laboratory rats and mice, with the exception of the saprophyte STR, belonged to four serotypes: 17, mostly from the respiratory tract of both rats and mice, probably corresponded to *M. pulmonis*, 5, from rats, to *M. arthritidis* and one, from mouse brain, to *M. neurolyticum*. A fourth type, represented by R38, R42A (2) and RME (2) from rats with purulent infections, was partially related to *M. arthritidis*.

Strains from cattle and goats

Eight cultures from cattle and goats were compared (Table 6). A 20-year-old strain, pp. cattle, from contagious bovine pleuropneumonia (CBPP) could not be distinguished from Gladysdale (*M. mycoides* var. *mycoides*), a more recently isolated strain from CBPP. A similar observation was made by Yoshida (1961), who found that CBPP strains kept in his laboratory retained their reactivity with rabbit and goat antisera although they no longer reacted with positive sera from cattle infected with the CBPP organism. Some antigenic loss obviously occurs

after prolonged subculture, but it is not apparent in C.F. tests using sera from immunized animals.

Two strains, G 1/61 and G 11, isolated by C. Pillai in the Sudan from goats with contagious caprine pleuropneumonia (CCPP), were indistinguishable from the two CBPP strains (Table 6). Hudson & Cottew (personal communication) also found that Pillai's strain G 1/61 was indistinguishable from *M. mycoides* var. *mycoides*, but distinct from 2 strains reputed to be *M. mycoides* var. *capri*.

Another CCPP strain, pp. goat, isolated by Beveridge and Chu in Turkey, was distinct from *M. mycoides* var. *mycoides* (Table 6). Although this organism is the same as the one described by Edward (1954) as *M. mycoides* var. *capri*, it seems doubtful whether the name is justified in view of the serological difference between it and *M. mycoides* var. *mycoides*, and the fact that strains serologically identical with or very closely related to the latter have been isolated from CCPP.

Of the other three cultures, Agalactia from infected milk of a goat with agalactia and PG 11 (*M. bovigenitalium*) were distinct from one another and from the CBPP and CCPP organisms (Table 6). Agalactia has the cultural and morphological properties described for *M. agalactiae* (Edward, 1954; Freundt, 1957). Strain 37, from bovine mastitis (Davidson & Stuart, 1960), was related to *M. bovigenitalium*. This is in agreement with the findings of Edward & Leach, reported by Stuart *et al.* (1963).

In summary, four species or serotypes were distinguishable among 8 cultures from cattle and goats, *M. mycoides* var. *mycoides*, *M. bovigenitalium*, *M. agalactiae* and a CCPP organism, pp. goat.

Saprophytic strains

Laidlaw's strains A and B were not distinguishable by this test. Earlier reports (Laidlaw & Elford, 1936; Klieneberger, 1940) indicate that A and B are related to some extent antigenically and Edward & Freundt (1956) name only one species, *M. laidlawii*. As already noted, only two cultures besides Laidlaw B, TC 7277 and STR, reacted to high titre with Laidlaw A antiserum.

DISCUSSION

The results from this and other laboratories of typing mycoplasma strains from the human genital tract show that most of them, at least in Europe, belong to one broad serological group, the species *M. hominis type* 1. There may be slight antigenic differences between strains within this species, as suggested by the observations of Nicol & Edward (1953), Card (1959), Oates, Whittington & Wilkinson (1959) and Taylor-Robinson *et al.* (1963). These might account for the differences in sensitivity between the 3 strains H 34, MJW and H 27 in detecting specific antibody in human sera (Lemcke & Csonka, 1962).

Although *M. hominis type* 1 has been isolated from the apparently healthy genital tract (Freundt, 1956; Nicol & Edward, 1953), most of the strains examined in this laboratory were isolated from patients with evidence of genital infection. In some instances (e.g. NGU patients) only PPLO were found; in others, another

Table 6. *Homologous and cross-*C.F. *titres of* Mycoplasma *strains from cattle and goats*

Antigen of strain	Origin of strain	C.F. titre with antiserum: pp. cattle *M. mycoides* var. *mycoides*	Gladysdale *M. mycoides* var. *mycoides*	G 1/61	pp. goat	Agalactia *M. agalactiae*	PG 11 *M. bovigenitalium*
		Homologous titre					
		640	**5120**	**1280**	**1280**	**2560**	**5120**
pp. cattle	Contagious bovine pleuropneumonia (CBPP)	**640**	10240	NT	NT	NT	*80*
Gladysdale	CBPP	640	**5120**	1280	*80*	*20*	*20*
G 1/61, G 11	Contagious caprine pleuropneumonia (CCPP) in Sudan	NT	2560	**1280**	*40*	*< 20*	*20–40*
pp. goat	CCPP in Turkey	NT	*80*	*40*	**1280**	*20*	*40*
Agalactia	Milk of goat with agalactia	*40*	*40*	*20*	*80*	**2560**	*80*
PG 11	Bovine genital tract	*80*	*80*	*40*	*80*	*20*	**5120**
37	Bovine mastitis	*80*	*40*	NT	NT	NT	1280

NT, not tested.

pathogen such as *Trichomonas vaginalis*, *Neisseria gonorrhoeae* or *Treponema pallidum* was present. *M. hominis type* 1 seems to be a potential pathogen which can proliferate in the urogenital tract under certain conditions, especially where tissues have been damaged by some other pathogen or by trauma.

Only one of the human strains, Ruiter & Wentholt's strain Navel, was not related to one of the four types or species *M. hominis type* 1, *M. fermentans*, *M. pneumoniae* and the human oral type. Since it was distinguishable from the 16 other serotypes, it may represent a hitherto unrecognized species.

The antigenic similarity of '*M. hominis type* 2' to *M. arthritidis* suggests that the former should no longer be recognized as a distinct species. *M. arthritidis* is found much more frequently in rats than 'type 2' strains are found in man and it is a recognized rat pathogen, whereas the pathogenicity of 'type 2' strains for man is obscure. It is probable, therefore, that the natural host of this serotype is the rat, and that 'type 2' strains occur in man as commensals or saprophytic contaminants.

There are no reports of the isolation of 'type 2' from the human genital tract in Europe, but in the U.S.A. a few other type 2 genital strains besides Campo and O 7 have been isolated (Norman *et al.* 1950; Bailey *et al.* 1961). There seems little justification, however, for the statement of Corriell, Fabrizio & Wilson (1960), that this is the genital type commonly found in the U.S.A. There has been no survey in America comparable to those carried out in Europe, where a large number of strains from the human genital tract has been identified serologically. Type 1 strains undoubtedly occur in America, as indicated by the identification of the NGU strain 4387 P in the present work and the reports of Nicol & Edward (1953) and Bailey *et al.* (1961). They may, therefore, be just as prevalent there as in Europe.

The results of this investigation as well as those of Collier (1957), Corriell *et al.* (1960) and Bailey *et al.* (1961) establish that a large proportion of PPLO from tissue cultures are *M. hominis type* 1. Barile, Malizia & Riggs (1962), using a fluorescent antibody technique, found that 48 strains from tissue cultures were antigenically related, but did not identify the serotype. One of these, H.Ep 2, proved in this present survey to be *M. hominis type* 1—further evidence of the widespread contamination of cell lines by this species. The suggestion of Collier (1957) that this type of *Mycoplasma* was present in the HeLa cells when originally isolated from a carcinoma of the human cervix, seems the most probable of all those proposed to account for the contamination. Cell lines other than HeLa could have become contaminated from HeLa lines maintained in the same laboratory, as the manipulations involved in cultivating cells probably predispose to such cross-contamination.

The isolation of *M. laidlawii* from a tissue culture has not previously been reported. Like the 'type 2' strain found by Bailey *et al.* (1961) it may just be an isolated instance.

The group of 5 tissue culture strains represented by 823 did not belong to any of the species of known origin with which it was compared, so that there is no indication of their origin. Culturally and morphologically they had the characters

of PPLO, not of authentic L-forms. All 5 strains were either isolated in the U.S.A. or came from material which had been passaged in tissue cultures there. The contamination of the cell lines involved might therefore be traceable to a common source, possibly a single supplier of cell cultures.

As the majority of PPLO from tissue cultures clearly belong to recognized species of *Mycoplasma*, it seems unlikely that they are, as has been frequently suggested, L-forms derived from contaminating bacteria under the influence of antibiotics in the cell-culture medium.

As regards avian strains, the isolation of AM and PSU 4 from embryonated eggs and their antigenic relationship to A 36 indicate that non-pathogenic avian strains can pass into the egg. Kleckner (1960) reported that non-pathogenic avian PPLO could be egg-transmitted, but did not specify which types. It is established that the PPLO associated with chronic respiratory disease (C.R.D.) in poultry, *M. gallisepticum* or 'Nelson's coccobacilliform bodies', can be transmitted through the egg (Fahey & Crawley, 1954). Isolation of the C.R.D. organism during the passage of infective agents or pathological material through embryonated eggs has been reported several times (Van Herick & Eaton, 1945; Klieneberger-Nobel, 1962; Marmion & Hers, 1963).

The demonstration in embryonated eggs of another serotype, A 36, besides *M. gallisepticum* emphasizes the necessity of identifying serologically any strain of *Mycoplasma* isolated from infective material passaged through eggs. The same applies to tissue culture passage, since BM, from a line of Eaton Agent maintained in tissue cultures proved to be antigenically related not to *M. pneumoniae*, but to a type of *Mycoplasma* previously found only in tissue cultures. Failure to identify strains properly could result in the erroneous implication, as pathogens in man or animals, of innocuous strains of avian or tissue culture origin. A similar mistake could be made with PPLO from animals, since strains resembling the saprophytic *M. laidlawii* are occasionally found, like STR from a rat and the bovine genital 'S' strains found by Edward (1950).

The widespread occurrence of mycoplasma infection in laboratory rats and mice is illustrated by the fact that the cultures examined came from 12 different stocks or colonies in 5 different countries. Most of the PPLO isolated from rats and mice seemed to belong to species recognized as pathogens of these animals, *M. pulmonis*, *M. arthritidis* and *M. neurolyticum*. The partial antigenic relationship to *M. arthritidis* of a fourth type, R 38, suggests that it is not a distinct species but an antigenic variant of *M. arthritidis*. It is distinguishable from *M. arthritidis* by its lesser pathogenicity in the rat (Klieneberger-Nobel, 1960; Lemcke, 1961).

From the anomalous behaviour in the C.F.T. of antigens prepared with what proved to be mixed cultures, the existence in two rat lesions of more than one serotype was detected. This demonstrates the sensitivity of the C.F.T. used in these investigations, and emphasizes the importance both of establishing clones from single colonies for tests and of isolating more than one colony from the primary plate, if mixed infections are to be detected. Mixed infections may indeed occur more frequently than is suspected. Immunofluorescence techniques, as applied to *Mycoplasma* (Malizia, Barile & Riggs, 1961; Chanock, Hayflick & Barile, 1962;

Clark, Bailey, Fowler & Brown, 1963; Marmion & Hers, 1963) should afford a method of detecting mixtures of serotypes in primary agar cultures. The tendency of mycoplasma species to participate in mixed infections is well illustrated in rats as well as in human genital infections. Two *M. arthritidis* strains, DW and LX, were isolated in association with a bacterium, *Streptobacillus moniliformis*, from lesions in rats.

The similarity of the two contagious bovine pleuropneumonia strains is in accord with observations which suggest that there is only one species, *M. mycoides* var. *mycoides*, associated with contagious pleuropneumonia of cattle (Klieneberger-Nobel, 1962). There is, however, some confusion among the goat strains. Unless the disease was wrongly diagnosed in either case, two serotypes, one of which is indistinguishable from *M. mycoides* var. *mycoides*, were isolated from contagious pleuropneumonia in goats. Moreover, organisms identical with or related to either *M. mycoides* or *M. agalactiae* have been found in disease conditions different from classical CCPP and agalactia (Hudson, personal communication). Thus, recognized species appear to be associated with more than one type of disease in goats. Without more information about the types of *Mycoplasma* in different goat diseases, it would be premature to attribute a particular goat disease, such as CCPP, to any one species.

It is clear that much confusion can be avoided in regard both to the aetiological significance of mycoplasma species in disease conditions and to the taxonomy of the group when cultures are identified by suitable serological techniques. More detailed knowledge of the antigenic structure of the Mycoplasmataceae is obviously desirable. Extraction and purification of the antigens should result not only in the improvement of existing serological methods, like the C.F.T., for diagnosis and identification, but in the development of precipitin techniques. Coupled with immunodiffusion methods, the use of purified extracts should also elucidate both the finer apparent antigenic differences between strains, like those between strains of *M. hominis type* 1, and the partial antigenic relations observed between rat strains such as R38 and *M. arthritidis*.

SUMMARY

A complement fixation test with rabbit antisera was used to differentiate 82 cultures of *Mycoplasma* from man, mammalian cell cultures, laboratory rats and mice, cattle, goats, poultry, embryonated eggs and sewage.

Seventeen serotypes were distinguished, 5 from man, 1 from mammalian cell cultures, 4 from rats and mice, 4 from cattle and goats, 2 from poultry and one saprophytic. Most of these corresponded to recognized species of *Mycoplasma*, but 1 of human origin (represented by 1 strain, Navel), and 1 from tissue cultures (5 strains), may represent new species. R38, one of the serotypes from rats, could be distinguished from the species *M. arthritidis*, but is probably an antigenic variant rather than a distinct species. Two species hitherto recognized as distinct, *M. arthritidis* and *M. hominis type* 2, could not be distinguished and appear to constitute a single species. These findings illustrate the necessity, from the viewpoint of taxonomy, of comparing mycoplasma strains by serological methods.

The serotypes of human and animal origin were largely host-specific. Exceptions were the inclusion of *M. arthritidis* from rats and *M. hominis type* 2 from man in a single serotype, the finding of a bovine organism among the strains isolated from goats and of a saprophytic strain in a rat.

In relation to the aetiology of disease in man and animals, the isolation of an endogenous *Mycoplasma* from embryonated eggs used to passage infective material illustrates the importance of identifying these organisms serologically. The demonstration of mixed mycoplasma infections in lesions in two rats shows the necessity of adequately purifying all cultures of *Mycoplasma* before examination.

I would like to thank those workers mentioned in Table 1 who kindly provided the cultures which they isolated. In addition I wish to thank the following who sent cultures: Prof. A. C. Ruys and Dr D. Herderscheê (Amsterdam) for G2, Prof. H. E. Morton (Philadelphia) for Campo and O7, Dr D. G. ff. Edward (Beckenham) for Campo (PG27), *M. bovigenitalium* (PG11), *M. iners*, and *M. gallinarum*, Dr R. G. Wittler for H606 and Miss A. G. Newnham for A36, TU, PSU4 and TC7277.

My thanks are also due to Dr L. H. Collier of this Institute, Dr G. W. Csonka (London), Prof. A. E. Macdonald (Aberdeen), Dr J. Payne (Edmonton, Alberta), Mr A. A. Tuffrey (Carshalton) and Dr D. Weisinger (Basle) who provided material from which PPLO were isolated. I gratefully acknowledge all the information so generously supplied by these workers about the strains or material provided.

I am indebted to Miss A. G. Newnham for carrying out an agglutination test on the avian strains.

Part of this work was carried out by the author under the aegis of the Medical Research Council Working Party on Non-Specific Urethritis with the aid of a grant from the U.S. Public Health Service.

REFERENCES

Bailey, J. S., Clark, H. W., Felts, W. R., Fowler, R. C. & Brown, T. McP. (1961). Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. *J. Bact.* **82**, 542.

Barile, M. F., Malizia, W. F. & Riggs, D. B. (1962). Incidence and detection of pleuropneumonia-like organisms in cell cultures by fluorescent antibody and cultural procedures. *J. Bact.* **84**, 130.

Bercovici, B., Persky, S., Rozansky, R. & Razin, S. (1962). Mycoplasma (pleuropneumonia-like organisms) in vaginitis. *Amer. J. Obstet. Gynec.* **84**, 687.

Card, D. H. (1959). PPLO of human genital origin. Serological classification of strains and antibody distribution in man. *Brit. J. vener. Dis.* **35**, 27.

Chanock, R. M., Dienes, L., Eaton, M. D., Edward, D. G. ff., Freundt, E. A., Hayflick, L., Hers, J. F. ph., Jensen, K. E., Liu, C., Marmion, B. P., Morton, H. E., Mufson, M. A., Smith, P. F., Somerson, N. L. & Taylor-Robinson, D. (1963). *Mycoplasma pneumoniae*. Proposed nomenclature for atypical pneumonia organism (Eaton agent). *Science*, **140**, 662.

Chanock, R. M., Hayflick, L. & Barile, M. F. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. *Proc. nat. Acad. Sci., Wash.*, **48**, 41.

Clark, H. W., Bailey, J. S., Fowler, R. C. & Brown, T. Mc.P. (1963). Identification of Mycoplasmataceae by the fluorescent antibody method. *J. Bact.* **85**, 111.

COLLIER, L. H. (1957). Contamination of stock lines of human carcinoma cells by pleuropneumonia-like organisms. *Nature, Lond.*, **180**, 757.

COOMBES, R. R. A., DANIEL, M. R., GURNER, B. W. & KELUS, A. (1961). Species-characterizing antigens of 'L' and 'ERK' cells. *Nature, Lond.*, **189**, 503.

CORRIELL, L. L., FABRIZIO, D. P. & WILSON, S. R. (1960). Comparison of PPLO strains from tissue cultures by complement fixation. *Ann. N.Y. Acad. Sci.* **79**, 574.

CSONKA, G. W. & FURNESS, G. (1960). A study of the aetiology of non-gonococcal urethritis and Reiter's disease. *Brit. J. vener. Dis.* **36**, 181.

DAVIDSON, I. & STUART, P. (1960). *Vet. Rec.* **72**, 766 (Letter).

DIENES, L. & MADOFF, S. (1953). Differences between oral and genital strains of human pleuropneumonia-like organisms. *Proc. Soc. exp. Biol., N.Y.*, **82**, 36.

EDWARD, D. G. FF. (1950). An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract. *J. gen. Microbiol.* **4**, 4.

EDWARD, D. G. FF. (1954). The pleuropneumonia group of organisms: a review, together with some new observations. *J. gen. Microbiol.* **10**, 27.

EDWARD, D. G. FF. & FITZGERALD, W. A. (1951). The isolation of organisms of the pleuropneumonia group from dogs. *J. gen. Microbiol.* **5**, 566.

EDWARD, D. G. FF. & FITZGERALD, W. A. (1954). Inhibition of growth of pleuropneumonia-like organisms by antibody. *J. Path. Bact.* **68**, 23.

EDWARD, D. G. FF. & FREUNDT, A. E. (1956). The classification and nomenclature of organisms of the pleuropneumonia group. *J. gen. Microbiol.* **14**, 197.

EDWARD, D. G. FF. & KANAREK, A. D. (1960). Organisms of the pleuropneumonia group of avian origin: their classification into species. *Ann. N.Y. Acad. Sci.* **79**, 696.

FAHEY, J. E. & CRAWLEY, J. F. (1954). Studies on CRD of chickens. III. Egg transmission of a PPLO. *Canad. J. comp. Med.* **18**, 67.

FINDLAY, G. M., KLIENEBERGER, E., MACCALLUM, F. O. & MACKENZIE, R. D. (1938). 'Rolling disease'; new syndrome in mice associated with a pleuropneumonia-like organism. *Lancet*, ii, 1511.

FREUNDT, E. A. (1956). Occurrence and ecology of *Mycoplasma* species (pleuropneumonia-like organisms) in the male urethra. *Brit. J. vener. Dis.* **32**, 188.

FREUNDT, E. A. (1957). In *Bergey's Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall and Cox.

HUIJSMANS-EVERS, A. G. & RUYS, A. C. (1956). Micro-organisms of the pleuropneumonia group (Family of Mycoplasmataceae) in man. II. Serological identification and discussion of pathogenicity. *Leeuwenhoek ned. Tijdschr.* **22**, 377.

KLECKNER, A. L. (1960). Serotypes of avian pleuropneumonia-like organisms. *Amer. J. vet. Res.* **21**, 274–280.

KLIENEBERGER, E. (1938). Pleuropneumonia-like organisms of diverse provenance. *J. Hyg., Camb.*, **38**, 458.

KLIENEBERGER, E. (1940). The pleuropneumonia-like organisms: further comparative studies and a descriptive account of recently discovered types. *J. Hyg., Camb.*, **40**, 204.

KLIENEBERGER, E. & STEABBEN, D. B. (1937). On a pleuropneumonia-like organism in lung lesions of rats, with notes on the clinical and pathological features of the underlying condition. *J. Hyg., Camb.*, **37**, 143.

KLIENEBERGER, E. & STEABBEN, D. B. (1940). On the association of the pleuropneumonia-like organism L3 with bronchiectatic lesions in rats. *J. Hyg., Camb.*, **40**, 223.

KLIENEBERGER-NOBEL, E. (1960). Pathogenicity and immunology of organisms of the pleuropneumonia group. *Ann. N.Y. Acad. Sci.* **79**, 615.

KLIENEBERGER-NOBEL, E. (1962). *Pleuropneumonia-like organisms (PPLO) Mycoplasmataceae*. London: Academic Press.

LAIDLAW, P. P. & ELFORD, W. J. (1936). A new group of filterable organisms. *Proc. roy. Soc.* B, **120**, 292.

LECCE, J. G. & MORTON, H. E. (1954). Metabolic studies on three strains of PPLO isolated from man. *J. Bact.* **67**, 62.

LEMCKE, R. M. (1961). Association of PPLO infection and antibody response in rats and mice. *J. Hyg., Camb.*, **59**, 401.

LEMCKE, R. M. & CSONKA, G. W. (1962). Antibodies against pleuropneumonia-like organisms in patients with salpingitis. *Brit. J. vener. Dis.* **38**, 212.

MALIZIA, W. F., BARILE, M. F. & RIGGS, D. B. (1961). Immunofluorescence of pleuropneumonia-like organisms isolated from tissue cell cultures. *Nature, Lond.*, **191**, 190.

MARMION, B. P. & HERS, PH. J. F. (1963). Proceedings of the conference on newer respiratory viruses. Washington, October, 1962. *Amer. Rev. resp. Dis.* **88** (3), Part 2, 198.

MORTON, H. E., LECCE, J. G., OSKAY, J. J. & COY, N. H. (1954). Electron microscope studies of pleuropneumonia-like organisms isolated from man and chickens. *J. Bact.* **68**, 697.

NICOL, C. S. & EDWARD, D. G. FF. (1953). Role of organisms of the pleuropneumonia group in human genital infections. *Brit. J. vener. Dis.* **29**, 141.

NORMAN, M. C., SASLAW, S. & KUHN, L. R. (1950). Antigenic similarity of five human strains of pleuropneumonia-like organisms. *Proc. Soc. exp. Biol., N.Y.*, **75**, 718.

OATES, J. K., WHITTINGTON, M. J. & WILKINSON, A. E. (1959). A note on the results of cultural and serological tests for pleuropneumonia-like organisms in Reiter's disease. *Brit. J. vener. Dis.* **35**, 27.

RUITER, M. & WENTHOLT, H. M. M. (1952). The occurrence of a pleuropneumonia-like organism in fuso-spirillary infections of the human genital mucosa. *J. invest. Derm.* **18**, 313.

RUITER, M. & WENTHOLT, H. M. M. (1955). Isolation of a pleuropneumonia-like organism from a skin lesion associated with a fuso-spirochetal flora. *J. invest. Derm.* **24**, 31.

STUART, P., DAVIDSON, I., SLAVIN, G., EDGSON, F. A. & HOWELL, D. (1963). Bovine mastitis caused by *Mycoplasma*. *Vet. Rec.* **75**, 59.

TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. & CHANOCK, R. M. (1963). Serological relationships among human Mycoplasmas as shown by complement-fixation and gel diffusion. *J. Bact.* **85**, 1261.

TOURTELLOTTE, M. E. & JACOBS, R. E. (1960). Physiological and serologic comparisons of PPLO from various sources. *Ann. N.Y. Acad. Sci.* **79**, 521.

VAN HERICK, W. & EATON, M. D. (1945). An unidentified pleuropneumonia-like organism isolated during passages in chick embryos. *J. Bact.* **50**, 47.

VILLEMOT, J. M. & PROVOST, A. (1959). Recherches immunologiques sur la peripneumonie. V. Relations antigeniques entre *Mycoplasma mycoides* var. *mycoides*, *M. mycoides* var. *capri* et d'autres micro-organismes du genre *Mycoplasma* (souches génitales bovines et humaines). *Rev. Élev.* **12**, 251.

WARREN, J. & SABIN, A. (1942). Some biologic and immunologic characteristics of a pleuropneumonia-like organism of human origin. *Proc. Soc. exp. Biol., N.Y.*, **51**, 24.

YOSHIDA, T. (1961). Antigenicity of cell components of antigenic variants of contagious bovine pleuropneumonia organisms in serological tests. *Nat. Inst. Ann. Health Quart. (Tokyo)*, **1**, 199.

J. Hyg., Camb. (1964), **62**, 221
Printed in Great Britain

Neomycin-resistant *Staphylococcus aureus* in a burns unit

By E. J. L. LOWBURY, J. R. BABB, VIVIEN I. BROWN and B. J. COLLINS

Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

(*Received* 9 *January* 1964)

Hospital strains of *Staphylococcus aureus* resistant to neomycin have been reported in the United States (Quie, Collin & Cardle, 1960; Griffith, Ostrander, Smith & Beswick, 1961; Cohen, Fekety & Clough, 1962) and more recently in Great Britain (Robertson, 1963; Jacobs & Willis, 1963). Many of the strains isolated in this country have proved difficult to type and are associated with patterns of inhibition by various phages of group III at 1000 R.T.D. (routine test dilution); strains showing this pattern have been designated 'type A' (Temple & Blackburn, 1963; Jacobs, Willis, Ludlam & Goodburn, 1963). They differ from the American strains, which were typable by phage 54 at 1000 R.T.D.

From 1954 to 1957 and for a period in 1961 all strains of *Staph. aureus* isolated from burns in this unit were tested for sensitivity to neomycin; although many patients were treated with local application of the antibiotic in three controlled trials, resistant strains were not found (Lowbury, 1955; Cason & Lowbury, 1960; Lowbury, Miller, Cason & Jackson, 1962). In 1963 routine neomycin sensitivity tests on staphylococci from burns were started again, and a large proportion of the strains were found to be resistant. We describe here some studies on the neomycin-resistant staphylococci and discuss the emergence and spread of these organisms.

MATERIALS AND METHODS

Strains of Staphylococcus aureus

Staph. aureus was isolated by methods described elsewhere (Cason & Lowbury, 1960) from swabs taken from the burns of patients in the Burns Unit. After the detection of neomycin-resistant staphylococci in burns, nasal swabs were taken also from the nurses in the burns wards and in all the other wards of the Accident Hospital, and from a series of 309 patients attending the Casualty Department; 320 strains of *Staph. aureus* isolated in the hospital from miscellaneous infective lesions other than burns were kindly supplied by Dr S. Sevitt. From each site a single colony of each colonial form was picked and subcultured on blood agar; recognition of *Staph. aureus* was determined by a tube coagulase test with 10% human plasma broth examined after overnight incubation.

Antibiotic sensitivity tests

Strains of *Staph. aureus* were tested for sensitivity to a range of antibiotics by a ditch plate test, with 10 μg. neomycin sulphate per ml. nutrient agar in the

ditch for tests of that antibiotic. Some of the strains were also tested for sensitivity by a tube dilution method, with an inoculum of 0·02 ml. of a 1/1000 dilution of overnight broth cultures of the organisms added to nutrient broth in tubes containing doubling dilutions of neomycin.

Phage typing

Staphylococci were typed with phages kindly supplied by the Central Public Health Laboratory, Colindale (Blair & Williams, 1960).

Method of study

From 5 June 1963, when neomycin-resistant staphylococci were first observed in the unit, all strains of *Staph. aureus* were tested for sensitivity to neomycin, and phage type was determined on one strain from each patient at each sampling (swabs being taken daily from exposed burns and at all changes of dressings from those treated by the covered method). For many months before this time staphylococci from burns and from the nares of patients in the burns ward had been preserved for further study; on detection of the first neomycin-resistant staphylococci in June, these preserved strains were tested for sensitivity to neomycin and phage typed.

EMERGENCE OF NEOMYCIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

A series of 829 strains isolated between 4 December 1962 and 4 June 1963 was tested for sensitivity to neomycin. The first resistant strain was isolated on 23 February (week 8) 1963, and from that time a rapidly increasing proportion of the swabs from burns was found to be carrying neomycin-resistant *Staph. aureus*, reaching a peak of 74% in the week of 9–15 April (week 15) (see Fig. 1), after which the proportion of resistant strains fluctuated and then fell gradually. Neomycin-resistant strains were resistant also to kanamycin. The proportion of patients carrying neomycin-resistant strains increased more gradually, reaching a peak of 42% in the week 2–8 July (week 27), after which it fluctuated and fell. Since 15 November (week 47) until the present time (7 December), no neomycin-resistant staphylococci have been isolated in the Burns Unit.

Resistant strains from other sources

Table 1 shows the neomycin sensitivity of strains of *Staph. aureus* isolated between 11 June and 2 September from the nursing staff of all wards in the hospital, from 134 patients attending the Casualty Department, and from 320 miscellaneous lesions other than burns examined between 25 May and 15 October. One nurse in the burns ward and one specimen (urine) examined in the pathology department were found to be colonized by neomycin-resistant staphylococci.

Relation of neomycin resistance to clinical use of the antibiotic

Three trials of chemotherapy were in progress at the time when neomycin-resistant strains were detected: (1) a comparison of neomycin–chlorhexidine tulle gras (Lowbury *et al.* 1962) with chlorhexidine tulle gras for local prophylaxis of

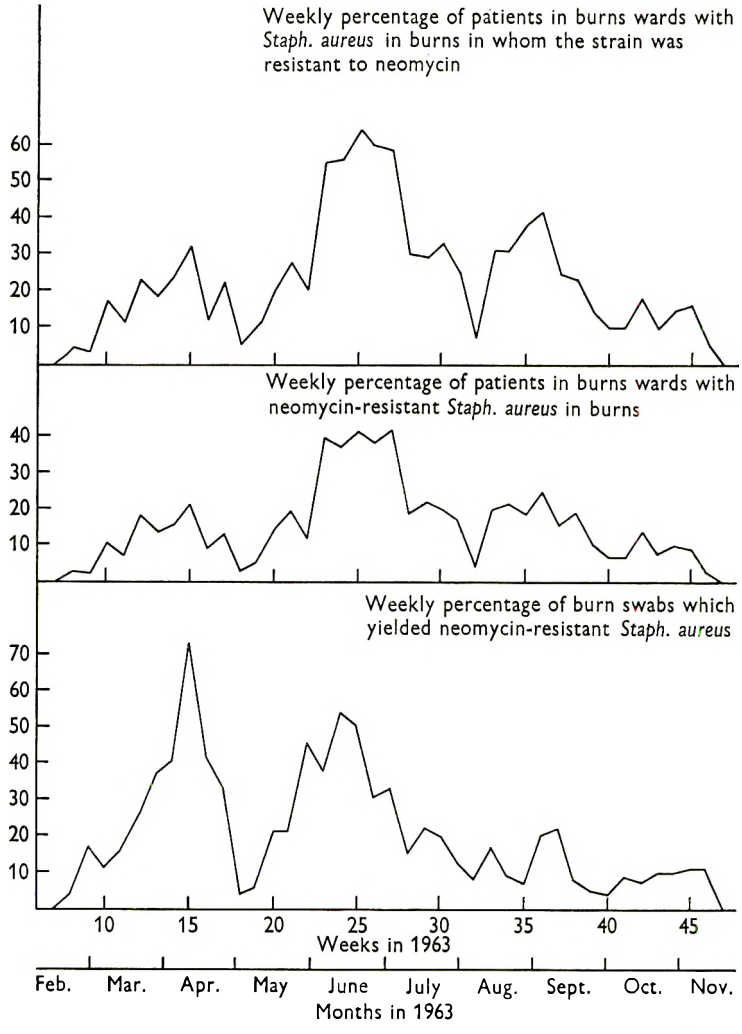


Fig. 1. Weekly percentage of strains of *Staph. aureus* from burns of in-patients which were resistant to neomycin between 18 February and 7 December.

Table 1. *Neomycin resistance of* Staphylococcus aureus *from various sources other than burns*

Sources	Samples	Number of samples yielding *Staph. aureus*	Number of samples yielding Neomycin-resistant strains
Nares of nurses in Burns Unit	13	10	1
Other wards	76	24	0
Nares of patients attending Casualty Department	309	134	0
Miscellaneous infections (not burns)	320	320	1

covered burns involving less than 20% of the body surface. Neomycin was first used in this trial on 25 September 1962; (2) a comparison of 'polybactrin' spray (polymyxin, neomycin and bacitracin in a volatile suspending fluid) with chlorhexidine powder for local prophylaxis of exposed burns; the trial began on 21 January 1963; and (3) a therapeutic trial in patients with severe burns (more than 35% of the body surface) of systemic kanamycin, colistine methane sulphonate and cloxacillin; this trial began on 6 September 1962.

The first resistant strain was isolated on 23 February 1963 in a patient who was being treated with polybactrin. The proportion of resistant strains fluctuated greatly during the trials, but the rise in the proportion of resistant strains occurred during a period when neomycin and kanamycin were in use; after the withdrawal of these antibiotics from the ward when resistant staphylococci were detected (in June 1963), there was a fluctuating fall and eventually an elimination of resistant strains from the ward. Patients continued to carry resistant strains for long periods in hospital and also after discharge from hospital.

Table 2. *Neomycin-resistant* Staphylococcus aureus *in burns during controlled trials of neomycin and kanamycin*

Trials	Total swabs	Swabs with *Staph. aureus*	Swabs with neomycin-resistant *Staph. aureus*
Kanamycin	811	318	148
Control	320	191	7
Polybactrin	299	196	69
Control	170	76	4
Neomycin–chlorhexidine tulle gras	328	166	55
Control	41	24	0

Table 2 shows the numbers of neomycin-resistant staphylococci isolated from the burns of patients treated with neomycin or kanamycin and from the control patients who were not treated with these antibiotics. The admission of patients to control or treatment groups in the prophylactic trials on the basis of odd or even hospital numbers led (by chance) to a disproportionately small number of swabs in the control series, but there was consistently a much larger proportion of swabs yielding resistant staphylococci from the burns of patients who were in the series treated with neomycin or kanamycin.

Phage type of resistant strains

All the strains of neomycin-resistant *Staph. aureus* showed an almost identical pattern of inhibition by certain phages of group III (6, 47, 54, 77, and often also 7, 53, 75 and 75B) at 1000 R.T.D. None of the neomycin-sensitive staphylococci isolated before or during the early weeks after resistant strains appeared showed the same pattern of inhibition by phages (see Table 3). At a later stage (on 17 and 19 March) 2 patients were found to be carrying neomycin-sensitive strains with this

phage inhibition pattern, one of them being the patient in whose burns resistant strains were first isolated here, and from whom they were still being isolated; strains of this description were not found in later specimens from the ward.

Table 3. *Phage group and inhibition pattern of neomycin-sensitive and neomycin-resistant* Staphylococcus aureus *in Burns Unit*

Time of isolation	Phage group or inhibition pattern 6/47/54/77, etc.	Strains of *Staph. aureus*		
		Neomycin sensitive	Neomycin resistant	Total
Week before first neomycin-resistant strain appeared (week 7 in 1963)	Group I	6	0	6
	Group II	1	0	1
	Group III	37	0	37
	Miscellaneous	0	0	0
	Not typable	7	0	7
	Inhibition pattern 6/47/54/77, etc.	0	0	0
Week when first neomycin resistant strain appeared (week 8 in 1963)	Group I	3	0	3
	Group II	0	0	0
	Group III	28	0	28
	Miscellaneous	1	0	1
	Not typable	11	0	11
	Inhibition pattern 6/47/54/77, etc.	0	2	2
Twelfth week after first neomycin resistant strain appeared (week 20 in 1963)	Group I	11	0	11
	Group II	1	0	1
	Group III	24	0	24
	Miscellaneous	9	0	9
	Not typable	2	0	2
	Inhibition pattern 6/47/54/77, etc.	0	30	30

Degree of resistance of neomycin-resistant staphylococci

The minimal inhibitory concentration (M.I.C.) of neomycin for two strains tested by a tube dilution method was 64 μg./ml.

Stability of resistance

Cultures of neomycin-resistant staphylococci were subcultured daily for a period of 2 months. The strains were still fully resistant after this procedure.

A resistant culture was spread on a blood agar plate and 100 single colonies obtained after overnight incubation were tested for sensitivity to neomycin. All the colonies yielded fully resistant subcultures.

In vitro *habituation of sensitive strains to neomycin*

A preliminary experiment was made with two neomycin-sensitive strains showing the same phage inhibition pattern as the resistant staphylococci (63/2882B and 63/2535A), and with two other neomycin-sensitive staphylococci (63/8747, type 80 and 63/9025, type 80/81). The M.I.C. was determined by a tube dilution test. The tubes containing the highest concentration of neomycin in which the

staphylococci had grown were subcultured to broth tubes containing a range of doubling dilutions of neomycin. After ten transfers, each of the strains showed the appearance of small-colony forms with approximately the same increase in resistance to neomycin (the M.I.C. rising from 0·25 or 0·5 μg./ml. to 32 or 64 μg./ml.). One of the strains which showed the same phage inhibition pattern as the naturally occurring neomycin-resistant staphylococci (63/2882B) showed a small proportion of normal full-sized staphylococcal colonies together with the small colonies. In a replicate experiment strain 63/2882B was again the only one from which colonies of increased resistance but normal size and appearance were obtained after a series of 7 transfers in medium containing neomycin; two other control strains were used in this experiment (63/2892, type 75/75B/77 and 63/2884, type 52/52A/80).

DISCUSSION

The neomycin-resistant staphylococci which appeared in the Burns Unit all showed the same pattern of phage inhibition. In tests kindly made by Dr M. T. Parker of the Central Public Health Laboratory, Colindale, these strains were lysed by certain experimental phages and could be described as an atypical type 83A; in this respect they are similar to those isolated in other parts of Britain in the past year. Since none of the many neomycin-sensitive staphylococci isolated in the period when the resistant strain emerged have shown this inhibition pattern it seems likely that the strain was brought into the hospital by a patient or a visitor; alternatively, it is possible that a loss or a change in phage susceptibility may have appeared on the emergence of a neomycin-resistant mutation (e.g. Harrison, Beavon & Griffin, 1959).

A striking feature was the emergence of a single strain as the dominant staphylococcus in the Burns Unit (cf. Lowbury & Collins, 1964). The emergence of this staphylococcus was clearly determined by the use of neomycin and kanamycin; it appeared during trials of these antibiotics, and it dwindled and disappeared after they were withdrawn from the ward. Patients who were not treated with neomycin or kanamycin were much less often colonized by the resistant strain, and the staff in other wards were never found to carry it in their nares; indeed, only one of the nurses in the Burns Unit carried this strain (probably a strain acquired from the contaminated environment rather than the source of that contamination).

Although a few neomycin-sensitive staphylococci showing the phage inhibition pattern of the resistant strain have appeared, including one in a patient who also carried resistant strains, there was evidence that resistance to neomycin was stable. Patients tended to continue carrying resistant strains for prolonged periods (including periods while they were at home), and resistant strains preserved and subcultured in the laboratory kept their full resistance without exposure to neomycin.

Eleven years elapsed between the introduction of neomycin (Waksman & Lechevalier, 1949) and the description of resistant pathogenic staphylococci by Quie *et al.* (1960). In our unit there was a lag of 9 years between our first use of the

antibiotic in 1954 and the first resistant strains which we detected in 1963—a surprising lag in a burns unit, where the emergence of resistant strains is a notorious hazard. It would appear that a neomycin-resistant mutant capable of growing in human wounds or nares is a great rarity, but one which can rapidly spread in environments where the antibiotic is much used and where cross-infection occurs readily. In view of these hazards it is recommended that neomycin and the related antibiotics kanamycin and framycetin should be applied in combination with another unrelated agent in the nares or on wounds when used for prophylaxis, and that sensitivity tests should be made whenever neomycin-containing applications are used.

SUMMARY

Early in 1963 neomycin-resistant *Staph. aureus* appeared in the burns of patients in a burns unit; after a period of 7 weeks three-quarters of the strains of *Staph. aureus* isolated from patients in the unit were resistant to neomycin, and after 22 weeks almost half of the patients in the burns wards were carrying the organism on their burns. When treatment with neomycin and kanamycin was stopped in the Burns Unit, neomycin-resistant strains gradually diminished in numbers and were no longer found in the ward after 6 months.

The neomycin-resistant staphylococci appeared during controlled trials of local neomycin and systemic kanamycin, and were much more frequently isolated from the burns of patients treated with these antibiotics than from patients in the control series.

During the previous 9 years local neomycin application had been used on many patients; though all staphylococci were tested for sensitivity to neomycin for a considerable part of this time, no resistant staphylococci were found.

All the neomycin-resistant staphylococci showed a pattern of inhibition by phages 6, 47, 54 and 77, and many also by phages 7, 53, 75 and 75B at 1000 R.T.D. No neomycin-sensitive staphylococci with this phage pattern were found at the time when resistant strains first appeared (though two such strains were found later); it seemed likely, therefore, that the resistant strain was introduced from outside the hospital. Preliminary tests of habituation to neomycin of sensitive strains with the phage inhibition pattern are described.

Back mutation to sensitivity was not found in tests on neomycin-resistant staphylococci.

REFERENCES

Blair, J. E. & Williams, R. E. O. (1960). Phage typing of staphylococci. *Bull. World Hlth Org.* **24**, 771.

Cason, J. S. & Lowbury, E. J. L. (1960). Prophylactic chemotherapy for burns. *Lancet*, ii, 501.

Cohen, L. S., Fekety, F. R. & Clough, L. E. (1962). Studies on the epidemiology of staphylococcal infection. IV. The changing ecology of hospital staphylococci. *New Engl. J. Med.* **266**, 367.

Griffith, L. J., Ostrander, W. E., Smith, Z. F. & Beswick, D. E. (1961). Appearance of kanamycin resistance in a single phage type of staphylococcus. *J. Bact.* **81**, 157.

Harrison, K. J., Beavon, J. & Griffin, E. (1959). The effect of neomycin on phage typing of staphylococci. *Lancet*, i, 908.

JACOBS, S. I. & WILLIS, A. T. (1963). Neomycin resistance in newly recognised strains of *Staphylococcus aureus*. *Lancet*, ii, 459.

JACOBS, S. I., WILLIS, A. T., LUDLAM, G. B. & GOODBURN, G. M. (1963). Studies on newly recognised strains of *Staph. aureus* associated with hospital infection. *Lancet*, i, 972.

LOWBURY, E. J. L. (1955). Cross infection of wounds with antibiotic-resistant organisms. *Brit. med. J.* i, 985.

LOWBURY, E. J. L. & COLLINS, B. J. (1964). The egg yolk reaction of *Staphylococcus aureus* isolated from burns. *J. Hyg., Camb.*, **62**, 229.

LOWBURY, E. J. L., MILLER, R. W. S., CASON, J. S. & JACKSON, D. M. (1962). Local prophylactic chemotherapy for burns treated with tulle gras and by the exposure method. *Lancet*, ii, 958.

QUIE, P. G., COLLIN, M. & CARDLE, J. B. (1960). Neomycin-resistant staphylococci. *Lancet*, ii, 124.

ROBERTSON, J. J. (1963). Neomycin-resistance in a newly recognised strain of *Staphylococcus aureus*. *Lancet*, ii, 33.

TEMPLE, N. E. I. & BLACKBURN, E. A. (1963). A newly recognised strain of *Staph. aureus* associated with epidemics in six hospitals. *Lancet*, i, 581.

WAKSMAN, S. A. & LECHEVALIER, H. A. (1949). Neomycin, a new antibiotic active against streptomycin-resistant bacteria. *Science*, **109**, 305.

J. Hyg., Camb. (1964), **62**, 229
Printed in Great Britain

The egg yolk reaction of *Staphylococcus aureus* isolated from burns

By E. J. L. LOWBURY and B. J. COLLINS
Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

(*Received* 9 *January* 1964)

A large majority of strains of *Staphylococcus aureus* were found by Gillespie & Alder (1952) to produce opacity when grown in egg yolk broth, apparently through the action of a lipase on certain triglycerides. Of the strains isolated from out-patients, 82 % were 'egg yolk positive' (EY^+), but only 45 % of those isolated from in-patients gave a positive reaction; there was a strong association between penicillin-resistance and a negative egg yolk (EY^-) reaction. Parker (1958) confirmed the association of negative egg yolk reaction with penicillin resistance, and found that such strains were particularly common in superficial lesions, including wounds; strains which caused deep lesions usually showed a positive egg yolk reaction.

We have tested strains of *Staph. aureus* isolated from burns in three periods (1958, 1960 and 1962) and from several other sources for egg yolk reaction; the association of this reaction with some other features, including phage type, antibiotic sensitivity and mercuric chloride resistance (Moore, 1960) has been examined. The relevance of these data to the epidemiology of staphylococcal infection is discussed.

MATERIALS AND METHODS

Tests for egg yolk reaction

A liquid egg yolk medium was prepared in the manner described by Gillespie & Alder (1952).

To facilitate the examination of large numbers of strains, a solid egg yolk medium was developed by one of the authors (B.J.C.); similar media have been described by Colbeck (1956), Carter (1960), Innes (1960) and Baird-Parker (1962). The formula which was found to give optimal results in tests which will be described separately (Collins, to be published) was yeastrel agar containing 5 % egg yolk and 5 % horse serum. Yeastrel agar contained the following ingredients: Yeastrel, 3 g.; peptone (Oxoid), 5 g.; sodium chloride, 5 g.; sodium dihydrogen phosphate, 3 g.; agar (Oxoid No. 3), 8 g.; and distilled water, 1000 ml. The mixture was steamed, filtered, adjusted to pH 7·4–7·6 and autoclaved at 121° C. for 15 min. To 18 ml. amounts of this medium cooled to approximately 50° C. were added 1 ml. of horse serum and 1 ml. of egg yolk (obtained aseptically from fresh farm eggs). The medium was poured into sterile plastic Petri dishes, 10 ml. per plate.

Plates of egg yolk agar were spot-inoculated with *Staph. aureus*, 16 strains per

plate, from overnight infusion broth cultures; a standard platinum loop was used. After overnight incubation at 37° C., growth of EY+ strains was surrounded by an opalescent zone of surface precipitation, about 0·5 mm. wide, surrounded by a sharply demarcated halo of clearing in the turbid medium, extending from 1 to 5 mm. from the edge of the disk of growth; the opalescent precipitate was absent when zones of clearing were narrow. EY− strains showed neither the opalescent precipitate nor the halo of clearing. About 98 % of a series of 825 strains gave the same results on solid egg yolk medium examined after overnight incubation and in liquid egg yolk medium examined, in the manner described by Gillespie & Alder (1952), after 3 days' incubation; a small proportion of strains gave a weak egg yolk reaction. After this comparison of the two media further tests were made only on the solid medium, which had the advantage of speed and convenience.

Strains of Staph. aureus *tested*

Tests for egg yolk reaction were made on strains of coagulase-producing staphylococci isolated from burns of in-patients during periods in 1958 (662 strains), in 1960 (457 strains) and in 1962 (329 strains); one strain was selected from each patient at each dressing. We also examined 97 strains from the noses of patients in the burns ward in 1959–60, 202 strains from the noses of patients attending the Casualty Department in 1958, and 134 strains from miscellaneous infections other than burns in 1958.

Other tests on staphylococci

The isolation and examination of these organisms was carried out as described elsewhere (Lowbury, 1960). Staphylococci were tested for phage type by the method described by Blair & Williams (1961) and for sensitivity to antibiotics by a ditch plate method with 10 units of penicillin, 50 μg. of tetracycline and 10 μg. of erythromycin per ml. nutrient agar in the ditches. Mercuric chloride sensitivity was tested in the manner described by Moore (1960).

RESULTS

Table 1 shows the frequency of isolation of egg yolk positive and egg yolk negative strains of *Staph. aureus* from burns in 1958, 1960 and 1962, and from other sources, mostly in 1958; a small number of strains gave weak or doubtful positive reactions, shown separately in this table. In 1958 and again in 1960 a large majority of the strains from burns were EY−, in contrast with the predominantly EY+ strains from miscellaneous infections and from the noses of the general public (represented by patients attending the Casualty Department); the staphylococci from the noses of patients in the burns wards during 1959 and 1960 showed an intermediate proportion of EY− strains, reflecting a replacement of the normal predominantly EY+ staphylococci by strains from burns in a proportion of the patients.

From these data it seemed likely that burns offer some selective advantage to EY− staphylococci. The strains isolated from burns in 1962, however, showed a

different pattern with a preponderance of EY+ strains (though EY− strains were still more common in burns than they were found to be in other environments during 1958).

Phage type and egg yolk reaction

A representative selection of the strains of staphylococci (one strain per patient at each sampling) was phage-typed; the results are presented in Table 2. This shows a preponderance of group III strains in burns during 1958 and 1962, but a slightly greater proportion of group I strains in 1960; the typable staphylococci from other sources were predominantly group I. Staphylococci of group I were predominantly EY+, except those isolated from burns in 1960, 78 % of which were EY−. Staphylococci of group II were rarely found in burns and were all EY+. Staphylococci of group III were usually EY− when isolated from burns, but less often EY− in 1962 than in 1958 and in 1960; the strains of group III from other sources were only slightly more often EY− than strains of group I.

Table 1. *The egg yolk reaction of* Staphylococcus aureus *from burns and from other sources*

Sources of *Staph. aureus*	Numbers of strains tested	Numbers of strains			% strains EY−
		EY+	?EY+	EY−	
Burns in ward, 1958	662	130	2	530	80
Burns in ward, 1960	457	77	11	369	81
Burns in ward, 1962	329	198	11	120	36
Nose and throat of patients in burns ward, 1959–60	97	39	1	57	59
Nose of patients attending Casualty Department, 1958	202	157	1	44	21
Miscellaneous infections in hospital other than burns, 1958	134	106	0	28	21

Table 3 shows the egg yolk reaction of the commoner phage types and patterns (those represented five times or more) of *Staph. aureus* included in Table 1. A striking feature is the presence in burns of 176 strains of type 52; 139 of these were EY−, all of them isolated in 1960 during which year 141 strains of type 52 staphylococci were isolated. This preponderance of one strain explains the high proportion of EY− staphylococci in phage group I during the year 1960 (Table 2); the strains of type 52 in Table 3 which were EY+ were all isolated in 1958 or in 1962. Most of the strains of group I during 1962 were of phage type 80 (or related patterns), and these were EY+. Strains of group II were hardly ever found in burns. In group III there was a preponderance of phage patterns which were predominantly EY−, but also some patterns (e.g. type 53) which were usually EY+.

Antibiotic sensitivity and egg yolk reaction

Table 4 shows the egg yolk reaction and sensitivity or resistance to penicillin, tetracycline and erythromycin of 240 strains of *Staph. aureus* isolated from burns of in-patients in 1958. EY− reactions were more commonly found in strains

Table 2. *Egg yolk reaction and phage group*

Sources of *Staph. aureus*	Strains of *Staph. aureus* in Phage group I Number	Phage group I % EY⁻	Phage group II Number	Phage group II % EY⁻	Phage group III Number	Phage group III % EY⁻	Other types Number	Other types % EY⁻	Not typable Number	Not typable % EY⁻	Total strains
Noses of patients attending Casualty Department	60	19	36	6	12	27	8	27	84	33	200
Miscellaneous infections other than burns	43	17	16	0	14	22	5	0	22	13	100
Burns in ward, 1958	21	0	0	0	99	97	5	40	13	54	138
Burns in ward, 1960	192	78	3	0	170	90	13	61	79	73	457
Burns in ward, 1962	119	1	3	0	165	66	23	13	19	8	329

resistant to each of the antibiotics; the proportion of antibiotic-sensitive strains was greater among EY$^+$ than among EY$^-$ staphylococci, and the majority of EY$^+$ strains were sensitive to tetracycline and to erythromycin.

In Table 3 one phage type of group I (type 52) was predominantly EY$^-$, while other related patterns in the group (52/80, 52/52A/80 and 80) were usually EY$^+$.

Table 3. *Egg yolk reaction of staphylococci from burns and other sources showing the common phage patterns*

Phage pattern	Phage group	Strains from burns		Strains from other sources	
		EY$^+$	EY$^-$	EY$^+$	EY$^-$
29	I	0	0	14	1
29/52	I	0	0	5	1
52	I	35 (2)*	139	24	3
52/52A	I	3	0	4	0
52/80	I	19	1	0	0
52/52A/80	I	31	1	19	2
52A	I	2	6	6	5
52A/79	I	1	0	20	0
52A/80	I	4 (1)	0	4	0
79	I	3	0	19	1
80	I	48 (2)	0	11	0
3B/55/71	II	1	0	4	0
55	II	1	0	8	0
55/71	II	1	0	14	2
71	II	0	0	5	0
6/7/47/53/54/75/75B/77	III	27	6	10	4
53	III	24 (1)	1	2	4
53/75/75B	III	1	4	0	0
53/75/75B/77	III	0	18	0	0
53/75/77	III	0	5	0	0
53/75B/77	III	2 (1)	42	0	0
75/75B	III	1	12	0	2
75/75B/77	III	0 (1)	90	1	1
75B	III	0 (1)	18	3	3
75B/77	III	4 (3)	98	2	0
77	III	3 (1)	60	0	3

* Doubtful positive reactions in brackets.

Similarly, in group III the staphylococci of types 53 and 6/7/47/53/54/75/75B/77 were usually EY$^+$, while other types (e.g. 75/75B/77, 75B/77, 77) were predominantly EY$^-$. Table 5 shows the antibiotic sensitivity patterns of the staphylococci with these phage patterns; both in group I and in group III (especially in the latter) the EY$^-$ strains were more commonly resistant to penicillin, tetracycline and erythromycin than the EY$^+$ strains. Since antibiotic resistant strains were more common in burns than in the other environments sampled (Table 6), it seems likely that the frequent selection of EY$^-$ strains of group III in burns treated in hospital is due to the selective advantage of antibiotic resistant bacteria in this environment; EY$^+$ strains of type 80, which often show multiple resistance, seem to have a similar advantage.

Egg yolk reaction and mercuric chloride sensitivity

Egg yolk reaction and mercuric chloride sensitivity were tested on 329 strains of *Staph. aureus* from burns of in-patients in 1962.

Table 7 shows the results of these tests. The majority of the strains (255) were resistant to mercuric chloride; the proportion of mercuric chloride resistant ('epidemic') strains was higher among the EY$^-$ staphylococci (111/120) than among those which gave an EY$^+$ reaction (144/209). Out of 26 strains of type 53, only one was EY$^-$, and this was the only strain of type 53 which was mercuric chloride resistant.

Table 4. *Egg yolk reaction and antibiotic resistance*

Antibiotic	Sensitivity test results	240 strains of *Staph. aureus* from burns (1958) EY$^-$	EY$^+$
Penicillin	Resistant	170	60
	Sensitive	1	9
Tetracycline	Resistant	166	21
	Sensitive	5	48
Erythromycin	Resistant	163	24
	Sensitive	8	45

Egg yolk reaction and pigmentation

A large majority of the EY$^+$ strains of staphylococci produced a rich golden-yellow pigment, while EY$^-$ strains were almost always poorly pigmented.

To determine whether the egg yolk reaction and pigmentation were inter-dependent properties, we grew ten pigmented EY$^+$ strains in nutrient broth containing 0·5% (w/v) lithium chloride, a method by which white variants of pigmented *Staph. aureus* have been obtained (Barber, 1955). After repeated daily subcultures, white variants were obtained from a strain of phage type 80 (PS 80). Ten white colonies were picked and found to retain their sensitivity to phage 80 and to give the positive egg yolk reaction shown by the parent strain.

DISCUSSION

Like Gillespie & Alder (1952) and Parker (1958) we found that egg yolk negative strains of *Staph. aureus* were more commonly resistant to antibiotics than egg yolk positive strains, and were more commonly members of phage group III than of phage groups I and II. Staphylococci which colonize burns in hospital have been found predominantly resistant to penicillin, tetracycline and erythromycin, and the selection of these strains would involve the coincidental selection of group III strains and those giving a negative egg yolk reaction. In phage group III the strains which were predominantly egg yolk negative were also the ones showing the highest incidence of multiple resistance and the ones which appeared most frequently in burns. Group I strains which were EY$^+$ (e.g. phage type 80) showed

Table 5. *Antibiotic sensitivity of predominantly EY⁺ and EY⁻* Staphylococcus aureus *within the same phage group*

Phage group	Phage types	Egg yolk reaction	Strains examined	*Staph. aureus* resistant to: Penicillin Number	Penicillin %	Tetracycline Number	Tetracycline %	Erythromycin Number	Erythromycin %	All these antibiotics Number	All these antibiotics %
I	80; 52/80; 52/52A/80; 52A/80	Predominantly EY⁺	71	68	96	58	82	55	77	48	68
	52	Predominantly EY⁻	178	176	99	168	94	162	91	157	88
III	6/7/47/53/54/75/75B/77, and closely related patterns; 47/77; 53; 6	Predominantly EY⁺	39	30	77	16	41	19	49	10	26
	53/75/75B/77; 53/75/77; 53/75B/77; 75/75B/77; 75/75B; 75B	Predominantly EY⁻	245	240	98	185	76	232	95	175	71

Table 6. *Antibiotic resistance of staphylococci from different environments*

Source of *Staph. aureus*	Strains of *Staph. aureus* resistant to: Penicillin Number	Penicillin %	Tetracycline Number	Tetracycline %	Erythromycin Number	Erythromycin %
Burns, 1958	230	96	187	78	187	78
Casualty patients, 1958	18	9	2	1	0	0
Infections other than burns	43	43	30	30	2	2

multiple resistance to antibiotics as often as EY⁻ strains and appeared to colonize burns as successfully as any of the EY⁻ strains of group III; in 1962 these strains were slightly more common than the EY⁻ strains of group III and became the predominant staphylococci of the Burns Unit. An example of a change in which one phage type (probably a single strain) became predominant was the emergence of type 52 EY⁻ strains in 1960. These staphylococci were indistinguishable by phage typing from the smaller number of type 52 strains isolated from burns in 1958, but egg yolk testing showed the latter to be all EY⁺. In differentiating between strains of the same phage type or between strains which are untypable, the egg yolk reaction appears to have some value as a test in epidemiological studies of staphylococcal infection.

Table 7. *Egg yolk reaction and mercuric chloride resistance*

	Numbers of strains of *Staph. aureus*			
Egg yolk reaction	Mercury resistant	Mercury sensitive	Doubtful reaction	Total
Positive	134	54	10	198
Doubtful positive	10	1	0	11
Negative	111	5	4	120
Total	255	60	14	329

The relevance of the egg yolk reaction to virulence and epidemicity is uncertain. Gillespie & Alder (1952) found that EY⁺ strains were more commonly associated with invasive infection, and EY⁻ strains were more commonly associated with superficial lesions such as impetigo and open wounds. Jessen, Faber, Rosendal & Eriksen (1959) found that EY⁻ strains were the commonest of the antibiotic-resistant staphylococci causing bacteraemia. These authors agree in classifying EY⁻ strains as pathogens, while apparently differing in their assessment of the invasiveness of such strains; a different view is implicit in the use by some authors of the egg yolk reaction as a diagnostic criterion for pathogenic staphylococci (e.g. Baird-Parker, 1962). Staphylococci-colonizing burns usually cause no obvious pathological effects, but on occasions they may invade the blood stream causing septicaemia or pyaemia; both EY⁻ and EY⁺ strains have been isolated from blood cultures in such cases. The majority of strains isolated from burns were mercury-resistant, and therefore 'presumptive epidemic strains'; of the mercury-sensitive strains a larger proportion gave EY⁺ than EY⁻ reaction.

These studies suggest that neither the egg yolk reaction (i.e. the production of a specific lipase) nor the phage group in themselves confer a selective advantage on particular staphylococci in burned tissue. Certain staphylococci, especially those of group III (Barber & Whitehead, 1949), appear to produce more mutations than others, and mutants which are better adapted because of antibiotic resistance or other features can be expected to flourish and eventually displace the less well adapted strains. A surprisingly large proportion of the staphylococci isolated from burns at certain times appeared to be 'subcultures' of the same strain; the pre-

ponderance of a single strain accounts for the fact that most staphylococci of group I were EY$^-$ in 1960 and that EY$^+$ staphylococci became the commonest burn staphylococci in 1962.

SUMMARY

Staph. aureus from burns of in-patients were tested for egg yolk reaction during three periods; in 1958 and in 1960 approximately 80% of the strains gave a negative reaction (EY$^-$), but in 1962 only 36% of the strains were egg yolk negative.

Staphylococci of phage group III were more commonly EY$^-$ than those of other groups isolated from burns. Within each of groups I and III, however, there were patterns predominantly EY$^-$ and others predominantly egg yolk positive (EY$^+$); in group I the majority of strains isolated in 1960 were of phage type 52 and EY$^-$, while those isolated in 1962 were predominantly of phage type 80 or related patterns which were always EY$^+$.

Most of the staphylococci in burns were resistant to penicillin, tetracycline and erythromycin; within groups I and III, the staphylococci which were EY$^-$ were also more commonly resistant than EY$^+$ strains to these three antibiotics.

Most of the staphylococci from burns were mercuric chloride resistant (presumptive epidemic strains); of the mercuric chloride sensitive staphylococci, the proportion of EY$^+$ strains was greater than that of EY$^-$ strains.

REFERENCES

Baird-Parker, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. appl. Bact.* **5**, 12.

Barber, M. (1955). Pigment production by staphylococci. *J. gen. Microbiol.* **13**, 338.

Barber, M. & Whitehead, J. E. M. (1949). Bacteriophage types in penicillin-resistant staphylococcal infection. *Brit. med. J.* ii, 565.

Blair, J. E. & Williams, R. E. O. (1961). Phage typing of staphylococci. *Bull. World Hlth Org.* **24**, 771.

Carter, C. H. (1960). Egg yolk agar for isolation of coagulase positive staphylococci. *J. Bact.* **79**, 753.

Colbeck, J. C. (1956). Studies in hospital infections. 1. The importance of fomites in the spread of staphylococcal infections, with particular reference to mattress and washing facilities. *Canad. Serv. med. J.* **12**, 563.

Gillespie, W. A. & Alder, V. G. (1952). Production of opacity in egg-yolk media by coagulase-positive staphylococci. *J. Path. Bact.* **64**, 187.

Innes, A. G. (1960). Tellurite-egg agar, a selective and differential medium for the isolation of coagulase positive staphylococci. *J. appl. Bact.* **23**, 108.

Jessen, O., Faber, V., Rosendal, K. & Eriksen, K. R. (1959). Some properties of *Staph. aureus* possibly related to pathogenicity. I and II. *Acta path. microbiol. scand.* **47**, 316, 327.

Lowbury, E. J. L. (1960). Infection of burns. *Brit. med. J.* i, 994.

Moore, B. (1960). A new screen test for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet*, ii, 453.

Parker, M. T. (1958). Some cultural characteristics of *Staphylococcus aureus* strains from superficial skin infections. *J. Hyg., Camb.*, **56**, 238.

J. Hyg., Camb. (1964), **62**, 239
With 3 plates
Printed in Great Britain

Physical and serological investigation of Rift Valley fever antigens

By JULIA LEVITT and A. POLSON
C.S.I.R. and U.C.T. Virus Research Unit, Medical School, Cape Town, South Africa

(*Received* 13 *December* 1963)

INTRODUCTION

When the infective particles have been centrifuged from preparations of MEF_1 poliovirus, rabies, Rift Valley fever, bluetongue and horse sickness viruses, complement-fixing 'soluble' antigens remain in the supernatant fluids (Kipps *et al.* 1957). By centrifugation in the preparative ultracentrifuge, it was found that the soluble antigens of MEF_1 poliovirus and neurotropic Rift Valley fever virus are approximately 12 mμ in diameter and that the complement-fixing antigen of bluetongue is associated with several components of which the smallest is 8 mμ in diameter.

Several new techniques which lend themselves to the study of soluble antigens are now available. Filtration through columns of granulated agar gel (Polson, 1961*a*; Andrews, 1962; Steere & Ackers, 1962) provides an additional method of particle size determination, and zone electrophoresis in a density gradient has proved effective in the purification (and classification) of certain viruses (van Regenmortel, 1961; Polson & Deeks, 1962). The application of these and other established techniques to the non-infective antigens of pantropic and neurotropic Rift Valley fever virus is now described.

MATERIALS AND METHODS

Virus strains

One pantropic and three neurotropic strains of Rift Valley fever virus were kindly supplied by the Division of Veterinary Services, Onderstepoort. The neurotropic strains consisted of neurotropic Rift Valley fever (NRVF) derived from Smithburn's original neurotropic virus (Smithburn, 1949) which had had 103 intracerebral passages in adult mice; a mouse-egg-mouse strain (MEM) which had had 102 mouse, 56 egg and an additional 17 mouse passages; and a tissue-culture strain (R120) derived from the serum of a lamb infected with a fully virulent field strain of virus passaged serially in lamb kidney tissue culture. After 50 passages in tissue culture, this strain was no longer hepatotropic but remained neurotropic (Weiss, personal communication).

In our laboratories, these viruses were injected intracerebrally into 3- to 5-day-old mice and the brains of all sick mice were harvested after 48 hr. Stock virus suspensions were prepared to eliminate the RVF virus inhibitor present in mouse

brain (Polson & Madsen, 1955). The diluent used was a mixture of 5 % (v/v) rabbit serum with 0·85 % (w/v) saline containing 200 units penicillin and 0·2 mg. of streptomycin per ml. A 10 % (w/v) suspension of infected brain tissue was clarified by centrifugation and then dialysed in a cellophane bag in saline at 4° C. for 2–3 days to remove the virus inactivator. After dialysis, the virus suspension was stored at 4° C. as stock virus. In an alternative method, the virus was removed from infected brain suspensions by alternate cycles of low- and high-speed centrifugation. Virus pellets were finally suspended in a convenient volume of serum-saline and stored at 4° C. as stock virus. Fresh stock virus suspensions were prepared about every 3 months.

The fully virulent pantropic virus (PRVF) was maintained by intraperitoneal mouse passage of infected liver or blood.

Phosphate buffered saline (PBS), 0·85 % (w/v) NaCl, 0·01 M-phosphate (pH 7).

Agarose was prepared from Ionagar no. 2 (Oxoid) by precipitation with polyethylene glycol (Russell, Mead & Polson, 1964).

Preparation of antigens

Antigens of the neurotropic strains were derived from the brain tissue of infant mice infected intracerebrally when 3–5 days old with 0·02 ml. stock virus diluted 1/10. The incubation period was 2–3 days. The pantropic antigen was derived from liver and spleen of mice approximately 16 days old infected by intraperitoneal injection of 0·1 ml. stock virus diluted 10^{-3}, which had an incubation period of 30 hr. Infected tissue was extracted by either of the following methods.

Method 1. About 10 g. of infected tissue was ground in a mortar with cold PBS (50 ml.) and the suspension in portions of 25 ml. was subjected to ultrasonic treatment at 20 kcyc./sec. in a M.S.E. Mullard ultrasonic disintegrator for 15–20 min. The suspension was centrifuged at 33,000 rev./min. for 60 min. in the no. 40 rotor of a Spinco model L ultracentrifuge to remove tissue debris and most of the virus. All centrifugations were done under a layer of liquid paraffin to trap lipoid floating to the surface. The supernatant fluid was pervaporated to half its volume, dialysed against PBS which had been diluted 1/2 with distilled water and pervaporated further to about one-eighth of the original volume. A precipitate which formed during pervaporation was removed by centrifuging at 12,000 rev./min. for 15 min. The solution was finally dialysed against undiluted PBS, stored at 4° C. in the presence of penicillin and streptomycin, and used with the least possible delay.

Method 2. Infected tissues were homogenized in a chilled Waring blender and extracted with acetone and ether (at a temperature between 0° and 4° C.) as described by Clarke & Casals (1958). To obtain concentrated antigen extracts, the dried tissue was rehydrated in 2 ml. cold PBS for every 1 g. of original tissue, left overnight at 4° C., and centrifuged at 30,000 rev./min. for 30 min. The supernatant fluid was removed and the sediment, redispersed in the same volume of PBS as before, was subjected to ultrasonic treatment at 20 kcyc./sec. for 20 min. The suspension was centrifuged at 30,000 rev./min. for 40 min. The supernatant fluid was removed and the residue extracted once more in the same way but with ultra-

sonic treatment for 10 min. only. The three extracts were combined and dialysed overnight against distilled water at 4° C. to remove impurities such as salt. The solution was freeze-dried in 6 ml. amounts and stored at −20° C.

Preparation of antisera

Neurotropic strains. Adult mice received six or seven intraperitoneal 0·2 ml. injections of either a 10% emulsion of infected mouse brains or virus which had been centrifuged from a 10% emulsion at 30,000 rev./min. for 60 min. and re-suspended in saline. The injections were given at 5-day intervals and the mice were bled 14 days after the last dose.

Pantropic virus. Unimmunized mice do not survive intraperitoneal injection of the pantropic virus. The adult mice used for production of antisera were therefore protected by two intraperitoneal injections of either the neurotropic virus (Smithburn, 1949) or pantropic virus inactivated with formalin, before receiving six injections of the live pantropic virus. PRVF antiserum I was obtained from mice preimmunized with a neurotropic strain and PRVF antiserum II from mice preimmunized with formalin inactivated pantropic virus.

Gel diffusion-filtration

The columns (60 × 2 cm.) were prepared (Polson, 1961*a*) from granulated 7% (w/v) agarose which had passed a 100-mesh sieve. The displacement medium, 0·067 M-phosphate buffer and 0·073 M-NaCl, pH 7·2, was saturated with chloroform to inhibit microbial growth. The following were used as reference proteins (Polson, 1961*a*):

(*a*) Haemocyanin of *Burnupena cincta*, molecular weight 6,600,000 and diffusion coefficient $1{\cdot}24 \times 10^{-7}$ cm.^{2}/sec.

(*b*) Haemocyanin of *Jasus lalandii*, molecular weight 490,000 and diffusion coefficient $3{\cdot}4 \times 10^{-7}$ cm.^{2}/sec.

(*c*) Mouse haemoglobin, molecular weight 68,000 and diffusion coefficient $6{\cdot}5 \times 10^{-7}$ cm.^{2}/sec.

The effluent from the column passed through an LKB 'Uvicord' apparatus which recorded its opacity to ultraviolet light of wavelengths near 2537 Å. and fractions were collected automatically in the apparatus of Polson (1961*b*).

Zone electrophoresis in sucrose density gradients

The apparatus of Polson & Cramer (1958) was used. Experiments were done under standardized conditions of pH, potential and concentration gradients, and ionic strength as described by Polson & Deeks (1962). To facilitate exact comparison between the results of different runs, the mobility of the antigens was in each case compared with the mobilities of phenol red (added to the sample) and haemoglobin which the antigen preparations always contained. Electrophoresis was continued for about 24 hr. with a current of about 25 mA. and a potential gradient of 4·5 V./cm. During this period, the haemoglobin migrated about 4 cm. and the phenol red 20 cm. Afterwards the contents of the column were fractionated as described by Polson & Cramer (1958).

The 'protein' content of each fraction was measured by the light scattering method of Mead (1962). Three ml. dilute perchloric acid (66 ml. A.R. 60%, w/w, acid diluted to 500 ml. in distilled water) were added to 1 ml. of a 1/100 dilution in saline of each fraction. The mixtures were examined in the light-scattering apparatus and the galvanometer readings which are proportional to 'protein' content were recorded as turbidimetric units.

Fractions to be tested by complement fixation and gel precipitation were dialysed against three changes of PBS containing thiomersal 0·01% (w/v) over a period of 3–5 days to remove the sugar. Fractions were concentrated when required, by dialysis overnight against PBS diluted 1/4 in distilled water, followed by freeze drying and solution of the residue in 0·5 ml. distilled water.

Complement-fixation (CF) titrations

Dilutions of antigen were made in the solution recommended by Mayer, Osler, Bier & Heidelberger (1946). Antisera were inactivated at 56° C. for 30 min. Titration was by the method of Casals & Olitsky (1950). Complement was titrated by the method of Casals, Olitsky & Anslow (1951) and two units used in the test. The complement-fixing titre is the reciprocal of the highest dilution of antigen giving 50% fixation.

Ultracentrifugation

Approximate sedimentation constants were determined by the methods of Polson & Linder (1953) and Polson & Madsen (1954). In this technique, a protein of appropriate sedimentation constant is added to form a density gradient which hinders convection.

Determination of particle density

Densities were determined by centrifugation in a preformed gradient of caesium chloride by the method of Polson & Levitt (1963*a*).

Gel precipitation

Qualitative gel-precipitin tests (Ouchterlony, 1953) were done on flat glass plates in a 3 mm. layer of 1·5% (w/v) washed Difco agar dissolved in phosphate buffer containing sodium ethylene-diamine tetra-acetate and sodium azide (Allison & Humphrey, 1960). A cutter was used forming wells 4 mm. in diameter and 5 mm. apart, parallel to a trough 3 mm. wide and 4 mm. distant from the wells. Antigen samples were placed in the wells and antisera in the troughs. A hexagonal arrangement of wells 7 mm. in diameter and 4 mm. apart (Mansi, 1957) was also used.

Quantitative gel-precipitin tests were performed in a perspex apparatus as described by Polson (1958).

By means of a microcomparator, the distance of the precipitation bands from the antibody meniscus and the band widths after various time intervals were measured. The microcomparator consisted of a telescope mounted on the platform of a Hilger–Watts travelling microscope with a vernier scale. The perspex apparatus was held in place in a special adjustable clamp on an optical bench, at the focal point of the telescope. The cross-hairs of the telescope were focused on the perspex apparatus and the measurements made.

EXPERIMENTAL AND RESULTS

Gel diffusion-filtration through granulated 7 % agarose

To determine the elution position of PRVF antigen in relation to those of the three reference proteins, 2 ml. of antigen prepared by Method 1 which already contained haemoglobin was mixed with 0·5 ml. of a 4 % solution of *B. cincta* haemocyanin and 0·5 ml. *J. lalandii* haemocyanin, placed on the agarose column and washed through with 0·067 M-phosphate, 0·073 M-NaCl, pH 7·2. When about 60 ml. had passed through, 50 fractions of 2 ml. were collected.

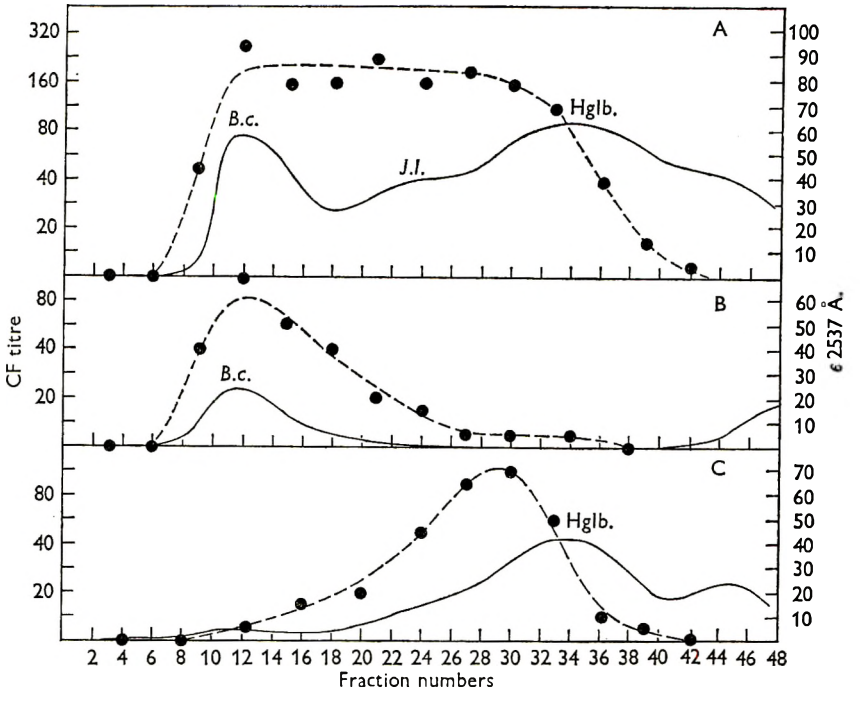


Fig. 1. A. Gel diffusion-filtration of PRVF antigen through granulated 7% agarose showing the complement-fixing titre of the filtrate (broken line) in relation to the peaks in the Uvicord trace (solid line) corresponding to *B. cincta* (*B.c.*) haemocyanin, *J. lalandii* (*J.l.*) haemocyanin and haemoglobin (Hglb.) respectively (reading from the left). B. Fractions 7–14 from A, pooled concentrated and refiltered through the column. C. Fractions 27–35 from A, pooled concentrated and refiltered through the column.

Every third fraction was titrated by complement fixation against the homologous antiserum diluted 1/50. Fig. 1 A shows the CF titre and the Uvicord trace. As the latter indicates opacity and not extinction it considerably underemphasizes the height and therefore the resolution of the protein peaks. The results show that while the haemocyanins and the haemoglobin came out in distinct though only partially separated peaks, the antigen emerged with an almost constant titre in a large volume comprising the two haemocyanins and about half the haemoglobin.

To confirm the inhomogeneity in particle size of the antigen suggested by this

result, fractions 7–14 containing antigen and *B. cincta* haemocyanin were mixed to form pool A and fractions 27–35 containing antigen and haemoglobin were mixed to form pool B. Pools A and B were separately dialysed against 0·017 M-phosphate, concentrated by pervaporation to 3 ml., clarified by centrifugation at 8000 rev./min. for 10 min. and run through agarose columns as described above. The elution curves of pool A (Fig. 1B) and pool B (Fig. 1C) confirm the particle size inhomogeneity and suggest that neither aggregation of the smaller particles nor dissociation of the larger ones was proceeding rapidly under the experimental conditions.

It appeared therefore that the soluble antigen of PRVF virus consists of particles ranging in diameter (or at least in diffusibility) from that of *B. cincta* haemocyanin (or greater) to a size slightly larger than that of haemoglobin. Partial classification of such a mixture on a particle size basis should be effected by centrifugation. To select the smaller particles, a solution of crude PRVF antigen was centrifuged four times successively under liquid paraffin in the no. 40 rotor at 33,000 rev./min. for 60 min. (cf. Polson & Levitt, 1963*b*). After each centrifugation, the supernatant fluid was removed by a pipette held initially half-way between the liquid paraffin layer and the bottom of the tube and lowered only enough to remove fluid to a level 1 cm. above the bottom of the tube. The remaining fluid was discarded. The pooled supernatants were centrifuged in clean tubes. The final supernatant fluid was pervaporated from 13 to 2 ml., dialysed against 0·034 M-phosphate and applied to the agarose column. The elution diagram (Fig. 2) confirmed that centrifugation had removed most of the larger particles of antigen.

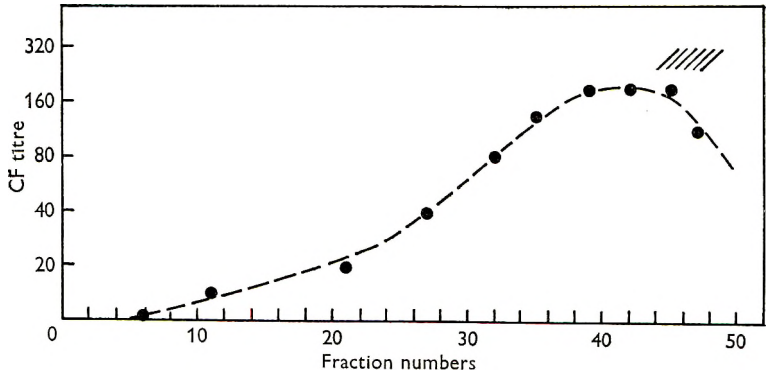


Fig. 2. Gel diffusion-filtration of the small particle fraction of PRVF antigen separated by centrifugation. The broken line shows the complement-fixing (CF) titre of the filtrate and the hatched area, the fraction in which haemoglobin was visible.

Antigen from a neurotropic strain (NRVF) mixed with the two haemocyanins and subjected to gel diffusion-filtration in the same way gave almost identical results (Fig. 3).

The CF titre curves in Figs. 1A and 3 appear to have three modest peaks suggesting the presence of three overlapping components. In an attempt to confirm this, a gel diffusion-filtration experiment on the pantropic antigen was performed

in which the dilutions in the CF test followed the series 1/50, 1/100, 1/150, 1/200. Although subsequent work with other methods supported the existence of three components, no confirmation was obtained on this occasion. The results are shown in Fig. 4. This experiment in which haemocyanins were not added indicates the elution characteristics of the ultraviolet absorbing constituents of the tissue extract and shows that some purification of the antigen could be achieved by this technique.

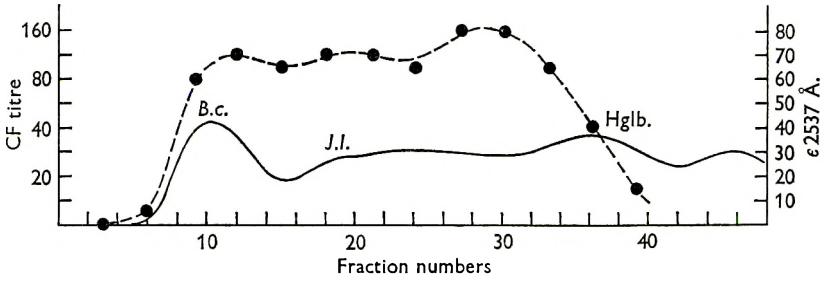


Fig. 3. Gel diffusion-filtration of NRVF antigen in relation to *B. cincta* (*B.c.*) and *J. lalandii* (*J.l.*) haemocyanins and haemoglobin (Hglb.). The solid line indicates the ultraviolet absorption and the broken line, the complement-fixing (CF) titre of the filtrate.

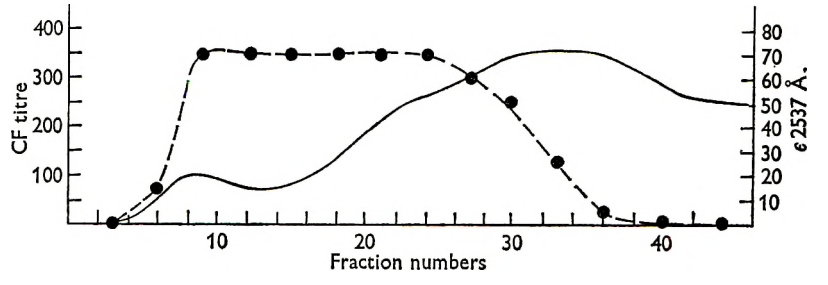


Fig. 4. Gel diffusion-filtration of PRVF antigen showing the ultraviolet absorption of the tissue extract in the absence of haemocyanins (solid line) and the CF titre (broken line).

Centrifugation in a protein density gradient

Ultracentrifugation experiments were performed in a further attempt to group the antigens according to size. Antigen suspensions were prepared by Method 1 but were pervaporated only to half their original volume before dialysis against PBS. Nine ml. of antigen suspension, mixed with 2 ml. *J. lalandii* haemocyanin, were placed in a graduated centrifuge tube and spun at 33,000 rev./min. for 180 min. in the no. 40 rotor. After centrifugation, the contents of the tube were removed in 10 fractions as described by Polson & Linder (1953). The CF titres of the fractions were plotted against their positions in the tube to give the sedimentation diagram for the soluble antigen of the pantropic virus shown in Fig. 5. There appear to be three complement-fixing components, of which one (*c*) sedimented to the bottom of the tube. The two slower sedimenting components (*a* and *b*) are indicated by the two steps in the curve. The sedimentation constants of (*a*) and

(*b*) calculated by the method of Polson & Madsen (1954) were 7·8 and 28·8 Svedberg units respectively.

An attempt was made to estimate the sedimentation constant of the largest component (*c*) by centrifuging an antigen suspension at 30,000 rev./min. in the presence of *B. cincta* haemocyanin for 40 min. The results were inadequate for exact calculation but indicated that the sedimentation constant of the largest antigen particle may exceed 100 Svedberg units.

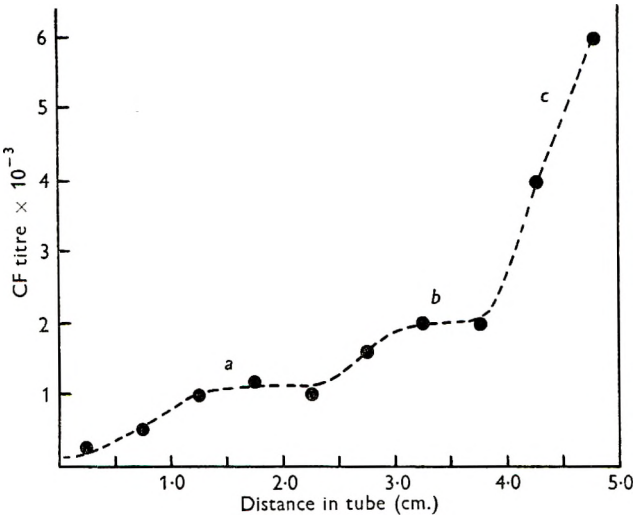


Fig. 5. The vertical distribution of CF titre in a tube containing PRVF antigen mixed with *J. lalandii* haemocyanin which had been centrifuged at 33,000 r.p.m. for 180 min.

A similar experiment with an antigen suspension prepared from a neurotropic strain (NRVF) yielded sedimentation constants of 8·4 and 28·9 Svedberg units for components (*a*) and (*b*) respectively. Here again there was evidence of a component of sedimentation constant greater than 100 Svedberg units.

Gel precipitation and immunological identity

The fractions obtained from gel diffusion-filtration experiments on the pantropic and neurotropic virus antigens (cf. Figs. 1 A and 3) were pooled in groups of three (6 ml.), freed of excess salt by dialysis against PBS which had been diluted 1/5 in distilled water, and freeze-dried to concentrate them for gel precipitation tests. The dry residues were redissolved in 0·2 ml. distilled water and tested for immunological identity against both a pantropic and neurotropic antiserum diluted 1/2 with the results shown in Plate 1*a*. The precipitation lines are continuous except at the terminal wells where line splitting and crossing occurred. These effects were found to be artifacts caused by the complex diffusion system at the ends of the rows and disappeared when the samples showing the effect were placed in wells in the central region. The continuity of the lines indicates that the antigens of the different size groups are immunologically identical.

It was further shown that antigen preparations of all four strains of the virus were indistinguishable when tested in neighbouring wells. Plate 1*b* demonstrates this and also reveals the presence of a second smaller antigen. Normal liver and brain preparations were also included in the experiment and showed no precipitation lines.

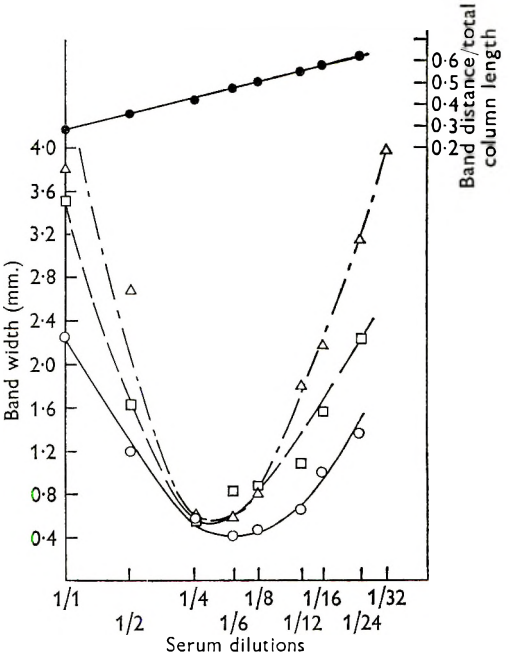


Fig. 6. Results of a quantitative gel precipitin test of polydisperse PRVF antigen against PRVF antiserum (I) diluted 1/2. The parabolic curves relate antigen dilution to band width and the diagonal curve to band distance from the antibody meniscus after 8 days (○ and ●), 14 days (□) and 21 days (△).

Quantitative gel-precipitin tests

To determine the diffusion constant of the pantropic antigen, several quantitative gel-diffusion experiments (Polson, 1958) were performed. Fig. 6 shows a typical diagram obtained with antigens prepared by Method 2. Similar results were obtained from antigens prepared by Method 1. These antigen preparations are now known to be inhomogeneous with regard to particle size and it should be noted that the band width curves are parabolic in contrast to the V-shaped curves obtained by van Regenmortel (1959) and Polson & Hampton (1960) using monodisperse systems. The former curves do not allow the diffusion coefficient to be accurately determined.

To obtain a suspension of more nearly monodisperse PRVF antigen, a preparation made by method 1 was centrifuged for 5 hr. at 33,000 rev./min. under liquid paraffin. The upper portion of the supernatant fluid was removed and tested against PRVF antiserum (II) diluted 1/10. The results are shown in Fig. 7. In this instance the band width measurements produced V-shaped curves indicating at their intersection the position that the precipitation line would occupy if derived

from antigen and antibody in exactly optimal proportions. The distance (x_b) of such a line from the antibody meniscus could be obtained by interpolation. By subtracting x_b from the total column length, the distance from the band to the antigen meniscus (x_g) could be determined. The diffusion coefficient of the antigen (D_g) was calculated from the equation

$$\frac{D_g}{D_b} = \frac{x_g^2}{x_b^2}.$$

There is unfortunately some doubt about D_b, the diffusion coefficient of mouse γ-globulin, which is stated by Allison & Humphrey (1959, 1960) to be the same as

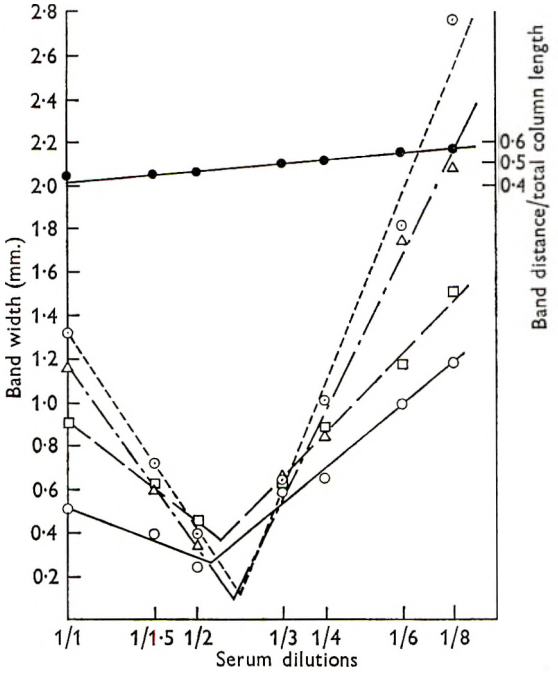


Fig. 7. Results of a quantitative gel precipitin test of the small fraction of PRVF antigen against PRVF antiserum (II), diluted 1/10. The V-shaped curves relate antigen dilution to band width and the diagonal curve to band distance from the antibody meniscus after 6 days (○ and ●), 8 days (□), 11 days (△) and 12 days (⊙).

that of human and rabbit γ-globulin for which they give a value of $3{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. A higher value, $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. was obtained by Largier (1959) for horse tetanus antitoxin which was shown to be free of traces of macroglobulins. This value is also similar to that calculated for the diffusion coefficient of the precipitating antibody against a haemocyanin prepared in the rat, the rabbit and the guinea-pig (Polson & Deeks, 1960). In addition, a value of $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. for macroglobulin-free human γ-globulin has been obtained by Polson & Potgieter (1964), who attributed the lower results of earlier investigators to the presence of macroglobulin in their preparations.

If the diffusion coefficient of mouse γ-globulin is provisionally assumed to have the higher value of $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec., the smallest particles of PRVF antigen

have a diffusion coefficient of $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. for which a particle diameter of 7 mμ is obtained by the Stokes–Einstein equation. If the diffusion coefficient of mouse γ-globulin has the lower value $3{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. given by Allison & Humphrey, the diffusion coefficient of the smallest antigen particles is $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. and the corresponding diameter, 9 mμ.

Plate 2*a* shows the appearance of precipitation bands from which measurements were made, 7 days after the experiment had been set up. After a further 25 days, the precipitation bands on either side of the optimal proportions region had split as shown in Plate 2*b*. This splitting occurred both with unfractionated antigen and with the more homodisperse fraction obtained by centrifugation. Plate 2*c* shows an Ouchterlony test set up with a series of twofold dilutions of PRVF antigen prepared by Method 2. It may be seen that a single band of precipitate split into two on dilution.

The density of PRVF antigen

To determine the density of the most rapidly sedimenting fraction, PRVF antigen prepared by Method 1 (but without pervaporation or dialysis) was centrifuged at 33,000 rev./min. for 90 min. in the no. 40 rotor. The pellets were resuspended in 3 ml. buffer and clarified at 8000 rev./min. for 8 min. Small portions were adjusted to densities of 1·08, 1·22 and 1·33 for insertion at appropriate levels in preformed density gradients of caesium chloride in separate tubes as described by Polson & Levitt (1963*a*). The tubes were centrifuged for 4 hr. at 33,000 rev./min. The contents of each tube were divided into 10 fractions by pipetting from the top and the fractions tested by gel precipitation. The average density of all fractions containing antigen was 1·27. The density of the antigen is therefore very close to 1·27 g./ml. During centrifugation, a visible band of a non-antigenic fraction formed at a density level of 1·19 g./ml. in each tube.

Another experiment was performed to obtain the densities of the slower sedimenting antigens. A preparation of PRVF antigen was separated on 7% agarose as described above (cf. Fig. 1) and the fractions containing *B. cincta* haemocyanin and haemoglobin were pooled separately. These will be referred to as *A* and *C* respectively since pool *B* was formed by the intermediate fractions in this instance. The three pools were concentrated to 0·5 ml. by pervaporation and dialysis as described and each was placed in the middle of a preformed caesium chloride gradient at a density level of 1·22 g./ml. Centrifugation was for 16 hr. at 30,000 rev./min. Ten fractions were taken from each tube and tested by gel precipitation with the results shown in Plate 2*d*. The average density of the antigen from each pool was again close to 1·27 g./ml.

Zone electrophoresis

Antigen extracts prepared by Method 2 were submitted to zone electrophoresis in a sucrose gradient to find out whether antigens from different strains differed in mobility and to investigate electrophoresis as a means of purification.

The results obtained with the three neurotropic strains and the pantropic strain are presented in Fig. 8*a* and *b* respectively. In all cases the complement-fixing

antigen had approximately the same migration rate as the haemoglobin which was contained in the 'protein' peak 3–5 cm. from the origin in the light scattering histograms. A second 'protein' peak occurs between 9–13 cm. from the origin. The complement-fixing antigens of the four strains are therefore indistinguishable by zone electrophoresis.

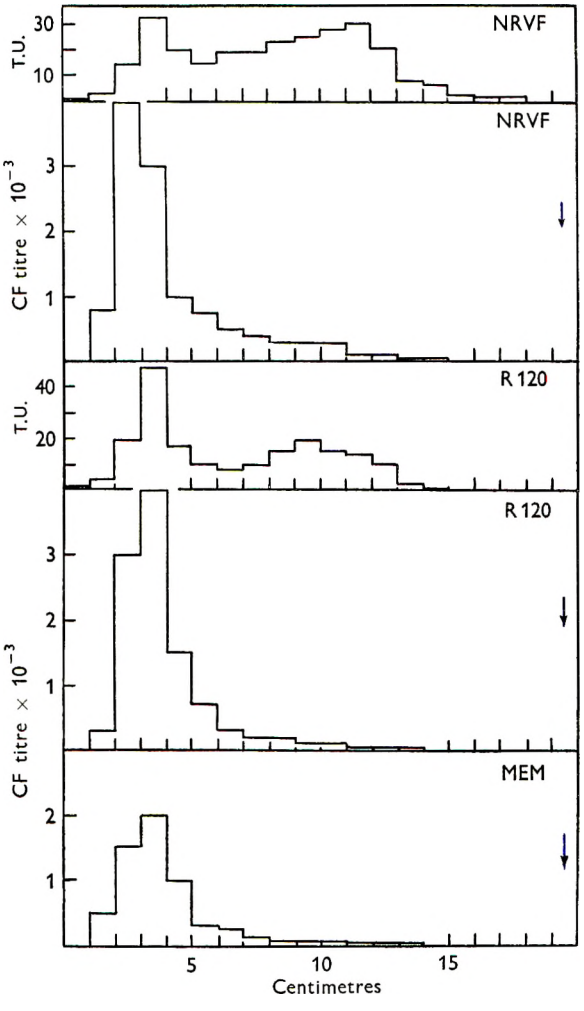


Fig. 8*a*. Zone electrophoresis diagrams of the antigens of the neurotropic strains. The CF titres and 'protein' content (turbidimetric units, T.U.) of successive fractions are plotted as histograms. The haemoglobin is contained in the 'protein' peak 3–5 cm. from the origin. The arrow indicates the position of the phenol red band.

Consecutive 1 cm. samples from the electrophoresis column were also tested by gel precipitation against antisera to all the strains. With all four strains of RVF, precipitation bands occurred in the same region as the peak complement-fixing activity. A second faint line of precipitation was detected when fractions 11 and 12 of PRVF were tested against the homologous antiserum (Plate 3*a*). This faint line of precipitation was detectable when fractions 11–15 of NRVF antigen were tested

against antisera to R 120 and PRVF and when fractions 11–14 of MEM antigen and fractions 11–13 of R 120 antigen were tested against PRVF and the homologous antiserum. Fractions 9–13 of R 120 antigen showed this precipitation band when tested against MEM antiserum. This precipitation line appears to be due to a second faster migrating antigen which may account for the 'tailing' of the CF titre into this region of the electrophoresis diagrams. Further evidence for the existence of this second antigen will be given.

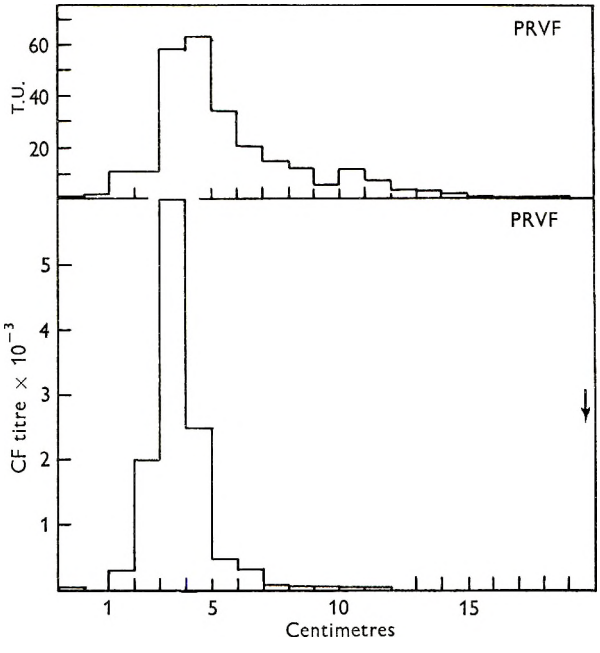


Fig. 8*b*. Zone electrophoresis diagram of the pantropic antigen. The curves have the same significance as in Fig. 8*a*.

The neurotropic antigen fractions having the highest CF titre showed splitting of the precipitation bands when tested with various antisera. A greater degree of splitting was shown by the pantropic antigen (Plate 3*a*), but there is however no other evidence for the presence of more than one antigen in this region.

The faster migrating antigen was not demonstrable when fractions from the electrophoresis column were tested in wells 7 mm. in diameter except with the R 120 strain. A precipitation band occurred with fractions 8–15 which coincided with the inner band formed by antigen which had not been submitted to electrophoresis. However, the faster migrating component of PRVF antigen was demonstrated by testing in wells 2 mm. in diameter against the homologous antiserum (Plate 3*b*). This test also showed that the bands produced by fractions 6 and 11 are not continuous indicating that two distinct antigens are involved.

A preparation of PRVF antigen which had not been exposed to acetone and ether was also shown to migrate with the haemoglobin.

DISCUSSION

Extracts from organs of mice infected with pantropic Rift Valley fever or with any of three strains of neurotropic Rift Valley fever contain, in addition to virus which may be removed by centrifuging, at least two 'soluble antigens'. These antigens are detectable and distinguishable from each other by the Ouchterlony test with mouse antisera and at least one of them is titratable by complement fixation. One of these antigens which appears to be quantitatively less important since it is not easily detectable has a higher electrophoretic mobility at pH 8·6 than the principal antigen but too little is known about its properties to merit further discussion.

Filtration of virus-free antigen preparations from infected mice through granulated 7 % agarose suggested the presence of antigenic particles ranging in size or diffusibility from that of haemoglobin to at least that of *B. cincta* haemocyanin. Early fractions (presumed to contain large or slowly diffusing antigen particles) and later fractions (presumed to contain smaller antigen particles) retained their properties when reconcentrated and refiltered separately through the column. This not only confirms the polydisperse nature of the antigen, but shows that neither aggregation of the smaller particles nor breakdown of the large ones took place perceptibly under the conditions of the experiment.

Centrifugal analysis indicated that the antigen though polydisperse is not a mixture of particles varying randomly in size but of particles in three size groups defined by sedimentation constants of about 8, 29 and more than 100 Svedberg units. Particles of the smallest size group of PRVF antigen prepared by repeated centrifugation showed, on diffusion-filtration through granulated 7 % agarose, peak CF activity in fractions which came out just before the haemoglobin, corresponding to an antigen fraction to which a sedimentation constant of 8 had been assigned by Polson & Levitt (1963*b*).

Densities of the antigens in the three particle size groups were determined by prolonged centrifugation in a preformed gradient of caesium chloride. The value of 1·27 g./ml. found for all the antigens may indicate the presence of lipid as most protein molecules have densities of about 1·33 g./ml. Spherical particles of sedimentation constant 100 and density 1·27 would be about 26 mμ in diameter according to the modified Stokes equation. The smaller particles having the same density would have diameters, if spherical, of 7–8 mμ and 14 mμ. There is no evidence to suggest that aggregation or dissociation of antigen molecules took place under the particular experimental conditions, but the possibility that the larger antigenic components are composed of 7–8 mμ diameter units is not ruled out.

A value of $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. was obtained for the diffusion coefficient of the smallest particles of antigen (from PRVF virus) separated by differential centrifugation when the diffusion coefficient of mouse γ-globulin was assumed to be $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. Substitution of this result in the Stokes–Einstein equation gives a particle diameter of the order of 7 mμ in good agreement with the value estimated by centrifugal analysis. If, however, the diffusion coefficient of mouse

γ-globulin given by Allison & Humphrey (1960) is assumed to be correct, the diffusion coefficient of PRVF antigen becomes $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. and the corresponding particle size is 9 mμ.

Band splitting occurred after about 2 weeks in quantitative gel precipitin tests (including one on a preparation rendered at least approximately homodisperse by differential centrifuging) at concentrations of antigen higher and lower than that at the optimal proportions region. This was not observed in experiments with haemocyanin (Polson & Deeks, 1960) or with turnip yellow mosaic virus (van Regenmortel, 1959) but with poliovirus, band splitting occurred after a prolonged period (Polson & Hampton, 1960). This was also demonstrated with RVF antigens in Ouchterlony tests when antigen concentrations were not at optimal proportions. The formation of multiple precipitation lines with a single antigen–antibody system in the presence of an excess of one of the reactants has also been observed amongst others by Wilson & Pringle (1954) and Grabar (1957).

Zone electrophoresis of the antigens from three neurotropic and a pantropic strain of RVF virus showed that in each instance the complement-fixing activity migrated together with or slightly behind the haemoglobin at pH 8·6. This technique therefore did not serve as a means of separating the antigens from haemoglobin which is invariably found in preparations from infected mouse brain or liver. However, separation from impurities at or near the origin of electrophoresis and midway between the origin and the phenol red band had occurred. The mobility of infective pantropic RVF virus in zone electrophoresis is approximately half that of phenol red under the same conditions (Levitt, Naudé & Polson, 1963). The infective virus of the neurotropic strains also migrates faster than haemoglobin (Polson, unpublished results). Thus the antigens of RVF virus migrate at a rate different from that of the infectious virus, whereas with MEF_1 poliovirus, both virus and its soluble antigen migrate at the same rate (Polson, Selzer & van den Ende, 1957).

A close correlation between complement fixation and gel precipitation was established, both tests showing peak antigen activity in the haemoglobin region of the electrophoresis column. Similarity between the gel diffusion reaction and the pattern produced in complement fixation was also demonstrated by Pereira, Pereira & Allison (1959) with adenovirus.

The antigens from pantropic and neurotropic strains of RVF could be distinguished neither by gel precipitin tests nor by electrophoresis. Antigen from the pantropic strain however had a higher complement-fixing titre and showed a greater degree of band splitting in gel precipitin tests. It appears therefore that greater amounts of pantropic antigen are produced in the liver than neurotropic antigen in the brain and that any difference between the antigens from the two sources is quantitative rather than immunological.

SUMMARY

1. Extracts containing non-infective soluble antigens have been prepared from the tissues of mice infected with a pantropic or any of three neurotropic strains of Rift Valley fever virus. The antigens were detectable by complement-fixation and gel-precipitin tests using antisera prepared in mice.

2. The extracts appeared to contain at least two antigens separable by electrophoresis and distinguishable by Ouchterlony tests. The faster migrating minor antigen occurs sparsely and was not further examined.

3. No distinction, physical or immunological, was observed between the major antigen derived from the four strains.

4. In gel diffusion-filtration experiments with granulated 7% agarose, the major antigen appeared to be polydisperse containing particles with diffusion rates ranging from at least as low as that of *Burnupena cincta* haemocyanin to almost as high as that of haemoglobin.

5. Centrifugal analysis indicated the presence of particles having sedimentation constants of about 8, 29 and greater than 100 Svedberg units. These particles have a density of about 1·27 g./ml. If all the particles of the major antigen are spherical, with a density of 1·27 g./ml., the three size groups have diameters of about 7–8, 14 and > 26 mμ.

6. The diffusion coefficient of the smallest particle was estimated to be $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. corresponding to a sphere 7 mμ in diameter, or $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. corresponding to a sphere 9 mμ in diameter depending on which of two values for the diffusion coefficient of mouse γ-globulin is used in the calculation.

7. In density gradient zone electrophoresis at pH 8·6, the principal antigen migrated at about the same rate as haemoglobin and appreciably slower than the virus.

The authors are indebted to Prof. A. Kipps for his continued interest in this work, and to Dr T. H. Mead for valuable criticism of this paper. This investigation was supported in part by a Public Health Service research grant AI 04044–02 from the National Institute of Health, Bethesda, U.S.A.

REFERENCES

ALLISON, A. C. & HUMPHREY, J. H. (1959). Estimation of the size of antigens by gel diffusion methods. *Nature, Lond.*, **183**, 1590.

ALLISON, A. C. & HUMPHREY, J. H. (1960). A theoretical and experimental analysis of double diffusion precipitation reactions in agar and its application to characterization of antigens. *Immunology*, **3**, 95.

ANDREWS, P. (1962). Estimation of molecular weights of proteins by gel filtration. *Nature, Lond.*, **196**, 36.

CASALS, J. & OLITSKY, P. K. (1950). A complement-fixation test for poliomyelitis virus. *Proc. Soc. exp. Biol., N.Y.*, **75**, 315.

CASALS, J., OLITSKY, P. K. & ANSLOW, R. O. (1951). A specific complement-fixation test for infection with poliomyelitis virus. *J. exp. Med.* **94**, 123.

CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. trop. Med. Hyg.* **7**, 561.

GRABAR, P. (1957). Agar-gel diffusion and immunoelectrophoretic analysis. *Ann. N.Y. Acad. Sci.* **69**, 591.

KIPPS, A., NAUDÉ, W. DU T., POLSON, A., SELZER, G. & VAN DEN ENDE, M. (1957). The size distribution of specific antigens in virus-infected tissues and their significance. *Ciba Foundation symposium on the nature of viruses*, p. 224. Ed. G. E. W. Wolstenholme and E. C. P. Millar. London: J. and A. Churchill Ltd.

LARGIER, J. F. (1959). A purification and investigation of tetanus antitoxin. *Arch. Biochem. Biophys.* **77**, 350.

LEVITT, J., NAUDÉ, W. DU T. & POLSON, A. (1963). Purification and electron microscopy of pantropic Rift Valley fever virus. *Virology*, **20**, 530.

MANSI, W. (1957). The study of some viruses by the plate gel diffusion precipitin test. *J. comp. Path.* **67**, 297.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1946). The activating effect of magnesium and other cations on the hemolytic function of complement. *J. exp. Med.* **84**, 535.

MEAD, T. H. (1962). Purification of rabies soluble antigen. *J. gen. Microbiol.* **27**, 397.

OUCHTERLONY, C. F. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta path. microbiol. scand.* **32**, 231.

PEREIRA, M. S., PEREIRA, H. G. & ALLISON, A. C. (1959). Use of gel diffusion precipitation test in the diagnosis of adenovirus infections. *Lancet*, i, 551.

POLSON, A. (1958). A method for determination of diffusion coefficients by the gel precipitin technique. *Biochim. biophys. Acta*, **29**, 426.

POLSON, A. (1961*a*). Fractionation of protein mixtures on columns of granulated agar. *Biochim. biophys. Acta*, **50**, 565.

POLSON, A. (1961*b*). A simplified fraction collector for gradient elution chromatography. *J. Chromatog.* **5**, 116.

POLSON, A. & CRAMER, R. (1958). Zone electrophoresis of type 1 poliomyelitis virus. *Biochim. biophys. Acta*, **29**, 188.

POLSON, A. & DEEKS, D. (1960). The diffusion constants of rabbit, guinea-pig and rat antibodies to a single common antigen. *Biochim. biophys. Acta*, **39**, 208.

POLSON, A. & DEEKS, D. (1962). Zone electrophoresis of entero-viruses. *J. Hyg., Camb.*, **60**, 217.

POLSON, A. & HAMPTON, J. W. F. (1960). Determination of the diffusion constant of poliovirus by the gel precipitin technique. *Biochim. biophys. Acta*, **44**, 18.

POLSON, A. & LEVITT, J. (1963*a*). Density determination in a preformed gradient of caesium chloride. *Biochim. biophys. Acta*, **75**, 88.

POLSON, A. & LEVITT, J. (1963*b*). A slowly sedimenting infectious component of Rift Valley fever virus. *J. Hyg., Camb.*, **61**, 451.

POLSON, A. & LINDER, A. M. (1953). The determination of sedimentation constants of proteins and viruses with the help of the Spinco preparative ultracentrifuge. *Biochim. biophys. Acta*, **11**, 199.

POLSON, A. & MADSEN, T. (1954). Particle size distribution of African horsesickness virus. *Biochim. biophys. Acta*, **14**, 366.

POLSON, A. & MADSEN, T. (1955). A brain factor influencing the viability of neurotropic Rift Valley fever. *Nature, Lond.*, **176**, 645.

POLSON, A. & POTGIETER, G. M. (1964). Determination of diffusion constants by a multiunit analytical method. *Nature, Lond.* (in the Press).

POLSON, A., SELZER, G. & VAN DEN ENDE, M. (1957). The electrophoretic mobilities of adapted MEF_1 poliomyelitis virus and its soluble antigen. *Biochim. biophys. Acta*, **24**, 600.

RUSSELL, B., MEAD, T. H. & POLSON, A. (1964). A method of preparing agarose. *Biochim. biophys. Acta* (in the Press).

SMITHBURN, K. C. (1949). Rift Valley fever. Neurotropic adaptation of the virus and the experimental use of the modified virus as a vaccine. *Brit. J. exp. Path.* **30**, 1.

STEERE, R. L. & ACKERS, G. K. (1962). Restricted-diffusion chromatography through calibrated columns of granulated agar gel; a simple method for particle size determination. *Nature, Lond.*, **196**, 475.

VAN REGENMORTEL, M. H. V. (1959). Determination of diffusion coefficient of turnip yellow mosaic virus by the gel precipitin technique. *Biochim. biophys. Acta*, **34**, 553.

VAN REGENMORTEL, M. H. V. (1961). Zone electrophoresis and particle size of cucumber mosaic virus. *Virology*, **15**, 221.

WILSON, M. W. & PRINGLE, B. H. (1954). Experimental studies of the agar-plate precipitin test of Ouchterlony. *J. Immunol.* **73**, 232.

EXPLANATION OF PLATES

PLATE 1

a. Gel precipitation test on pantropic and neurotropic antigens after gel diffusion-filtration. The fractions pooled in groups of three are in the wells. The antisera in the troughs are diluted 1/2.

b. Gel precipitation test on (1) PRVF, (2) NRVF, (3) MEM and (4) R120 antigens showing immunological identity of the main antigens and the presence of a second probably smaller antigen. Wells 5 and 6 contained normal brain and liver extracts respectively. PRVF antiserum (II) was used.

PLATE 2

a, *b*. Quantitative gel precipitin test on dilutions of PRVF antigen against NRVF antiserum diluted 1/4 showing the appearance of the bands after (*a*) 7 and (*b*) 32 days. C = control tube containing normal liver antigen.

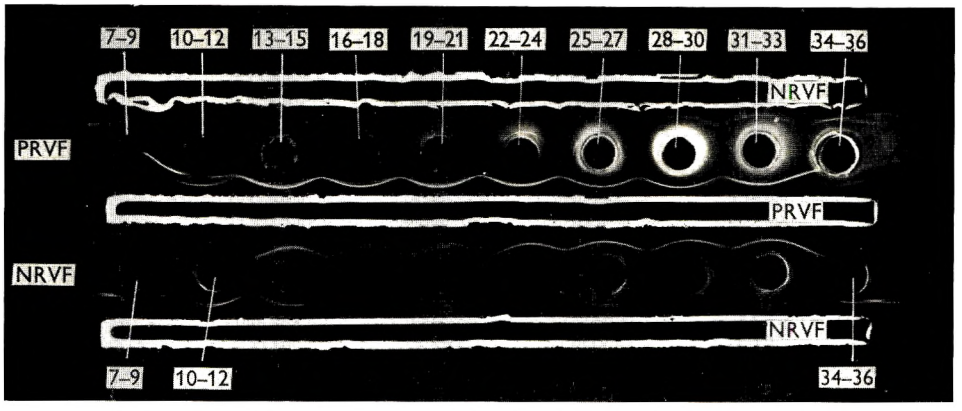
c. Gel precipitin test of doubling dilutions of PRVF antigen against PRVF antiserum (I) showing line splitting.

d. Estimation of the particle density of PRVF antigen. Pools *A*, *B* and *C* (see text) of antigen were introduced into preformed density gradients as fraction 5 (density 1·22) and centrifuged for 16 hr. at 30,000 rev./min. Successive fractions from each tube were submitted to the gel precipitin test with the results shown. After centrifugation the relevant fractions had the following densities: Pool *A*, fraction 7, 1·278; pool *B*, fraction 6, 1·256, fraction 7, 1·286; and pool *C*, fraction 5, 1·238, fraction 6, 1·278 and fraction 7, 1·286. The average density is close to 1·27.

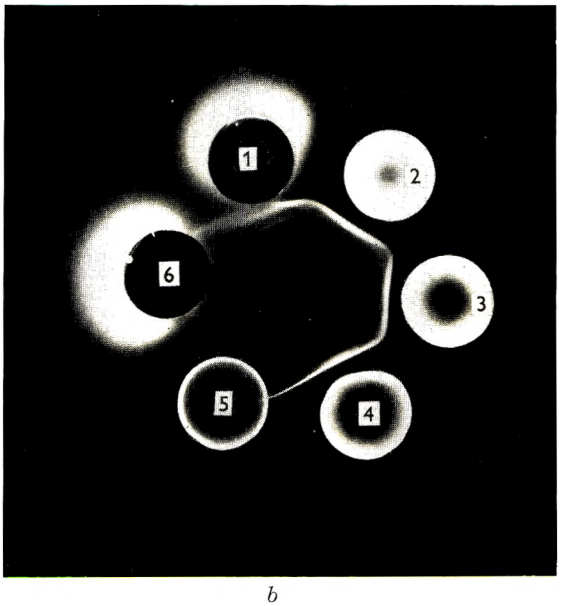
PLATE 3

a. Consecutive 1 cm. fractions of PRVF antigen after zone electrophoresis, tested by gel precipitation. The various antisera in the troughs are diluted 1/2 except R 120[1] and MEM[1] which are undiluted. Sample 0 containing opalescent material was taken 1 cm. below the origin of electrophoresis.

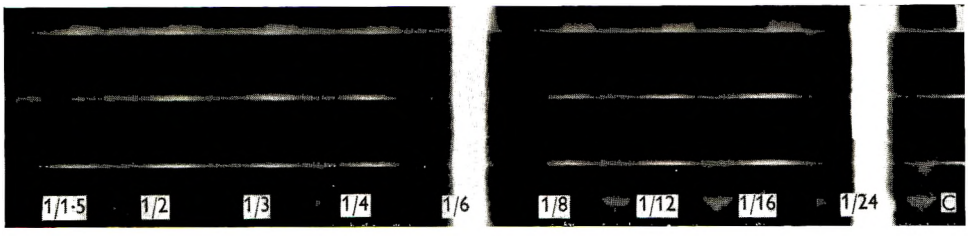
b. Gel precipitation test of fractions from zone electrophoresis, showing non-identity of the antigens in fractions 6 and 11.


7–9
10–12
13–15
16–18
19–21
22–24
25–27
28–30
31–33
34–36
NRVF
PRVF
PRVF
NRVF
NRVF
7–9
10–12
34–36

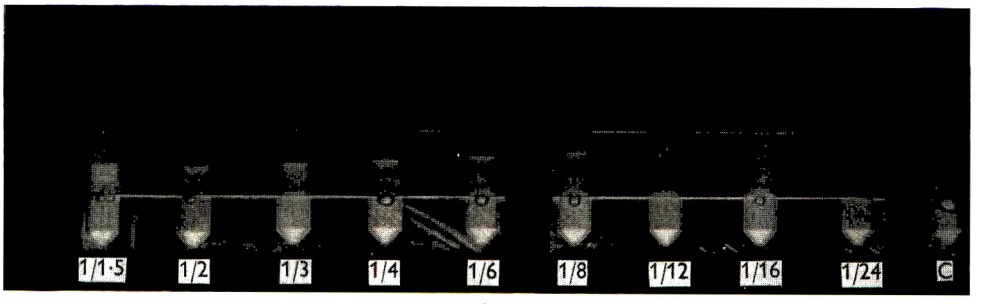
a


1
2
3
4
5
6

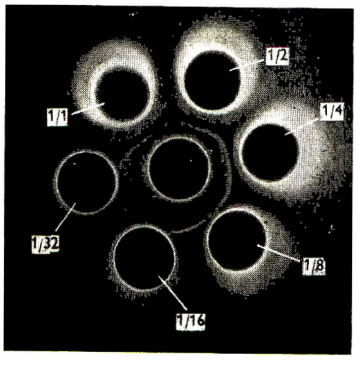
b



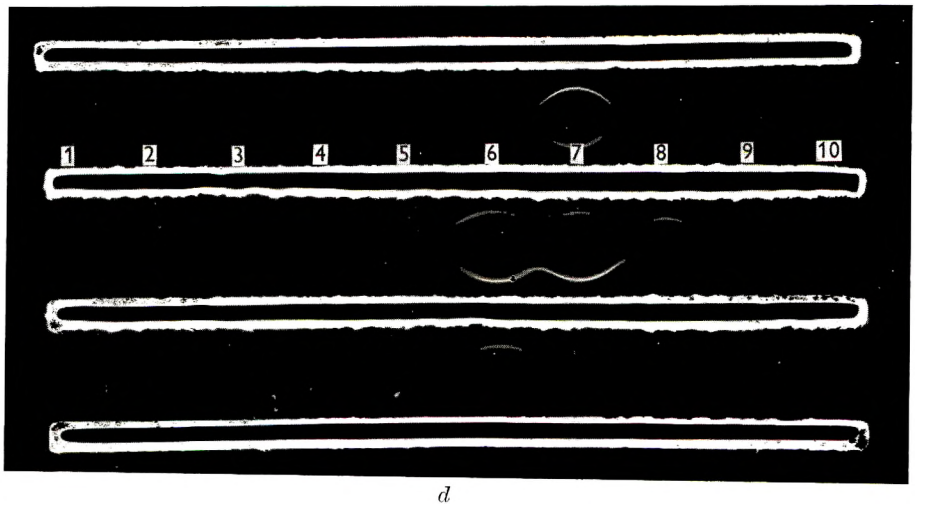
a



b

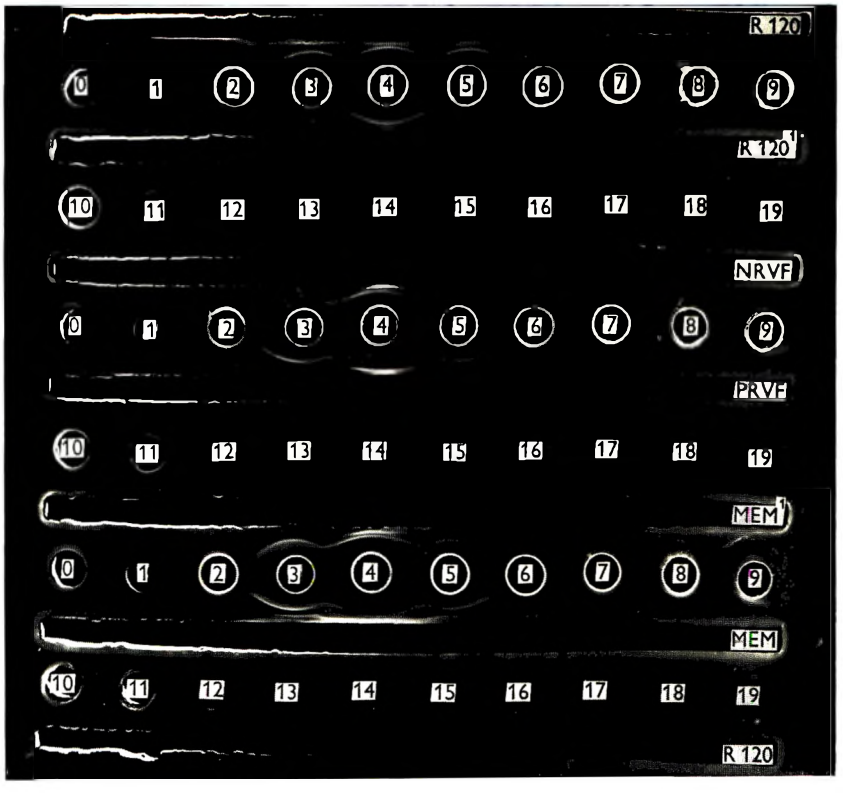


c

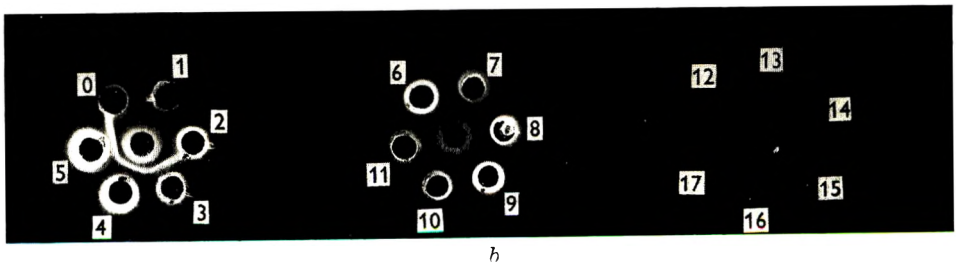


d

JULIA LEVITT AND A. POLSON


R 120
0 1 2 3 4 5 6 7 8 9
R 120
10 11 12 13 14 15 16 17 18 19
NRVF
0 1 2 3 4 5 6 7 8 9
PRVF
10 11 12 13 14 15 16 17 18 19
MEM
0 1 2 3 4 5 6 7 8 9
MEM
10 11 12 13 14 15 16 17 18 19
R 120

a


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

b

J. Hyg., Camb. (1964), **62**, 257
Printed in Great Britain

Studies on the pathogenesis of rinderpest in experimental cattle

II. Proliferation of the virus in different tissues following intranasal infection

By W. PLOWRIGHT

East African Veterinary Research Organization, Muguga, P.O. Box 32, *Kikuyu, Kenya*

(*Received* 24 *January* 1964)

The first paper of this series (Liess & Plowright, 1964) described the results of experiments designed to provide quantitative data on the routes of excretion of virulent rinderpest virus, in cattle infected by a number of different methods. An attempt was made to correlate virus excretion with the appearance and development of clinical signs and viraemia. However, no adequate explanation of the phenomena could be offered without detailed knowledge of the quantitative distribution of virus in various tissues, at increasing intervals after infection. The experiments to be described in this paper were performed to provide the necessary data.

All cattle were infected by the intranasal route, with the same strain of virus as that employed previously (RGK/1). It was considered that these animals would probably behave in a manner closely resembling that following natural infection and hence it should be possible to provide an account of the normal pathogenesis of the disease. The information obtained would be of practical significance in helping to formulate recommendations for the laboratory diagnosis of rinderpest, by the recovery of infectious virus from the tissues of animals in various stages of the disease. It should also, incidentally, be fundamental in reaching a scientific assessment of the dangers of the presence and persistence of rinderpest virus in the meat and offals from cattle, which might be slaughtered in the preclinical or clinical phases of the disease. There is little evidence that the need for such data is properly appreciated at present, or furthermore that the potential value of tissue culture techniques, in providing economical answers to these problems, has even begun to be recognized (see, for example, Provost, 1960).

Finally, it was hoped that an elucidation of the pathogenesis of rinderpest in cattle might be of comparative interest to those concerned with human measles and canine distemper. In this connexion, Downie (1963) has remarked on the advantages of the veterinary virologist in being able to study his diseases in the definitive host, and not in another experimental one which is regarded as more or less comparable.

MATERIALS AND METHODS

Experimental animals

A total of twenty-nine grade cattle was used. All of them were approximately 2 years old and twenty-two of them had, apparently, a large proportion of the blood of the highly susceptible Channel Islands breeds (Guernsey and Jersey). Their sera were found to be negative in screening tests for rinderpest-neutralizing antibody (Plowright & Ferris, 1961) and they were housed in isolation units which precluded the possibility of accidental infection. They were fed with hay only and provided with water *ad lib.*

Rectal temperatures were recorded every morning and any rise to 102·0° F. or above was regarded as abnormal. Daily clinical examinations were performed to ascertain the time to appearance of mouth lesions and diarrhoea or dysentery.

Virus strain

The origin of the RGK/1 strain of rinderpest virus has been described in a previous paper (Liess & Plowright, 1964). It was stored at −70° C. as untreated fragments of spleen tissue derived from an ox (no. 8994), which represented the fifth laboratory passage, three of which were in primary calf kidney monolayers, and two in cattle. Pieces of frozen spleen were thawed immediately before use and 10% (w/v) suspensions were prepared in Ten Broeck grinders, using culture maintenance medium as a diluent.

Infection of cattle

All animals were infected by dropping 2 ml. of a 10% spleen suspension into the nostrils, with the head held in such a position that the material flowed quickly over the turbinates towards the nasopharynx; thereafter the nostrils were compressed with the hand to produce a short period of noisy, forcible inspiration. Cattle did not cough as a result of this treatment and it was assumed that the inoculum did not enter the larynx. Care was taken that no mechanical injury to mucosae took place during the manipulations.

The inoculum was always prepared with culture maintenance medium as a diluent and samples were titrated in primary calf kidney cultures, by the method to be described later. The figures obtained were pooled to give an estimate of the reliability of the titration technique as well as to ascertain the size of the infecting dose for cattle. The mean and standard deviation for twelve separate titrations over a period of 87 days was $10^{5\cdot37\pm0\cdot19}$ TCD 50/g. Hence each animal received about $10^{4\cdot7}$ TCD 50 intranasally.

Collection of tissues

Animals were killed by shooting at intervals of 1–16 days after intranasal infection (Table 1). The neck vessels were severed and a sample of *blood* for viraemia study was collected into one-third the final volume of 1·5% EDTA in 0·7% sodium chloride (Plowright & Ferris, 1962). In fourteen instances (see Table 1) blood was also collected for the separation of serum. Cattle were allowed to bleed out as fully

as possible and then subjected to immediate systematic autopsy; in two cases, however (animals nos. 9287 and 9292; Table 1) death occurred spontaneously about 1 hr. before the post-mortem examination was begun.

Table 1. *Details of some cattle used in a study of rinderpest pathogenesis*

Animal no.	Incubation period*	Duration of pyrexia*	Day of max. temp.	Days to onset of		Day killed or died (D)	Titre† of serum
				Mouth lesions	Diarrhoea		
9284	4	N.D.	N.D.	N.D.	N.D.	4	N.D.
9296	N.D.	N.D.	N.D.	N.D.	N.D.	4	N.D.
9297	4	N.D.	N.D.	N.D.	N.D.	4	N.D.
9283	4	N.D.	N.D.	N.D.	N.D.	5	N.D.
9306	4	N.D.	N.D.	N.D.	N.D.	5	N.D.
9302	4	N.D.	N.D.	N.D.	N.D.	6	0·0
9305	4	N.D.	N.D.	N.D.	N.D.	6	N.D.
9308	4	N.D.	N.D.	N.D.	N.D.	6	N.D.
9304	4	N.D.	N.D.	7	N.D.	7	N.D.
9309	4	N.D.	N.D.	7	N.D.	7	N.D.
9303	5	N.D.	N.D.	8	N.D.	8	0·0
9307	4	N.D.	N.D.	6	8	8	0·0
9287	4	4	7	7	8	9 (D)	1·6
9301	4	N.D.	N.D.	7	9	9	0·6
9289	3	⩾ 8	8	7	9	10	0·8
9300	4	6	9	7	9	10	0·8
9292	4	8	8	7	9	12 (D)	0·8
9298	4	⩾ 9	8	8	8	12	2·2
9329	4	5	7	6	8	12	2·8
9326	5	6	7	8	8	14	2·6
9327	4	7	6	8	9	14	2·2
9288	5	5	8	7	9	16	1·8
9299	4	7	7	9	Absent	15	2·8
Range	3–5	4–9	6–9	6–9	8–9	—	—
Mean	4·1	6·5‡	7·5	7·3	8·5	—	—

* = Figures calculated from days with morning temperature ⩾ 102·0° F.
† = Log_{10} S.N. 50 at time of death or destruction.
‡ = Median.
N.D. = Not determined.

One each of the following lymph nodes was excised intact with separate sterile instruments and placed in individual Petri dishes, viz. *submaxillary* (mandibular), *pharyngeal* (supra-pharyngeal), *prescapular* (posterior superficial cervical), *mesenteric* (jejuno-ileal) and *ileocolic* (colic). The designations in brackets are those advocated by Sisson & Grossman (1940). One or more *haemolymph* (haemal) nodes were dissected out cleanly from the surrounding tissues of the prescapular groove or axilla. *Spleen pulp* was excised from the centre of the organ, free from capsule and larger trabeculae.

After washing free surfaces carefully with running water, portions of the *palatal*

tonsil, free of surrounding muscle and connective tissue, were excised with a scalpel. Similarly, a strip of mucosa was carefully dissected from the *base of the tongue* in the region lying just behind the vallate papillae; this latter, together with the pharyngeal mucosa, is a predilection site for the necrosis and erosion which is such a characteristic feature of rinderpest. Every effort was made to exclude tongue muscle fibres from the mucosal strip but many lobules of yellowish orange, mucous glands were closely adherent to the deep side of the epithelium and could not be removed effectively.

Nasal mucosa was stripped from a part of the washed middle third of the dorsal turbinate bone; separation in this region occurred cleanly, as a result of traction following outlining of an area by a scalpel incision. *Lung* tissue was excised from the left cardiac lobe and *myocardium* from the region of the papillary muscles of the left ventricle, excluding the endocardium. *Liver* and *kidney* were taken free of capsular connective tissue and '*brain*' consisted of portions of a cerebral hemisphere from which the meninges had been removed.

The *abomasal* (*pyloric*) *mucosa* was dissected with scissors from the underlying connective tissue, within an area situated not more than 3 in. from the pyloric protuberance. The surface was first washed liberally with running water to remove as much as possible of the food material and secretions; this precaution was also applied to other mucosae of the alimentary tract.

Ileal, *caecal* amd *colonic mucosae* were scraped off with a scalpel when the gut wall was held tightly stretched to eliminate folding; by this means it was possible to avoid the inclusion of elements deep to the mucosa. In the ileum areas were chosen which did not include any Peyer's patch, in the caecum harvests were taken from the segment within 6 in. of the apex, while colonic mucosa was obtained from the region near the abrupt bend in the *ansa spiralis*, i.e. near the mid-point of the viscus.

Bone marrow was collected from a number of posterior sternebrae, freed so far as possible of the surrounding muscle and connective tissues.

Further treatment of tissues

The nasal mucosa was always washed with phosphate-buffered saline (P.B.S.) (Dulbecco & Vogt, 1954), to free it from adherent blood-clot; nothing else required washing. Quantities of *ca.* 0·5–1·5 g. of each solid tissue were weighed in Petri dishes, chopped with crossed scalpels or scissors and transferred to Ten Broeck grinders. Sufficient culture maintenance medium to make a 10 % (w/v) preparation was added and the materials were reduced to uniform suspensions by vigorous grinding, without the use of abrasives. The suspensions were poured off, leaving any trabecular or interstitial connective tissue trapped between the plunger and the cylinder of the grinders. Tongue and nasal mucosae, together with the lung, were the most difficult tissues to disperse adequately. The mucosae of the abomasum, ileum, caecum and colon were all processed within about 1 hr. of removal from the body. This was to reduce to a minimum any inactivation of virus by proteolytic enzymes such as pepsin and trypsin. Free rinderpest virus particles are very rapidly inactivated by the latter (Plowright, 1964).

Bone marrow cells were obtained from fragments of red marrow, excised with bone forceps from the bodies of the posterior sternebrae. These were shaken with about 40 ml. of P.B.S. in a rubber-stoppered tube and the resulting suspension was decanted through muslin into a centrifuge tube. After spinning at 1000 rev./min. for 5 min. the fat formed a compact, solid layer on top of the fluid and both these fractions were poured off, leaving the cells as a deposit. The latter was resuspended in a smaller quantity of P.B.S. and packed by centrifuging in a graduated tube for 5 min. at 1000 rev./min. The deposit was then redispersed in maintenance medium to give a 10% (v/v) suspension, which was found by weighing to be practically the same as a 10% (w/v) preparation.

Tenfold dilutions of the different tissue suspensions were prepared in culture maintenance medium, with vigorous pipetting at each stage to break up cell aggregates. The range of dilutions extended to a minimum of 10^{-3} and a maximum of 10^{-8} (w/v). Dilutions from 10^{-2} upwards could be introduced into tissue cultures without adverse affects; at least two and usually four to five dilutions were inoculated into calf kidney monolayers, of which five were employed per dilution, the inoculum being 2·0 ml. each.

Blood with EDTA was tested for the presence of viraemia by the separation of leucocyte fractions from 20 ml. samples (Plowright & Ferris, 1962). These fractions were resuspended in 10 ml. of maintenance medium and inoculated in 2 ml. quantities into each of five tube cultures. Titrations of blood were performed in the manner already described (Plowright & Ferris, 1962).

All materials were inoculated into tissue cultures within 3 hr. of harvesting from the animal and during any period of extended delay they were placed at 4° C.

Representative blocks, from all solid tissues which were titrated, were also fixed in Zenker-formol and embedded in paraffin-wax for later study of the histopathology.

Preparation and maintenance of cell cultures

Primary calf kidney monolayers were prepared as already described in tubes of 160 × 15 mm. dimensions (Plowright & Ferris, 1959). They were used for titrations when 4–10 days old, all medium being decanted before the introduction of a 2 ml. inoculum for each tube and transfer to drums rotating at about 8 rev./hr. All tissues from one animal were titrated in tube cultures of the same batch.

On the following day the inoculum was decanted off and each tube was washed twice with 2 ml. of P.B.S. (Dulbecco & Vogt, 1954). The maintenance medium was then replaced, using 1 ml. only per tube and the cultures were returned to roller drums. Subsequent changes of medium were carried out on the 3rd, 5th and 7th days after inoculation.

Maintenance medium was as already detailed (Plowright & Ferris, 1959), with the addition of the following antibiotics per ml.: sodium penicillin, 100 units; streptomycin sulphate, 100μg.; neomycin sulphate, 25μg. and kanamycin sulphate, 50μg. No trouble was experienced with bacterial or fungal contaminants, even in titrations of mucosae of the alimentary or respiratory tracts. No cytopathic agent, other than rinderpest virus, was detected in any tissue.

Microscopic examination for the typical cytopathic effects of virulent strains of rinderpest virus (Plowright & Ferris, 1959, 1962) were carried out at intervals from the 3rd to the 9th days, final readings being taken on the latter day. Titres, expressed as $\log_{10}$ TCD 50/g. of solid tissues or per ml. of blood, were calculated by the method of Thompson (1947).

Evaluation of the results

The tissue suspensions from which tenfold dilutions were made consisted of viable and dead cells, either singly or in clumps, together with tissue fluids and blood. They therefore contained free virus and 'infective centres' which may have contained or released many more than one infectious particle. Some evidence that ultrasonic treatment released many infective particles from some cells in tissue suspensions was mentioned by Plowright (1962).

It might have been expected that titration results would have been irregular but in practice this was not the case. With the exception of a few tissues taken from animals 12 days after infection, reference to which will be made again later, titrations were as regular in their outcome as those performed with centrifuged culture fluids. The accuracy of replicate titrations of a tissue in different batches of calf kidney cells was shown by the small standard deviation observed in titrations of the inoculum for cattle (see above).

Since the minimum dilution inoculated into cultures was 10^{-2} and of this five tubes received 2 ml. each, the usual weight of solid tissues tested for the presence of virus was 0·1 g. and a nil result meant that virus was not detected in this amount of material. Negative outcome in the tests for detection of viraemia implied that virus was not present in *ca.* 13 ml. of blood.

An advantage of the use of inocula containing viable cells, whether from blood or solid tissues, was that it allowed the detection of virus in animals which had probably possessed circulating antibody for several days. It is doubtful if this infectivity would have been detected in cell-free suspensions. The possibility must also be mentioned that some of the first recoveries of virus, as from the cephalic lymph nodes, might have been accounted for by infected cells which were still in the phase of viral eclipse. This infectivity would also not have been demonstrable in cell-free extracts.

Serum neutralization tests

As shown in Table 1, quantitative neutralization tests were carried out on the sera of fourteen animals killed on the 6th to 16th days after infection. The technique employed was that described by Plowright & Ferris (1961) and all sera were tested against a 10^{-2} dilution of the same virus stock, providing a mean of $10^{2\cdot4}$ TCD 50 per tube.

RESULTS

Clinical signs in infected cattle

Table 1 gives some details for twenty-three cattle which were killed or died after the onset of fever. It will be seen that the incubation period varied from 3 to 5 days but that the majority of animals (19/23) showed a temperature reaction on

the 4th morning. The peak of the pyrexia was reached on the 6th–9th days after inoculation and the duration of fever was 4–9 days (median 6·5) in the ten animals which survived long enough for observations to be completed.

Mouth lesions, taking the form of focal necrosis and erosion of the epithelia and similar to those already described (Liess & Plowright, 1964), appeared on the 6th–9th days (mean 7·3) after infection, i.e. on the 3rd–6th days of the disease. Diarrhoea or dysentery was somewhat more delayed to 8 or 9 days (mean 8·5) after infection; it was absent in only one of the twelve animals which survived for an adequate time. Only two of the animals died, one on the 9th, and the other on the 12th day (see Table 1).

On the basis of these findings and those reported previously (Liess & Plowright, 1964), it was decided to subdivide the clinical course of the infection into four phases, these being successively:

(*a*) The incubation period, lasting about 4 days from the time of intranasal infection, and terminated by the first rise of temperature to 102° F.

(*b*) The prodromal phase, normally ending on the 7th day after infection with the appearance of mouth lesions. Its duration was thus approximately 3 days and the temperature of individual animals usually reached its maximum towards the end of this time or at the beginning of the next period. Other clinical signs during this phase included sero-mucoid ocular and nasal discharges, becoming muco-purulent later. Animals normally continued to eat but often became dull and apathetic.

(*c*) The mucosal phase, extending from the 8th to the 12th days. It was characterized at first by the increasing extent of mouth lesions and a little later by the sudden onset of diarrhoea or dysentery. Rectal temperatures declined and deaths often occurred on the 9th to 12th days. Animals which survived the 12th day after infection usually recovered and virtually all their temperatures had returned to normal by this time (see Table 1 and Liess & Plowright, 1964). Towards the end of this phase diarrhoea had often ceased or become less severe and mouth lesions were beginning to heal.

(*d*) The early convalescent phase, not clearly delineated but for the present purpose assumed to extend from the 13th to the 16th days, inclusive. Diarrhoea ceased during this period and mouth lesions were obliterated by rapid reconstitution of the epithelia.

It is not intended to claim that the figures proposed here for the duration of these phases have any strict, general application; they are reasonably accurate, but only in relation to the particular strain of virus and type of cattle employed in these experiments. In the description which follows, virological events will be correlated where possible with the four clinical phases.

Incubation period

Detailed titration figures for nine cattle killed on the first 4 days after infection are given in Table 2 and mean values for various tissues are shown in Figs. 1–6. One of two animals killed after 24 hr. (no. 9137) had virus in small quantities in a pharyngeal lymph node, but otherwise no infectivity was detected in either of

them. At 48 hr. one ox (no. 9135) showed appreciable amounts of virus in its cephalic lymph nodes and tonsil, while generalization had already occurred, as shown by a very low viraemia and by the presence of virus in a prescapular lymph node and spleen. The other animal (no. 9290) had virus in a submaxillary lymph node only. The difference between the site of primary proliferation in cattle nos. 9137 and 9290 suggested that virus could be absorbed and transported from the nasal mucosa to either the pharyngeal or submaxillary nodes. Such a deduction would not conflict with the known sources of their afferent vessels, which include the posterior part of the nasal cavity for the pharyngeal and the anterior part of the turbinates and *septum nasi* for the submaxillary nodes (Sisson & Grossman, 1940).

Table 2. *Titrations of the tissues of cattle killed on the first 4 days after infection*

Time after infection ...	1 day		2 days		3 days		4 days		
Cattle no.	9137	9291	9135	9290	9295	9360	9296	9297	9284
Submaxillary lymph node	0·0	0·0	2·2	2·2	N.T.	4·0	⩾ 6·2	6·6	6·6
Pharyngeal lymph node	2·2*	0·0	4·0	0·0	4·4	3·2	⩾ 6·2	7·2	6·2
Tonsil	0·0	0·0	3·4	0·0	5·4	4·0	⩾ 6·2	6·8	7·2
Prescapular lymph node	0·0	0·0	2·4	0·0	3·8	1·6	4·4	4·8	4·4
Mesenteric lymph node	N.T.	0·0	N.T.	0·0	3·0	0·0	4·8	5·8	4·8
Ileocolic lymph node	N.T.	N.T.	N.T.	N.T.	3·2	0·0	⩾ 5·2	4·8	5·6
Prescapular haemo-lymph node	N.T.	N.T.	N.T.	0·0	N.T.	1·8	⩾ 5·2	4·4	5·8
Spleen	0·0	0·0	2·2	0·0	4·0	3·0	⩾ 5·2	4·4	4·8
Blood	0·0	0·0	Tr.†	0·0	Tr.	Tr.	1·6	0·6	⩾ 2·2
Bone marrow	N.T.	N.T.	N.T.	N.T.	Tr.	Tr.	2·4	Tr.	2·2
Liver	N.T.	N.T.	N.T.	0·0	0·0	0·0	2·0	0·0	2·6
Kidney	N.T.	N.T.	N.T.	0·0	0·0	0·0	0·0	Tr.	0·0
Myocardium	N.T.	N.T.	N.T.	N.T.	N.T.	0·0	N.T.	N.T.	0·0
Brain	N.T.	N.T.	N.T.	N.T.	N.T.	0·0	N.T.	N.T.	0·0
Nasal mucosa	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Lung	N.T.	N.T.	N.T.	N.T.	2·0	0·0	2·0	0·0	2·4
Tongue mucosa	0·0	0·0	0·0	0·0	0·0	0·0	⩾ 4·2	3·6	5·4
Pyloric mucosa	N.T.	N.T.	N.T.	N.T.	0·0	0·0	2·8	0·0	3·2
Ileal mucosa	N.T.	N.T.	N.T.	N.T.	0·0	0·0	Tr.	0·0	2·8
Caecal mucosa	N.T.	N.T.	N.T.	N.T.	2·6	1·6	⩾ 4·2	3·0	4·2
Colonic mucosa	N.T.	N.T.	N.T.	N.T.	N.T.	Tr.	N.T.	3·2	3·4

* Titre in $\log_{10}$ TCD 50/g. or per ml. (blood). Where 2 or more tubes were infected with a 10^{-2} dilution of solid tissues it was assumed that all cultures would have been infected with a 10^{-1} dilution.

† Tr. = Trace, i.e. one to three cultures were infected out of five inoculated with leucocyte fractions from blood, or one out of five inoculated with a 10^{-2} dilution of solid tissues.

N.T. = not tested.

On the 3rd day virus in considerable quantities was present in the cephalic nodes and tonsils of both animals killed. There was a low-level viraemia, only just

detectable in leucocyte fractions from about 13 ml. of blood, while virus had begun to proliferate in the spleen and prescapular lymph nodes. It was also present in minimal quantities in the bone marrow and a haemolymph node of ox no. 9360. It was surprising to find that virus was already detectable in the caecal mucosa of both animals and in the colonic mucosa of one; the relatively late onset of diarrhoea had erroneously led to the expectation that virus would probably not reach these tissues until a relatively late stage of the infection. Visceral lymph nodes were infected in ox no. 9295 only and this animal also showed localization in the lung.

At this point it became clear that virus was not detectable in the nasal or tongue mucosae during the first 3 days after infection. This was in spite of the demonstration of a low-level viraemia in three of the six animals and the use of a large initial inoculum, part of which might have persisted in the nasal mucosa. No macroscopic abnormalities were noted in the latter and a preliminary examination of fixed and stained sections of turbinate bone also failed to reveal significant changes.

Of three cattle killed on the 4th day, two were already febrile, whereas the other, no. 9296, had not yet reacted. All three animals showed large amounts of virus in the cephalic lymph nodes and tonsils, titres varying between $10^{6\cdot2}$ and $10^{7\cdot2}$ TCD 50/g. There were also considerable but lower levels of infectivity in the prescapular and visceral lymph nodes, the spleen and haemolymph nodes and in the mucosa of the base of the tongue. Since the latter contains moderate numbers of large, aggregated lymphoid follicles, it was impossible in this experiment to determine whether the increase of virus was due to proliferation in these structures and/or in the surface epithelium.

In two cattle the level of viraemia had increased markedly and, possibly associated with this, measurable quantities of virus appeared in the bone marrow, liver and lung. The third animal, no. 9297, had a lower viraemia and virus was either absent or present in trace quantities in these tissues; it also showed a minimal quantity of virus in the kidney. Virus was proliferating in the caecum, probably also in the colonic mucosa of all animals, while in two of them (excluding no. 9297) it had appeared for the first time in the pyloric and ileal mucosae. The nasal mucosa was still negative.

Comment and conclusions on virological events during the incubation period

Summarizing the results for the incubation period it can be stated that virus in the inoculum almost certainly passed through the nasal mucosa without proliferating and without causing a local lesion. It was transferred to the associated pharyngeal or submaxillary lymph nodes, where it began to multiply within 24–48 hr. Some virus, probably in the form of infected cells, spilled over into the efferent lymph stream and gave rise to a low-level viraemia on the 2nd to 3rd days after infection. Secondary foci of proliferation were quickly established in the superficial and visceral lymph nodes, in the spleen and haemolymph nodes and in mucosal lymphoid follicles, such as those of the tongue and probably Peyer's patches. Finally, virus began to multiply in the mucosae of the alimentary tract,

beginning with the large intestine. The onset of fever was probably associated with the simultaneous proliferation of virus at many scattered sites and with its release in moderate quantities into the blood stream.

The prodromal period (days 5–7 inclusive)

From the beginning to the end of this phase virus titres in the cephalic lymph nodes and tonsils maintained very high levels, usually between $10^{7\cdot0}$ and $10^{8\cdot0}$ TCD50/g. (Fig. 1 and Table 3). Titres of virus in the prescapular and visceral nodes were commonly lower by 1–2 log units, so that they exceeded $10^{6\cdot6}$ in only three of twenty-one instances. This was reflected in the depression of mean values shown graphically in Figs. 2 and 3.

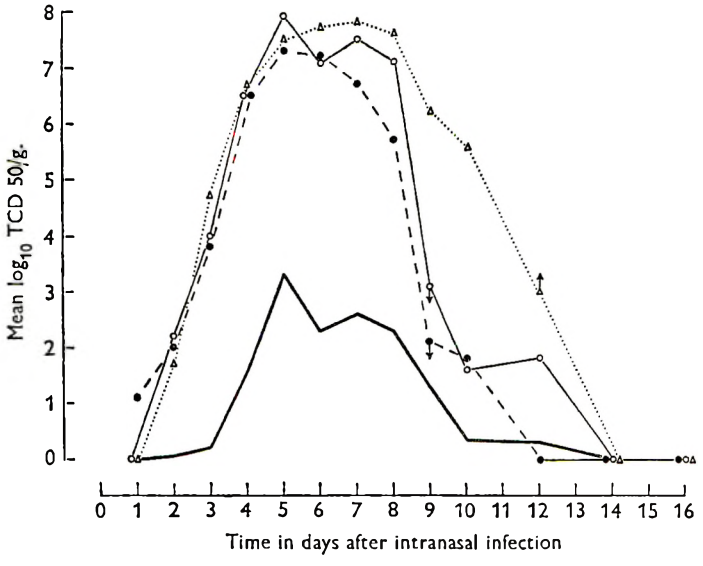


Fig. 1. Proliferation of virulent rinderpest virus, strain RGK/1, in lymphoid tissues of the head of cattle: correlation with viraemia. ○—○, Submaxillary lymph nodes; ●– –●, pharyngeal lymph nodes; △. . . . . △, palatal tonsil; ——, blood.

The level of virus in the spleen was lower still, a peak mean titre of $10^{6\cdot2}$ TCD50/g. being attained on the 5th day after infection. The latter was also, incidentally, the day of peak viraemia (mean $10^{3\cdot3}$ TCD50/ml., see Fig. 2). The haemolymph nodes developed considerably more virus than the spleen, to which they have many structural similarities (Trautmann & Fiebiger, 1952); titres on the 6th and 7th days were always $10^{7\cdot0}$ or higher. Fig. 2 shows quite clearly that virus proliferation commenced earlier in the spleen than in the haemolymph nodes and also began to decline sooner.

Mean figures for virus in the bone marrow (Fig. 2) did not exceed those for blood until the 6th day and the maximum was attained on the 7th day. Individual titres during this period varied widely from $10^{1\cdot8}$ to $10^{5\cdot2}$ TCD50/g., being usually 1·2–2·2 log units lower than those for spleen. The tongue mucosa, with its associated lymphoid follicles, maintained a plateau of mean values around $10^{6\cdot0}$ during the

Table 3. *Titrations of the tissues of cattle killed on the 5th to 7th days after infection*

Time after infection	5 days		6 days			7 days	
Animal no.	9306	9283	9308	9305	9302	9309	9304
Submaxillary lymph node	⩾ 8·2*	7·6	6·6	7·6	7·2	8·0	7·0
Pharyngeal lymph node	7·2	7·4	7·4	7·0	7·2	7·0	6·4
Palatal tonsil	7·8	7·2	7·8	7·6	7·8	8·0	7·6
Prescapular lymph node	6·4	⩾ 8·2	5·4	6·2	6·6	6·4	6·8
Mesenteric lymph node	6·2	6·6	5·8	6·4	7·2	6·6	6·4
Ileocolic lymph node	6·8	6·2	5·9	5·8	5·6	7·2	6·2
Prescapular haemolymph node	6·7	6·9	7·0	7·2	7·6	7·8	7·6
Spleen	6·2	6·2	4·4	5·4	5·8	6·4	5·6
Blood	3·2	3·4	1·6	2·4	3·0	3·0	2·2
Bone marrow	1·8	4·8	2·5	4·0	3·6	5·2	3·6
Liver	3·4	4·0	0·0	3·2	4·6	4·0	3·2
Kidney	1·8	2·6	0·0	2·2	0·0	3·2	Tr.
Brain	N.T.	N.T.	N.T.	N.T.	0·0	1·8	0·0
Myocardium	N.T.	0·0	0·0	0·0	0·0	Tr.†	0·0
Nasal mucosa	2·4	2·6	0·0	5·0	1·6	5·0	2·6
Lung	⩾ 4·2	⩾ 4·8	< 2·4	6·0	5·6	5·4	5·4
Tongue mucosa	6·2	6·6	5·8	6·6	5·4	7·0	5·8
Pyloric mucosa	4·8	4·8	3·4	4·0	4·2	5·2	4·6
Ileal mucosa	4·0	4·8	< 2·4	4·8	5·6	4·6	4·2
Caecal mucosa	5·4	5·0	4·0	5·8	5·4	6·6	5·6
Colonic mucosa	4·6	4·6	⩽ 2·4	5·4	4·6	5·4	5·0

* Titre in $\log_{10}$ TCD 50/g. or per ml. (blood). Where two or more tubes were infected with a 10^{-2} dilution of solid tissues it was assumed that all cultures would have been infected with a 10^{-1} dilution.

† Tr. = Trace, i.e. one to three cultures were infected out of five inoculated with leucocyte fractions from blood, or one out of five inoculated with a 10^{-2} dilution of solid tissues.

N.T. = not tested.

whole of the prodromal phase (Fig. 4); in different animals titres ranged rather narrowly between $10^{5\cdot4}$ and $10^{7\cdot0}$.

All the mucosae of the gastro-intestinal tract supported a considerable multiplication of virus, with mean titres, except in the case of the pylorus, reaching a maximum towards the end of the period. There was an unexpected gradient in the amount of infectivity demonstrable in the intestinal mucosae; the caecum almost invariably showed the highest titres, followed by the colon and then by the ileum. This is clearly shown by the mean lines for these tissues, presented in Fig. 4. With one exception (ox no. 9308), individual figures for the caecum ranged from $10^{5\cdot0}$ to $10^{6\cdot6}$, for the colon from $10^{4\cdot6}$ to $10^{5\cdot4}$, for the ileum from $10^{4\cdot0}$ to $10^{5\cdot6}$ and for the pylorus from $10^{4\cdot0}$ to $10^{5\cdot2}$.

The exceptional animal, no. 9308, which was killed on the 6th day after infection, showed good average titres for its lymphoid tissues but very small quantities of virus in the pyloric, ileal and colonic mucosae. It also had a low-level viraemia and little or no infectivity in its liver, lung and nasal mucosa, whereas other animals killed during this period commonly showed considerable amounts of virus in these tissues. It is possible that this ox was one which would have rapidly controlled

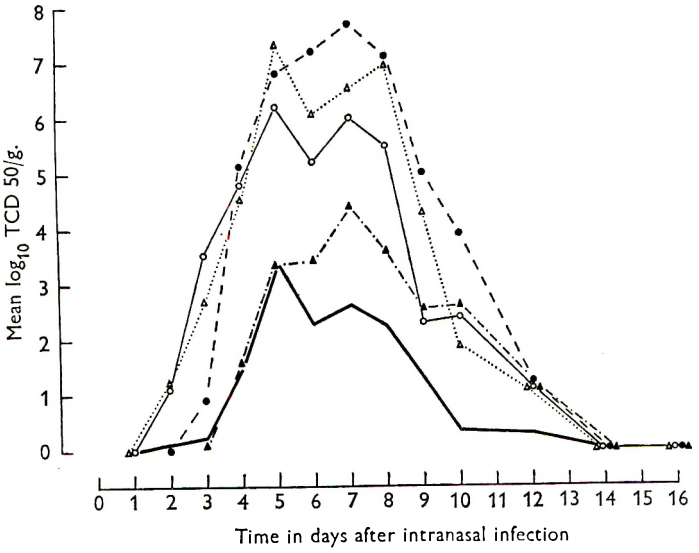


Fig. 2. Proliferation of virulent rinderpest virus, strain RGK/1, in the haemopoietic tissues of cattle: correlation with viraemia. ○—○, Spleen; ●– –●, prescapular haemolymph nodes; △. . . . . △, prescapular lymph nodes; ▲–·–▲, bone marrow; ——, blood.

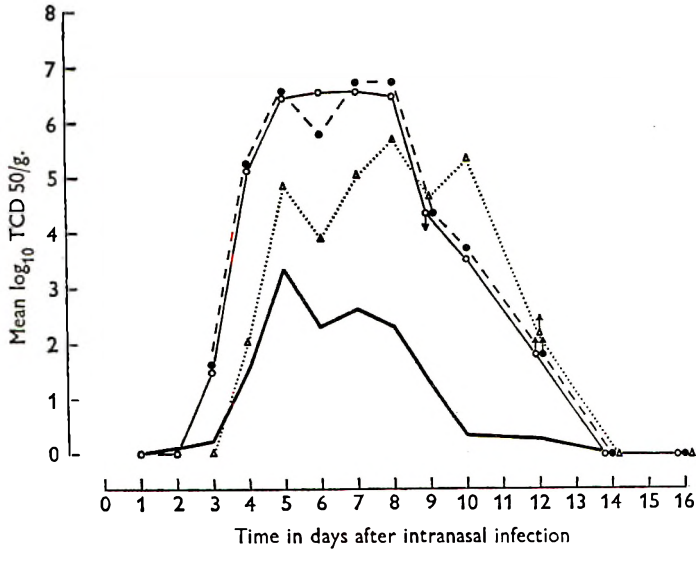


Fig. 3. Proliferation of virulent rinderpest virus, strain RGK/1, in the alimentary tract of cattle I: correlation with viraemia. ○—○, Mesenteric lymph nodes; ●– –●, ileocolic lymph nodes; ▲ . . . . ▲, pyloric mucosa; ——, blood.

virus proliferation by antibody production and thus recovered quickly after showing only mild clinical signs.

In the respiratory tract, virus first became detectable in the nasal mucosa on the 5th day. With the exception of ox no. 9308, already mentioned, all animals thereafter showed some virus but in greatly varying quantities. Thus individual titres ranged from $10^{2\cdot4}$ to $10^{5\cdot0}$ TCD 50/g.; these figures were surprisingly low and it was

only on the 7th day that the mean titre began to exceed that of the blood (Fig. 5). The lung tissue contained considerable quantities of virus in the absence of any macroscopic pathological changes. Titres on the 6th and 7th days, again with the exception of ox no. 9308, varied between $10^{5.4}$ and $10^{6.0}$, being always higher than those for the nasal mucosa, often by a wide margin (Fig. 5).

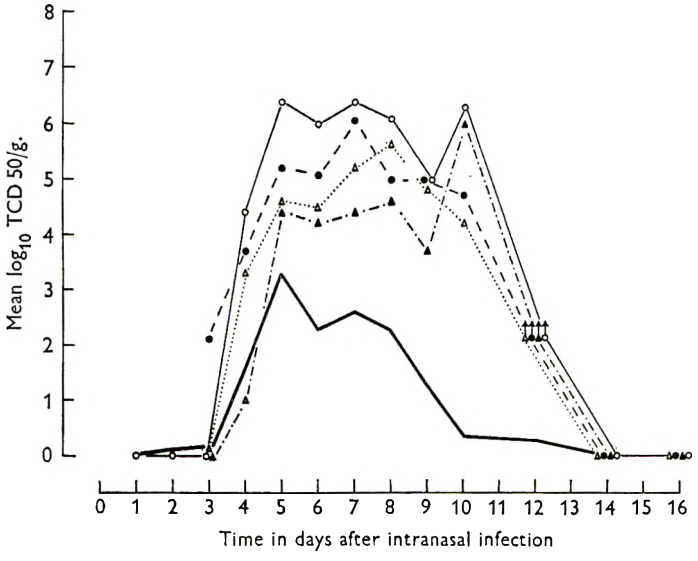


Fig. 4. Proliferation of virulent rinderpest virus, strain RGK/1, in the alimentary tract of cattle II: correlation with viraemia. ○—○, Mucosa of tongue; ●- -●, mucosa of caecum; △ . . . △, mucosa of colon; ▲-·-▲, mucosa of ileum; ——, blood.

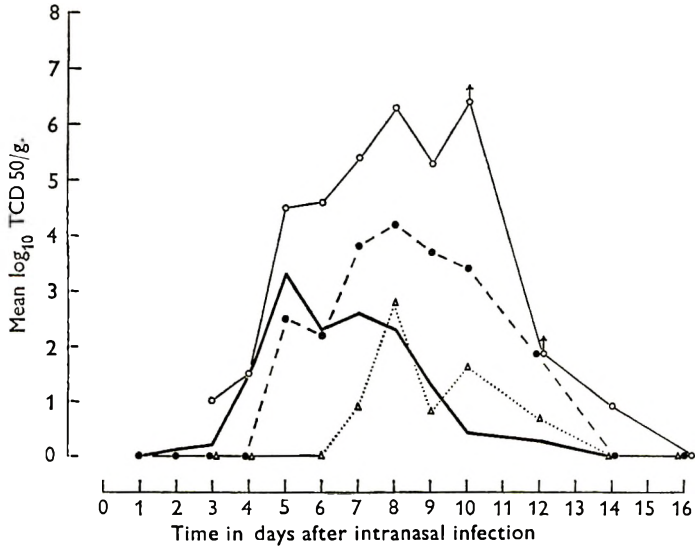


Fig. 5. Proliferation of virulent rinderpest virus, strain RGK/1, in the respiratory tract and brain of cattle: correlation with viraemia. ○—○, Lung; ●- -●, nasal mucosa; △ . . . . . △, brain; ——, blood.

Virus in the liver of individual animals increased considerably during the prodromal phase and exceeded the blood level by 0·6 to 1·6 log units on the 6th and 7th days; individual titres ranged from $10^{3·2}$ to $10^{4·6}$ TCD 50/g. (again excluding ox no. 9308). In the kidney virus was not always present and titres were invariably low during this period (see Fig. 6). No infectivity was demonstrable in the myocardium, with the exception of a trace in one animal on the 7th day. The same state of affairs was also found in the brain (Fig. 5).

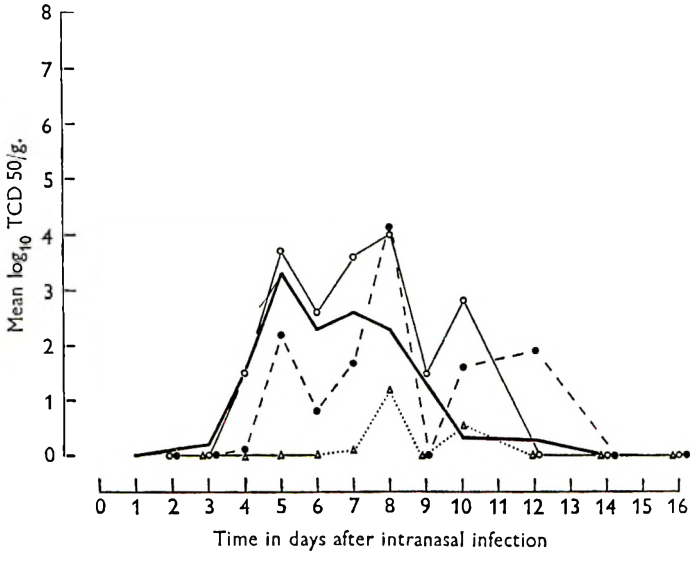


Fig. 6. Proliferation of virulent rinderpest virus, strain RGK/1, in the parenchymatous organs of cattle: correlation with viraemia. ○—○, Liver; ●- -●, kidney; △ . . . . . △, heart; ——, blood.

Comment and conclusions on virological events during the prodromal period

The prodromal period was, in general, characterized by a plateau of high virus titres in the lymphopoietic and lympho-epithelial tissues, with a gradient descending from the cephalic lymph nodes and tonsils to the superficial body nodes and haemolymph nodes, finally to the visceral lymph nodes and spleen. The level of viraemia was consistently high and reached a peak during this phase, while the bone marrow supported maximal virus proliferation towards its conclusion. Gastro-intestinal mucosae showed high virus contents throughout, peak values being reached towards the end of the period or early in the next phase; again there was a gradient, descending this time from caecum to colon, to ileum and pylorus.

Virus first appeared in the turbinate mucosa on the 5th day after infection and increased steadily in quantity during this period. Titres were, however, very variable and generally low. Virus in the lung increased slowly throughout and titres consistently exceeded those of the nasal mucosa. Although some local proliferation or accumulation of virus was probably demonstrated in the liver,

Table 4. *Titrations of the tissues of cattle harvested on the 8th to 12th days after infection*

Time after infection	8 days		9 days		10 days		12 days		
Cattle no. ...	9307	9303	9301	9287	9300	9289	9292	9298	9329
Submaxillary lymph node	7·0*	7·2	⩽ 3·2	3·0	⩽ 3·2	0·0	3·6†	0·0	1·8
Pharyngeal lymph node	4·0	7·4	⩽ 3·2	0·0	3·6	0·0	0·0	0·0	0·0
Palatal tonsil	7·0	⩾ 8·2	6·4	6·0	7·4	3·8	⩾ 6·2	2·8†	0·0
Prescapular lymph node	6·0	8·0	3·6	5·0	3·8	0·0	3·2†	0·0	Tr.†
Mesenteric lymph node	6·0	6·8	5·4	⩾ 3·2	7·0	0·0	⩾ 5·2	0·0	Tr.
Ileocolic lymph node	6·2	7·2	5·0	3·6	5·0	2·4	⩾ 5·2	0·0	0·0
Prescapular haemo-lymph node	6·4	7·8	4·2	5·8	7·8	0·0	0·0	3·6†	0·0
Spleen	4·8	6·2	2·4	2·2	4·8	0·0	3·2†	0·0	0·0
Blood	1·6	3·0	1·8	0·8	0·6	Tr.	0·8	0·0	0·0
Bone marrow	2·8	4·4	2·9	2·2	3·0	2·4	3·4	0·0	0·0
Liver	3·8	4·2	3·0	0·0	2·8	2·8	Tr.	0·0	0·0
Kidney	⩾ 4·2	4·0	0·0	0·0	3·2	0·0	2·8†	2·8	0·0
Myocardium	2·4	0·0	0·0	0·0	Tr.‡	0·0	0·0	0·0	0·0
Brain	2·8	2·8	1·6	0·0	3·2	Tr.	3·0	0·0	0·0
Nasal mucosa	4·0	4·4	4·2	3·2	3·2	3·6	3·8	1·8	0·0
Lung	6·4	6·2	6·6	4·0	⩾ 7·2	5·6	⩾ 5·2	0·0	Tr.†
Tongue mucosa	6·0	6·2	5·4	4·6	6·6	6·0	⩾ 6·2	0·0	0·0
Pyloric mucosa	5·2	6·0	5·0	4·2	6·6	4·0	⩾ 6·2	0·0	0·0
Ileal mucosa	4·8	4·4	4·8	2·6	7·0	4·9	⩾ 6·2	0·0	0·0
Caecal mucosa	5·8	6·2	5·8	4·2	6·4	3·0	⩾ 6·2	0·0	0·0
Colonic mucosa	5·2	6·0	5·2	4·4	5·2	3·2	⩾ 6·2	0·0	0·0

* Titre in $\log_{10}$ TCD 50/g. or per ml. (blood). Where two or more tubes were infected with a 10^{-2} dilution of solid tissues it was assumed that all cultures would have been infected with a 10^{-1} dilution.

† Irregular titration result, probably due to the simultaneous presence of virus and antibody.

‡ Tr. = Trace, i.e. one to three cultures were infected out of five inoculated with leucocyte fractions from blood, or one out of five inoculated with a 10^{-2} dilution of solid tissues.

there was no clear indication that it did so in the kidney during the prodromal period. The myocardium did not support virus multiplication.

The mucosal phase (days 8–12 inclusive)

This period of the disease, characterized clinically by the further development of mouth lesions and by the onset of diarrhoea and dysentery, began with very high levels of virus in all the major sites of proliferation, but these declined rapidly with the appearance of circulating antibody on the 9th day after infection (see Tables 1 and 4).

In the lymphopoietic tissues mean titres were always high on the 8th day (Figs. 1–3 inclusive) but, as shown in Table 4, a pharyngeal lymph node of ox no. 9307 already had a titre depressed to $10^{4\cdot0}$ TCD 50/g., whereas the submaxillary node

and tonsil contained 1000-fold more infectious virus ($10^{7\cdot0}$). The tendency for irregularity in the virus content of lymphoid tissues became more marked on the 9th and 10th days after infection, when the infectivity demonstrable in the cephalic lymph nodes fell dramatically, while titres in the visceral nodes remained considerable in two of four instances (cattle nos. 9301 and 9300); appreciable amounts of virus were also recovered from the prescapular nodes in three of four animals killed on the same days. It appeared, therefore, that infectivity began to decline rapidly in different lymph nodes in roughly the same sequence as it made its appearance.

Table 5. *Some anomalous results for the titrations of tissues from ox no.* 9292, *killed on day* 12 *post-infection*

Tissue	Number of cultures with cytopathic effects at tissue dilution				Estimate of titre
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Submaxillary lymph node	1/5	5/5	2/5	0/5	$10^{3\cdot6}$/g.
Prescapular lymph node	2/5	3/5	2/5	0/5	$10^{3\cdot2}$/g.
Spleen	4/5	3/5	2/5	0/5	$10^{3\cdot2}$/g.

On the 12th day results were made irregular by a series of unexpectedly high figures in one animal (no. 9292) from which tissues were harvested immediately after its death. Otherwise, it will be seen from Table 4 that virus was only detectable in minimal quantities, if at all, in the lymphoid tissues and titration results for all three individuals were sometimes anomalous. Detailed results for some of the irregular titrations of tissues of ox no. 9292 are given in Table 5. The wide range of tenfold dilutions giving less than 100% infection was never observed for tissues harvested before the 12th day and unfortunately rendered accurate calculation of 50% end-points impossible. The figures in the final column of Table 5 were estimated from the proportion of tubes infected with 10^{-3} and 10^{-4} dilutions; it was assumed that all cultures inoculated with 10^{-2} dilutions would have yielded virus if it had not been for the effect of neutralizing antibody, or possibly interferon, either present at the time of inoculation or produced afterwards by viable cells included in the suspensions. The visceral lymph nodes of ox no. 9292 showed very high titres, probably in excess of $10^{7\cdot0}$ TCD 50/g. and associated with comparable levels of virus in the intestinal mucosae (see below). The amount of virus in the tonsil was similarly very high.

Virus in the spleen had already begun to decline seriously on the 9th day, being absent from one of two animals killed on the 10th day, and from two of three on the 12th. Considerable amounts of infectivity persisted to the 9th day in the haemolymph nodes but had disappeared from one of two animals killed on each of the 10th and 12th days. Viraemia had declined to very low levels by the 10th and could not be demonstrated in two of three animals on the 12th day. As shown in Table 1, all animals killed or dying on the 9th day and later had measurable amounts of circulating antibody; hence, all infectivity in the blood must have been intracellular. Bone marrow titres declined steadily during this phase and virus

had disappeared from two of the three cattle killed on the 12th day. Its behaviour in this tissue was, therefore, very similar to that in the blood and spleen.

The behaviour of virus in the tonsil and tongue mucosa was somewhat different from that in the lymph nodes, since titres often remained high up to the 10th day. These tissues contain epithelial and lymphoid elements in both of which the virus produces cytopathic effects and therefore, presumably, multiplies. Predominantly epithelial tissues, such as the mucosae of the gastro-intestinal tract, also retained considerable amounts of infectious virus up to the 10th day. Excluding ox no. 9292, which died, virus had disappeared from the mucosae of the gastro-intestinal tract in two animals killed on the 12th day after infection. In one of the two, no. 9298, small amounts of infectivity were still recoverable from the lympho-epithelial tissue of the tonsil, whereas it had disappeared in the other. Ox no. 9292 had virus to extremely high titre in its intestinal mucosae; from the time to appearance of cytopathic effects in cultures inoculated with 10^{-6} (w/v) dilutions, it was estimated that titres for the ileal, caecal and colonic mucosae were probably of the order of $10^{7.5}$ to $10^{8.0}$ TCD 50/g.

In the nasal mucosa virus titres remained at moderately high levels ($10^{3.2}$ to $10^{4.4}$) up to and including the 10th day (Fig. 5). By the 12th, infectivity had disappeared from one of three animals, but was still fairly high ($10^{3.8}$) in ox no. 9292 and minimal ($10^{1.8}$) in the third (Table 3). Lung titres were consistently high, up to and including the 10th day; thereafter there was a rapid fall; only ox no. 9292 had appreciable amounts of virus in the lung on the 12th day, the titre probably exceeding $10^{6.0}$ TCD 50/g.

Liver and kidney both attained peak virus contents of about $10^{4.0}$ TCD 50/g. on the 8th day, but afterwards isolations were irregular and titres remained low, not exceeding $10^{3.0}$ (see Fig. 6). Two of three animals showed virus in the kidney on the 12th day. Recovery of infectivity from the myocardium was very irregular, the maximum titre being recorded on the 8th day ($10^{2.4}$ TCD 50/g. in ox no. 9307). All samples were negative on and after the 10th day (Fig. 6). In the brain a mean titre of $10^{2.8}$ was attained on the 8th day but thereafter recovery was irregular and the highest individual figure was $10^{3.2}$ TCD 50/g. in ox no. 9300 (day 10). One animal only (no. 9292) had virus in the brain on the 12th day.

Comment and conclusions on the virological events during the mucosal phase

On day 8, at the beginning of this stage of the disease, the virus content of all tissues was near maximal but within a very short time there were definite indications of a decline in titre, especially in those lymphopoietic tissues which had borne the brunt of the first virus attack. High titres were maintained longer—up to the 10th day—in tissues where the virus was probably proliferating in epithelial cells, e.g. the tonsils, tongue mucosa, lungs and gastro-intestinal mucosae. Nevertheless, by the 12th day considerable amounts of infectivity remained in only one animal of three examined and this had died with very severe clinical and pathological manifestations. The course of events in this ox (no. 9292) may have been determined by its slower development of neutralizing antibody, since the serum

titre ($\log_{10}$ S.N. 50) at death was only $10^{0.8}$ compared with $10^{2.2}$ and $10^{2.8}$ for cattle nos. 9298 and 9329, which were killed on the same day.

A factor which was probably of great significance in the decline of virus titres during this phase was the destruction of susceptible cells; thus, preliminary histological examination of lymphoid tissues showed an extreme degree of degeneration and depletion of cells of the lymphocytic series. Similar changes, but of less intensity, were seen in surface and glandular epithelia of the alimentary tract, including the tongue and palatal tonsil (Plowright, unpublished). The successive multiplication of the virus in the cephalic, followed by the body lymph nodes and spleen, then by the epithelia, would be likely to result in a similar sequence of exhaustion of cells capable of supporting virus proliferation.

In addition, the production and circulation of neutralizing antibody, perhaps also of interferon, could obviously have impeded the cell-to-cell transfer and even intracellular multiplication of the virus. The majority or all of the infectivity which was demonstrated on the 12th day was almost certainly intracellular and would not have been detected if cell-free extracts of tissues had been employed as inocula. It also seems logical to suppose that antibodies would have appeared first in significant concentration in those lympho-reticular structures which were first exposed to virus antigens. Since these were the cephalic lymph nodes it was not, therefore, surprising to find that an irregular but usually precipitous fall of virus titre occurred first in these situations.

The early convalescent period (days 13 to 16 inclusive)

Only four animals were killed during this period, two on the 14th and one each on the 15th and 16th days after infection. From a total of eighty-four tissues which were titrated, virus was recovered from only one, the lung of ox no. 9326. The titre was minimal, only a proportion of tubes being infected by 10^{-2} and 10^{-3} dilutions; the only lesion observed in the positive lung was an interstitial emphysema, which is common in animals autopsied at this period.

Failure to recover virus from the mucosae of the gastro-intestinal tract was somewhat surprising since no. 9326 still had severe, watery diarrhoea at the time of destruction and adherent necrotic deposits and/or unhealed erosions were still present in foci near the pylorus, on the Peyer's patches or in the caecum and colon of all 4 animals.

As shown in Table 1, all cattle had acquired high levels of circulating antibody by the 14th to 16th day after infection, log S.N. 50 titres ranging from $10^{1.8}$ to $10^{2.8}$.

Comment and conclusions on virological events during the early convalescent period

By the 14th day after infection virus had virtually disappeared from all the tissues examined. It is possible that small 'pockets' of virus do commonly persist to this time in situations where they are not exposed to antibody in significant quantities. In the lung, for example, this may include foci of tissue isolated by emphysematous bullae or virus-containing cells in aggregates within the bronchioli. In the gastro-intestinal tract, widespread necrosis and erosion of epithelia presum-

ably leads to the passage of dead and infected cells into the lumen, accompanied by fluid exudates containing antibody; hence virus would have little opportunity for prolonged persistence.

DISCUSSION

So far as is known to us, no systematic studies have ever been reported previously on the quantity of infectious rinderpest virus contained in different tissues of cattle at various intervals after infection. Such information as existed was usually fragmentary and acquired in the course of attempts to produce inactivated tissue vaccines of the greatest possible efficacy, consistent with economy.

Jacotot (1931), for example, killed cattle at the height of fever, 6–8 days after infection and titrated their tissues in calves and goats. He found that dilutions of abomasal mucosa were infective up to 1/300,000, whereas tracheal, buccal and vulvo-vaginal mucosae or skin were not usually infective beyond a 1/25,000 to 1/50,000 dilution; liver, kidney, blood, spleen, lymph nodes, thymus, tonsil and thyroid did not usually exceed 1/25,000 to 1/50,000, while lung failed to infect at a dilution of 1/50,000. Walker *et al.* (1946) stated that 1/10,000 dilutions of the lymph nodes, spleen and lungs of calves, killed on the 2nd or 3rd day of fever, were infective to cattle. Bergeon (1952) studied bone marrow, brain and spinal cord from cattle similar to those used by Jacotot (1931). He inoculated suspensions into cattle and found that brain had a low infectivity, not demonstrable in dilutions above 1/1000; no virus was detected in a 1/100 dilution of spinal cord but bone marrow was infective up to 1/20,000.

MacOwan (1956) stated that data had accumulated which showed that the titre of a virulent laboratory strain of virus was $10^{6\cdot0}$ in carcass lymph nodes, spleen and abomasal mucosa. The time of harvest was not mentioned but it was added 'Other tissues have considerably less virus'. Infectivity was demonstrable in the brains of cattle 4 days after infection, probably on the 1st to 2nd days of fever. The figures produced by MacOwan (1956) have apparently been accepted without comment by Provost (1960), who also asserted that bone marrow contained 10–100 infectious units of virus, probably per gramme.

It is quite evident, where details are provided, that the accuracy of these and many similar communications must be severely questioned, if only because they were based on the inoculation of relatively small numbers of cattle or goats, which may or may not have been susceptible and effectively isolated. In the case of each tissue it is usually found that one or, at best, a very few samples were titrated, at imprecisely determined or unstated intervals after infection. The details of preparation of the inocula, whether end-points were obtained, and the method of expressing the results, are matters often left in doubt. It is difficult, therefore, to compare the figures obtained with those presented here.

The Japanese workers Furuya and Fukusho were quoted by Mornet & Gilbert (1958) as having titrated rinderpest-infected cattle tissues by inoculating tenfold dilutions into rabbits, which were subsequently tested for immunity by challenge with the normally lethal lapinized rinderpest virus. This method gave figures which were said to be 10 to 100 times lower than those obtained by cattle inocu-

lations. Their titres were 10^{-4} to 10^{-6} (probably per gramme) for lymph nodes and ileum; 10^{-4} to 10^{-5} for caecal tonsil, spleen, tracheal mucosa and the posterior part of the nasal mucosa; 10^{-2} for the kidney, thyroid, submaxillary gland, oral and oesophageal mucosae; 10^{-1} for the adrenal, testis, skin, urinary bladder, gall-bladder, bone marrow, ruminal mucosa and C.S.F.; nil for the myocardium, voluntary muscles and spinal cord. These tissues were probably all taken at the height of the temperature reaction. With few exceptions the figures of Furuya and Fukusho would appear to fall within the ranges established for the same tissues in the present study, so long as 1 or 2 $\log_{10}$ units are added for the lower sensitivity of rabbits compared with cattle.

The primary phase of rinderpest infection was of comparative interest in that no evidence was obtained for virus proliferation at the presumed site of penetration, i.e. the nasal mucosa. As noted in the section on 'Materials and Methods', care was taken to wash off as much blood as possible from this mucosa and all samples were taken from the middle third of the dorsal turbinate bone. Hence primary sites of multiplication in the lower or upper thirds, of the same or other turbinates, may have been missed. However, rinderpest obviously differs from pox-virus infections, which have been the subject of many previous studies on the pathogenesis of generalized virus diseases and in which proliferation at the initial site of virus penetration is readily demonstrable. (See Fenner (1948*a*, *b*) for ectromelia; Fenner & Woodroofe (1953) for myxomatosis; Hahon & Wilson (1960) for monkey small-pox; Bedson & Duckworth (1963) for rabbit pox.)

Grist (1950), reviewing the position with respect to human measles, suggested that in this disease there may sometimes be a transient 'illness of infection', associated possibly with the production of a 'primary complex'. Robbins (1962) also concluded that measles virus probably invaded and proliferated in the respiratory epithelial cells. Neither of these opinions was supported by any experimental data for man, although Robbins quoted Sergiev, Ryazantseva & Shroit (1960) as having found considerable quantities of virus in the naso-pharyngeal mucosae of monkeys during the incubation period.

In a review of canine distemper, Gorham (1960) pointed out the discrepancy between different reports on the primary site of virus proliferation. Titration of ferret and mink tissues, at different times after infection, indicated that virus was present in the nasal tissues on the second day after exposure but this may have been due to the inclusion of viraemic blood (Crook, Gorham & McNutt, 1958). On the other hand, sequential studies in ferrets by the fluorescent antibody technique never revealed virus antigens in the epithelium covering the nasal turbinates, whereas the cervical lymph nodes showed specific fluorescence from the 2nd day onwards (Liu & Coffin, 1957). These experiments were extended to naturally infected dogs and it was found that the behaviour of this species was similar to that of ferrets, although nasal smears from dogs clinically sick with distemper were said to contain specific antigens (Coffin & Liu, 1957).

It is generally accepted that in prodromal measles there is a systemic involvement of the lympho-reticular tissues, beginning even in the incubation period (Grist, 1950; Robbins, 1962). The evidence on which this belief is based is largely

histopathological, particularly the presence of 'mesenchymal' or 'reticulo-endothelial' giant cells, especially in the upper respiratory tract, the lungs and parts of the alimentary tract. Grist (1950) considered it likely that the mesenchymal and epithelial reactions were manifestations of successive phases of viral attack, the onset of pyrexial illness coinciding with a flooding of the circulation by virus and products of cellular damage, which occurs towards the end of the first or lympho-reticular phase. The same reviewer concluded that widespread dissemination of virus occurred before symptoms appeared.

In distemper-infected ferrets, the work of Crook *et al.* (1958) showed that viraemia and generalization of virus to the spleen, liver and brain occurred before the onset of pyrexia. However, virus titres in these organs, as well as in the nasal tissue, lung and blood, continued to increase until the 5th to 7th day of estimated pyrexia; the course of events in mink was found, by the same authors, to be similar though somewhat slower. Liu & Coffin (1957) found specific fluorescence in the spleen of distemper-infected ferrets before the onset of fever (4th day after exposure) but they could not demonstrate viral antigens in the gastro-intestinal, respiratory and urinary tracts until the 6th or 7th days after infection.

Curasson (1932), in his monograph on rinderpest, remarked as follows: 'Pendant la période d'incubation, il semble que le virus cultive et se multiplie dans les tissus jusqu'à ce que en quantité suffisante, il puisse envahir la masse sanguine, la maladie explosant brusquement.' The results described in the present communication demonstrated unequivocally that generalization of rinderpest virus in cattle occurred during the late incubation and early prodromal phases. This process was presumably a sequel to the low-level viraemia which became demonstrable by the 2nd or 3rd days after infection, originating in the cephalic lymph nodes; it involved the lymph nodes of the body, the spleen, bone marrow, lung and alimentary mucosae. It can be seen, therefore, that generalization in measles, distemper and rinderpest infections occurs before the onset of clinical signs and, as pointed out by Fenner (1948*b*), the latter cannot be due to virus 'invasion' from a primary focus. In fact pox-virus titres are near-maximal at the time of the first rise of temperature but this was not true of rinderpest.

It was not possible, on the evidence obtained, to subdivide generalization in rinderpest into lympho-reticular and epithelial phases, as suggested by Grist (1950) for measles, since localization in the mucosae of the alimentary tract and in the lung occurred at about the same time as virus appeared in the lymphoid tissues of the body. A final decision on this question can be given only as a result of sequential studies with fluorescent antibody. In the tongue, as also in the gastro-intestinal mucosae, there was a distinct possibility that the first cells involved were local, nodular aggregations of lympho-reticular cells. The early appearance (3rd day) of virus in the lung could have been due to proliferation in either peribronchiolar lymphoid nodules or in epithelia. In the nasal mucosa there were diffusely scattered lymphoid cells and these could have supported the virus proliferation first detected on the 5th day; nevertheless, primary multiplication in this tissue may have been in the epithelium, especially as superficial necrosis and erosion became visible at this time near the nares (Liess & Plowright, 1964).

One of the most interesting aspects of the results described in rinderpest-infected cattle was the light which they threw on the phase of virus decline. There were clear-cut indications that titres began to fall first in those lymphoid tissues which supported initial virus multiplication. They were maintained longest in lympho-epithelial structures such as the tonsils, base of the tongue, the gastro-intestinal mucosae and the lung. An explanation of these findings would require further investigation, but it seems reasonable to suppose that the irregularity of lymph node titres, from the 8th day onwards, was due to local production of antibody and the exhaustion of cells capable of supporting virus proliferation. From the practical point of view it should be evident that tissues such as the tonsil or lung are more suitable than spleen or lymph nodes for virus recovery after the onset of diarrhoea or dysentery. Before this time cephalic lymph nodes are more likely to give positive results than are visceral or superficial nodes and spleen.

Nasal excretion of rinderpest virus was found, by Liess & Plowright (1964), to occur as early as 1–2 days preceding the onset of pyrexia in cattle, although most previous work had suggested that the nasal secretions became infective on the 2nd or 3rd days of thermal reaction. No virus was recovered by these authors from nasal swabs collected during the first 48 hr. following intranasal inoculation and this finding supports the contention that local multiplication of virus did not occur in the nasal tissues before infection of the local lymph nodes. Since no infectivity was detected in the nasal mucosa until the 5th day after infection, it is obvious that virus in nasal swabs taken before that time must have come from elsewhere.

Two alternative origins can be postulated, these being the palatal tonsils and the lungs. Virus in the tonsillar tissue reached moderately high titres by the 3rd day and some of this might have escaped into the epithelial crypts and thence to the nasopharynx; lung tissue was irregularly infective on the 3rd and 4th days after infection, titres being low. The quantity of virus found in nasal swabs taken during the later course of the disease (often $> 10^{4\cdot0}$, sometimes $> 10^{5\cdot0}$ TCD 50 (Liess & Plowright, 1964)) also militated against its exclusive origin from the nasal mucosa, where titres only occasionally exceeded $10^{4\cdot0}$ TCD 50/g. The disappearance of virus from nasal excretions collected on the 9th day of pyrexia, corresponding with the 11th to 13th days after infection (Liess & Plowright, 1964), agrees fairly well with the data obtained in this study, where two animals killed on the 12th day retained very little virus in their tissues.

The first urinary excretion of virus was noted on the 1st day of pyrexia and the rate increased to affect a maximum of 62·5 % of the animals on the 5th to 7th days of the disease (Liess & Plowright, 1964). These observations correlate very well with the irregular figures for virus in kidney tissue, as do also the variable and generally low titres for virus in the urine. It is possible, of course, that some urinary infectivity was derived from the epithelium of the lower urinary tract but there would not appear to have been any necessity for this, so long as virus was able to pass into the glomerular filtrate.

No virus was demonstrable in the faeces of cattle before the 3rd day of pyrexia, corresponding approximately to the 6th day after infection. The faecal excretion rate rose to a maximum of 40 % on the equivalent of the 10th day after infection

and titres were very variable (Liess & Plowright, 1964). It must be concluded that the consistent presence of moderately high-titre virus in the gastro-intestinal mucosae, lasting from the 5th to the 10th or 12th days after infection, did not automatically lead to appreciable levels of excretion in the faeces. The longevity of infectious virus liberated into the lumen of the alimentary tract would obviously depend on the presence and concentration of bile salts and enzymes capable of inactivating it. Free virus would also be killed rapidly at body temperature, especially in locations where pH values deviated widely from neutrality and where putrefactive bacteria were multiplying rapidly. The cessation of faecal excretion in animals surviving to the 9th day of the disease (Liess & Plowright, 1964) correlates well with the disappearance of virus from the alimentary tract of two animals killed on the 12th day after infection. Ox no. 9292, however, was probably excreting large quantities of virus in its faeces, when it died on the 12th day, since titres in all the gastro-intestinal mucosae were extremely high (see Table 4).

SUMMARY

The pathogenesis of rinderpest virus was studied in twenty-nine grade cattle, which were infected by the intranasal route with a virulent strain of virus recently isolated in East Africa (RGK/1). These animals were killed or died at intervals of 1–16 days after infection and a number of tissues from each of them (usually 21) was titrated for virus infectivity in monolayer cultures of primary bovine kidney cells.

Temperature reactions were first detected on the 3rd to 5th days (mean 4·1), mouth lesions on the 6th to 9th and diarrhoea on the 8th or 9th days, following infection. The course of the disease was divided into four phases, viz. incubation (days 1–4), prodromal (days 5–7), mucosal (days 8–12) and early convalescence (days 13–16). Virus proliferation in different tissues was related to these clinical phases, detailed results being presented in tabular and graphical form.

No primary multiplication was detected in the nasal mucosa but virus was demonstrable within 48 hr. in its associated lymph nodes. Low-level viraemia began on the 2nd or 3rd days after infection and generalization had occurred by the end of the incubation period. The virus had established itself at this time throughout the alimentary tract and, sometimes, in the lungs.

The prodromal phase was characterized by plateaux of high virus titres in the lymphopoietic and lympho-epithelial tissues; there was a descending gradient from the cephalic nodes to the superficial lymph and haemolymph nodes of the body, to the visceral lymph nodes and spleen. There was a similar gradient in the titre of virus in the mucosae of the gastro-intestinal tract—from caecum to colon, to ileum and pylorus. Virus first appeared in the turbinate mucosa on the 5th day, post-infection and lung titres were high towards the end of this period. Some virus proliferation may have occurred in the liver, but none was certainly demonstrable in the kidney, myocardium or brain.

The mucosal phase began with continuing high titres of virus in all the major sites of proliferation, but a decline set in from the 9th day onwards. This at first

involved the cephalic lymph nodes but soon extended to the spleen and other lymphopoietic tissues. It was most delayed in lympho-epithelial structures such as the tonsil, lung and gastro-intestinal mucosae. It was suggested that the decline in virus titres was due to the destruction of susceptible cells, accompanied by the local production and later circulation of antibody. Neutralizing antibody was present in the serum on and after the 9th day.

During the early convalescent period virus had disappeared from four animals, with the exception of one recovery from the lung tissue. Antibody titres were high during this time.

These results were discussed with reference to previously existing information on the distribution of rinderpest virus in infected cattle. An attempt was made to correlate them with published information on the pathogenesis of human measles and canine distemper. The data were also used to explain some previously reported observations on the excretion of rinderpest virus by experimentally infected cattle.

This study would not have been possible without the enthusiastic co-operation of Messrs C. S. Rampton, A.I.M.L.T., R. F. Staple, A.I.M.L.T., and R. Pillinger, F.I.M.L.T., in organizing and carrying out a very considerable number of laborious titrations. Dr B. Liess helped with some of the animals killed at earlier stages of the infection. Our junior African staff gave invaluable assistance with their careful and efficient handling of many thousands of tissue cultures; amongst them we should particularly like to mention Mr Francis Ngugi and Mr Harrison Agili.

This paper is published with the permission of the Director, E.A.V.R.O., Mr H. R. Binns, C.M.G., O.B.E.

REFERENCES

BEDSON, H. S. & DUCKWORTH, M. J. (1963). Rabbit pox: an experimental study of the pathways of infection in rabbits. *J. Path. Bact.* **85**, 1–20.

BERGEON, P. (1952). Peste bovine. Richesse en virus pestique des tissus nerveux et de la moelle osseuse des veaux atteints de peste bovine expérimentale. *Bull. Soc. Path. exot.* **45**, 148–52.

COFFIN, D. L. & LIU, C. (1957). Studies on canine distemper by means of fluorescein-labelled antibody. II. The pathology and diagnosis of the naturally-occurring disease in dogs and the antigenic nature of the inclusion body. *Virology*, **3**, 132–45.

CROOK, E., GORHAM, J. R. & MCNUTT, S. H. (1958). Experimental distemper in mink and ferrets. I. Pathogenesis. *Amer. J. vet. Res.* **19**, 955–7.

CURASSON, G. (1932). *La peste bovine.* Paris: Vigot Frères.

DOWNIE, A. W. (1963). Pathogenesis of generalised virus diseases. *Vet. Rec.* **75**, 1125–33.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167–83.

FENNER, F. (1948*a*). The clinical features and pathogenesis of mousepox (infectious ectromelia of mice). *J. Path. Bact.* **60**, 529–52.

FENNER, F. (1948*b*). The pathogenesis of the acute exanthems. An interpretation based on experimental investigations with mousepox (infectious ectromelia of mice). *Lancet*, ii, 915–20.

FENNER, F. & WOODROOFE, G. M. (1953). The pathogenesis of infectious myxomatosis. The mechanism of infection and the immunological response in the European rabbit (*Oryctolagus cuniculus*). *Brit. J. exp. Path.* **34**, 400–11.

GORHAM, J. R. (1960). Canine distemper (la maladie de Carré). *Adv. vet. Sci.* **6**, 287–351.

GRIST, N. R. (1950). The pathogenesis of measles. Review of the literature and discussion of the problem. *Glasgow med. J.* **31**, 431–41.

HAHON, N. & WILSON, B. J. (1960). Pathogenesis of variola in *Macaca irus* monkeys. *Amer. J. Hyg.* **71**, 69–80.

JACOTOT, H. (1931). Sur la teneur en virus de quelques tissus des veaux atteints de peste bovine expérimentale. *Bull. Soc. Path. exot.* **24**, 21–6.

LIESS, B. & PLOWRIGHT, W. (1964). Studies on the pathogenesis of rinderpest in experimental cattle. I. Correlation of clinical signs, viraemia and virus excretion by various routes. *J. Hyg., Camb.*, **62**, 81–100.

LIU, C. & COFFIN, D. L. (1957). Studies on canine distemper infection by means of fluorescein-labelled antibody. I. The pathogenesis, pathology and diagnosis of the disease in experimentally infected ferrets. *Virology*, **3**, 115–31.

MACOWAN, K. D. S. (1956). *Annu. Rep. Dep. vet. Serv., Kenya* (1955). Nairobi: Govt. Printer.

MORNET, P. & GILBERT, Y. (1958). Les méthodes actuelles de lutte contre la peste bovine. *Cah. méd. vét.* **27**, 1–52.

PLOWRIGHT, W. (1962). The application of monolayer tissue culture techniques in rinderpest research. I. Introduction. Use in serological investigations and diagnosis. *Bull. Off. int. Epiz.* **57**, 1–23.

PLOWRIGHT, W. (1964). The growth of virulent and attenuated strains of rinderpest virus in primary calf kidney cells. *Arch. ges. Virusforsch.* (in the Press).

PLOWRIGHT, W. & FERRIS, R. D. (1959). Studies with rinderpest virus in tissue culture. I. Growth and cytopathogenicity. *J. comp. Path.* **69**, 152–72.

PLOWRIGHT, W. & FERRIS, R. D. (1961). Studies with rinderpest virus in tissue culture. III. The stability of cultured virus and its use in virus neutralization tests. *Arch. ges. Virusforsch.* **11**, 516–33.

PLOWRIGHT, W. & FERRIS, R. D. (1962). Studies with rinderpest virus in tissue culture. A technique for the detection and titration of virulent virus in cattle tissues. *Res. vet. Sci.* **3**, 94–103.

PROVOST, A. (1960). *Virus bovipestique et viandes de boucherie. Documents sur la persistance des virus de certaines maladies animales dans la viande et produits animaux.* Paris: joint publication by the Office International des Epizooties (O.I.E.) and the Interafrican Bureau of Animal Health (I.B.A.H.).

ROBBINS, F. C. (1962). Measles: clinical features. Pathogenesis, pathology and complications. *Amer. J. Dis. Child.* **103**, 266–73.

SERGIEV, P. G., RYAZANTSEVA, N. E. & SHROIT, I. G. (1960). The dynamics of pathological processes in experimental measles in monkeys. *Acta virol., Prague*, **4**, 265. Quoted by Robbins, 1962.

SISSON, S. & GROSSMAN, J. D. (1940). *The Anatomy of the Domestic Animals.* Philadelphia and London: W. B. Saunders Co.

THOMPSON, W. R. (1947). Use of moving averages and interpolation to estimate median effective dose. I. Fundamental formulas, estimation of error and relation to other methods. *Bact. Rev.* **11**, 115–45.

TRAUTMAN, A. & FIEBIGER, J. (1952). *Fundamentals of the Histology of Domestic Animals.* 1st English edition. London: Ballière, Tindall and Cox.

WALKER, R. V. L., GRIFFITHS, H. J., SHOPE, R. E., MAURER, F. D. & JENKINS, D. L. (1946). Rinderpest. III. Immunization experiments with inactivated bovine tissue vaccines. *Amer. J. vet. Res.* **7**, 145–51.