EXPERIMENTS WITH THE CYPRUS STRAIN OF BLUE-TONGUE VIRUS: MULTIPLICATION IN THE CENTRAL NERVOUS SYSTEM OF MICE AND COMPLEMENT FIXATION

By M. van den Ende, Anne Linder and V. R. Kaschula

The C.S.I.R. and U.C.T. Virus Research Unit, Department of Pathology, University of Cape Town; and the Veterinary Research Laboratories, Onderstepoort

INTRODUCTION

The virus of blue-tongue has been adapted to eggs by Alexander, Haig & Adelaar (1947). These authors made the important observation that the temperature of incubation has a profound influence on the virus content of blue-tongue infected eggs. Alexander (1947), furthermore, observed certain irregularities in titrations which he suggested might be explained as an interference phenomenon.

Blue-tongue virus has since been successfully adapted to the brains of suckling mice, but adult mice are apparently resistant to this virus (Kaschula, 1953).

During experiments on the physical properties of the Cyprus strain of blue-tongue virus we were once more struck by the influence of temperature of incubation on virus titre. We also observed that the virus would persist in the brains of adult mice for many generations without eliciting symptoms.

We therefore undertook further studies of this virus, particularly on quantitative aspects of its multiplication and to attempt to elucidate the reasons for the differences in titre resulting from different temperatures of incubation.

It appeared possible, for instance, that at higher incubation temperatures in eggs and during multiplication in adult mouse brains as distinct from suckling mouse brains, virus multiplication may produce incomplete virus which under appropriate conditions might interfere with the formation of infective virus.

MATERIAL AND METHODS

The Cyprus strain of blue-tongue virus, adapted to eggs by Alexander, was used for most of the experiments. When these experiments were started it had already received 126 passages in eggs and was completely attenuated for sheep.

The Mimosa Park virus used was not egg adapted, but was transferred directly from sheep to suckling mice. When these experiments were made it had received thirty-one intracerebral passages in suckling mice.

Adaptation to baby mice

Suckling mice aged 1–10 days received intracerebral inocula of 0·02 ml. of a 10% emulsion of blue-tongue-infected chick embryo or suckling mouse brains and cords. During early passages two inocula, each of 0·02 ml. separated by an

https://doi.org/10.1017/S0022172400027364 Published online by Cambridge University Press

interval of 2–6 hr., were usually given, one into each cerebral hemisphere. The mice were killed for collection of the central nervous system within 24 hr. of developing symptoms.

Adaptation to 'adult' mice (3–4 weeks old)

For the first passage a 10% emulsion of the brains and cords of intracerebrally infected suckling mice was used. Mice aged 3–4 weeks showed no symptoms after inoculation of blue-tongue virus. For passage, mice were killed 4–7 days after inoculation, and a 10% emulsion of their brains and cords in broth was used as inoculum.

Titration in eggs

White leghorn eggs, pre-incubated for 8–9 days at 37·5° C., were used. Serial tenfold dilutions of the inocula were prepared in broth containing 100 u. of penicillin and 100 γ of streptomycin per ml. 0·5 ml. of each dilution was injected into the yolk sacs of eggs in groups of 4–6. These were further incubated at 35° C. for 18–24 hr., and then transferred to an incubator at 33–34° C. All eggs were candled daily and dead embryos examined before discarding. Eggs were incubated at the lower temperatures in ordinary bacteriological incubators without forced draught. Temperatures were checked daily; they unavoidably fluctuated as much as 1° C. Since comparative titrations were made at the same time, eggs in all such titrations were subjected to identical conditions.

Titrations in suckling mice

Serial tenfold dilutions were injected intracerebrally in 0·02 ml. doses into litters of suckling mice; litters of six or more were halved, one-half receiving one dilution and the remainder another. In a litter of five or less all mice received the same inoculum. We felt justified in this practice because we obtained no evidence of variations in litter susceptibility. The mice were examined daily for symptoms. The times of onset of illness and deaths were recorded.

Fifty per cent mortality end-points were determined by the method of Reed & Muench (1938).

Determination of multiplication rate in suckling and adult mice

Each of a group of mice of uniform age, either 3 to 4-day-old sucklings or 3 to 4-week-old 'adults', received an intracerebral inoculum of a 10% suspension of blue-tongue-infected brain and cord. Two or three mice were killed immediately after inoculation, and others at intervals thereafter. The brains of each group were removed immediately, a 10% emulsion prepared in broth and the virus content determined by titration in eggs.

Complement-fixation tests

Antigens for complement fixation were prepared from the brains and cords of blue-tongue-infected baby mice killed within 24 hr. of developing symptoms after intracerebral inoculation of virus. The pooled brains and cords from 30 to 100 animals were used for each batch of antigen. The antigen was prepared by acetone

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and ether extraction according to the method of Casals (1949). Similar antigens were also prepared from blue-tongue-infected adult mouse brains and from chick embryos infected with egg-adapted blue-tongue virus.

Crude saline extracts and saline extracts clarified by simple centrifugation as well as saline emulsions extracted with an equal volume of ether were also tested in complement-fixation tests.

Antisera were obtained from mice which had received three or more injections each of 0·5 ml. of a 10 % emulsion of blue-tongue-infected suckling mouse brain and cord in saline. They were bled from the heart 12–14 days after the last inoculation, and the serum was separated from the clot. Complement was also fixed with the sera from mice which had received a single intranasal inoculum of 0·1 ml. of fresh blue-tongue virus in 10 % suckling or 'adult' brain or in 10 % chick embryo emulsion.

The sera were inactivated at 56° C. for 50 min., and stored at − 20° C.

Complement-fixation tests were carried out by the method of Casals & Olitsky (1950).

RESULTS

Adaptation to suckling mice

In our first attempt at adaptation of blue-tongue virus from eggs, suckling mice aged 6 days were used, and in the first passage single intracerebral and intraperitoneal injections were given. None of the animals which survived for more than one day became ill. The passage to 4-day-old mice of pooled brain, cord and spleen from apparently healthy animals killed on the 11th day after inoculation resulted in illness or death of 90 % of the animals. Adaptation to sucklings thereafter was rapid, and by the fifth passage 90 % or more of the inoculated animals were sick on the 3rd day after inoculation and dead by the 5th day. It was soon apparent that the virus was present in large amounts in the central nervous system of passage mice, but that the virus content of the spleen was negligible. For further passages therefore only brain and cord were used.

In a subsequent adaptation with egg virus as starting material younger sucklings were used from the start, and for the earlier passages two intracerebral inocula, separated by an interval of 2–6 hr., were given to each animal. Even the first passage animals became ill on the 4th or 5th day, and all were dead on the 8th day after inoculation.

The most regular results were obtained with sucklings aged 2–4 days. In these younger mice, after nine or more passages, a single intracerebral injection of blue-tongue virus regularly produced illness within 2–3 days. Not infrequently mice died during the 3rd night after inoculation, and often the dead mice were partly or completely eaten before the next morning. When therefore large numbers of sucklings were inoculated for the preparation of antigen for complement-fixation tests, the mice were separated from their mothers on the 3rd evening after inoculation. They were killed in ether vapour the next morning and their brains and cords collected.

Mice older than 4 days but less than 13 days were found susceptible to intra-

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cerebral injection of suckling-adapted blue-tongue virus. In older mice, however, the latent interval following injection was longer and the mortality lower. Occasional mice of the older age groups survived the injection. We did not succeed in producing fatal infection in mice older than 12 days, even with virus repeatedly passaged in 10 to 12-day-old ('adolescent') mice.

Adaptation to the brains of 3 *to* 4-*week-old* (*'adult'*) *mice*

Although the intracerebral injection of fresh blue-tongue-infected suckling mouse-brain emulsion into adult mice gave rise to no illness, there was evidence of limited multiplication of the virus and persistence of virus for at least fourteen serial passages. The titre of virus as present in the adult mouse brain at the time of harvesting in routine passages never exceeded 10^4 LD_{50} per gram when titrated in sucklings. When titrated in eggs very irregular results were obtained so that the determination of the LD_{50} was often impossible. The titres were, however, consistently lower than in sucklings and usually lower than 10^2 LD_{50} per gram.

The results of titrations at various stages in the adaptation to suckling and adult brains are recorded in Table 1. These results show that the virus content of mouse brains, as determined by titration in suckling mice, appeared consistently higher than when the same emulsions were titrated in eggs. There did not, however, appear to be a significant reduction in the infectivity for eggs of the suckling mouse-adapted virus during twenty-nine passages.

Table 1. *Virus content of suckling and adult mouse brains, as determined by titrations in eggs and suckling mice*

Suckling C.N.S.	Titre in eggs	Titre in sucklings
Passage 5	5·8*	—
7	6·9	—
9	8·1	—
14	—	6·7
16	Brain 7·9	—
	Cord 5·2	—
29	5·8	8·0
7	6·4	7·3
	± 7·3	8·4
Adult brain		
Passage 4	1·6	3·7
12	—	3·7
13	2·0	—

* Log LD_{50} per gram of brain or C.N.S.

The rate of multiplication of blue-tongue virus in the brains of mice

Owing to short supply it was not possible to carry out many titrations in suckling mice. Reliance had therefore to be placed almost entirely on titrations of the virus in eggs.

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Alexander has already emphasized the difficulties encountered in quantitative experiments with blue-tongue virus in eggs. The irregularities in titrations such as Alexander encountered in work with egg-adapted 'Becker' virus appeared to be exaggerated in our experiments with the 'Cyprus' strain when mouse-adapted virus was titrated in eggs. The results of two typical experiments recorded in detail in Tables 2 and 3 exemplify the type of irregularities encountered, and which make

Table 2. *Multiplication of blue-tongue virus (Cyprus) in the brains of 2 to 3-day-old suckling mice*

Interval after inoculation of mice (hr.)	Titration in eggs							Log LD_{50} per gram brain
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Zero	5/5*	5/5	3/5	1/5	2/5	—	—	4·9
3	5/5	4/5	1/5	1/5	1/5	—	—	4·6
6	5/5	4/5	2/5	2/5	0/5	—	—	4·3
12	—	4/5	3/5	3/5	1/5	1/5	—	5·3
24	—	3/4	4/4	1/4	0/4	0/4	—	4·8
36	—	4/4	5/5	4/5	4/5	1/5	—	6·7
48	—	—	5/5	5/5	3/5	4/5	1/5	7·7
55	—	—	4/4	4/5	2/5	0/5	2/5	6·5
60	—	—	4/5	3/5	4/5	0/5	2/5	6·6
72	—	—	—	4/5	3/5	1/5	2/5	6·8
Original inoculum†	—	—	—	5/5	5/5	3/5	2/5	7·6

* Numerator, number of eggs dying in 7 days, denominator, number of eggs inoculated.
† Original inoculum = 10 % (10^{-1}) suspension of brains and cords of 11th passage sucklings. Dose per egg 0·5 ml.

Table 3. *Multiplication of blue-tongue virus in the brains of 3 to 4-week-old mice*

Interval after inoculation of mice (hr.)	Titrations in eggs									Log LD_{50} per gram brain
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
¼	4/4	1/4	0/4	—	—	—	—	—	—	2·0
2¼	2/4	1/4	2/4	0/4	—	—	—	—	—	1·9
4½	4/4	3/4	0/4	0/4	—	—	—	—	—	2·6
6	4/4	2/4	1/4	0/4	—	—	—	—	—	2·5
12	4/4	4/4	2/4	0/4	2/4	—	—	—	—	3·7
21	—	4/4	4/4	2/4	0/4	0/4	—	—	—	4·3
29	—	3/4	4/4	2/4	0/4	0/4	—	—	—	4·3
45	—	1/4	1/4	3/4	2/4	1/4	—	—	—	3·8
Original inoculum	—	—	—	—	4/4	4/4	4/4	1/4	1/4	8·1

Original inoculum is 1 % (10^{-2}) suspension of brains of 18th passage sucklings.

the interpretation of results extremely difficult. The results are those of experiments in which suckling or adult (3 to 4-week-old) mice received a single intracerebral injection of an emulsion of blue tongue-infected suckling mouse brains.

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Immediately after inoculation and at intervals thereafter three mice were killed, their brains collected, emulsified in broth, and the virus content titrated in eggs.

The experiments on the multiplication of suckling mouse-adapted virus in the brains of adult mice have been repeated at the Onderstepoort Laboratories using both Cyprus and Mimosa Park strains of blue-tongue virus. Titrations were carried out in eggs and in sucklings. A typical result obtained with the Mimosa Park strain recorded in Table 4 confirms the findings with the Cyprus virus.

Table 4. *Multiplication of blue-tongue virus (Mimosa Park) in the brains of 8-week-old mice*

Interval after inoculation of mice (hr.)	Titration in eggs						Log LD_{50} per gram brain
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
3	1/4	2/5	1/5	0/5	0/6	0/5	1·0
6	1/5	1/5	1/6	0/5	0/6	0/5	1·0
12	1/6	2/5	0/5	0/5	0/5	0/5	1·0
24	5/6	2/6	3/6	1/6	0/6	0/6	2·3
32	6/6	5/6	0/6	2/6	1/6	0/6	2·8
48	6/6	4/5	3/6	3/6	0/4	0/5	3·3
72	6/6	4/6	3/6	0/5	0/6	0/6	2·6
Original inoculum	6/6	6/6	4/6	2/5	0/6	0/6	3·4

Original inoculum is 10% (10^{-1}) suspension of brains of 31st passage sucklings.

The results show that there is definite multiplication of the virus in the brains of 3 to 4-week-old mice. Such 'adult' mice have, however, on no occasion shown any signs of illness.

Both in sucklings and adults the first evidence of multiplication was found after approximately 8–12 hr, and this would appear to be the time taken for a single multiplication cycle of this virus in mouse brain. We have not so far been able to establish the duration of a single cycle in eggs.

Attempts were made to demonstrate multiplication of blue-tongue virus in the chorio-allantoic membrane of chick embryos.

Eggs were inoculated into the allantoic cavity through a window according to a technique described to us by Dr J. Cairns. The inoculum consisted of a 1–10% emulsion of blue-tongue-infected chick embryo in saline containing penicillin and streptomycin. The window was covered with a glass cover-slip and wax. Through the same window samples of allantoic fluid were withdrawn at intervals and titrated in eggs. All manipulations were carried out in an incubator room at a temperature of approximately 36° C. Under the conditions of our experiments we have obtained no evidence of virus multiplication in the chorio-allantoic membranes. Technical difficulties and imperfect facilities have made it impossible to pursue this aspect of the work further at present.

The most striking feature of the experiments in mice, apart from the inability of the virus to elicit symptoms in older mice, is the fact that with brains removed 24 hr. or longer after inoculation irregular titration results are obtained, thus with less dilute inocula there are frequently fewer deaths in the titration eggs than with higher dilutions. This phenomenon, previously observed by Alexander, has been

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referred to by him as a hypothetical interference phenomenon. It is our impression that this type of irregular titration was more frequently obtained with virus propagated in adult mouse brains.

Attempts to demonstrate interference by virus in adult mouse brain and eggs incubated at 38° C

It was considered possible that, particularly in adult mouse brains and in eggs incubated at 38° C., there may be 'incomplete' virus, capable in high concentration of interfering with complete or infective virus.

Eggs which had been incubated for 7 or 8 days received primary inocula of chick embryo virus in various dilutions into the yolk sacs. Control eggs received equivalent volumes of diluent or no inoculum at all. They were then returned to an incubator at 38·5° C. and 1 or 2 days later they received challenge inocula into the yolk sac of fresh blue-tongue virus emulsion. After the challenge inoculum they were placed in an incubator at 33·5–34° C., and thereafter candled daily.

In some experiments emulsions of blue-tongue virus in adult or suckling mouse brains were used as primary inocula and egg virus was used for challenge.

Table 5. *The effect of preliminary incubation at higher temperature on susceptibility of eggs to subsequent infection*

Primary inoculum and temperature of incubation first 2 days	Challenge inoculum and temperature of incubation subsequent days	Dilution of virus in primary inoculum			
		10^{-1}	10^{-3}	10^{-5}	Nil
Virus, 38·5° C.	Nil, 38·5° C.	0/4	1/5	0/5	—
Virus, 38·5° C.	Nil, 34° C.	5/5	6/6	0/5	—
Virus, 38·5° C.	10^{-4} egg, 34° C.	5/5	5/6	5/6	—
Nil, 38·5° C.	10^{-4} egg, 34° C.	—	—	—	7/10

Titre of virus for primary inoculum $10^{5\cdot5}$ at 34° C. Titre of virus for challenge inoculum $10^{5\cdot1}$ at 34° C.

The results of a typical experiment are recorded in Table 5. The results show that not only has there been no demonstrable interference by the preliminary incubation at 38·5° C., but the virus of the primary inoculum has persisted to cause death of the embryos when the incubation was continued at 34° C., even when no additional virus was injected.

We have similarly failed to demonstrate interference with egg virus by preliminary injection of virus in adult or suckling mouse brain.

Complement-fixation tests

It was considered possible that more blue-tongue virus is in fact formed in eggs incubated at 38·5° C. than would appear from titrations of infectivity, but that the virus formed at the higher temperature is not infective for eggs, and does not interfere with the multiplication of infective virus at 34° C. Such virus might still be identifiable serologically and complement-fixation tests were therefore carried out in attempts to detect virus antigen in blue-tongue-infected eggs incubated at various temperatures.

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The serum used for the tests was obtained from adult mice which had receive four or more intraperitoneal injections of emulsions of blue-tongue-infected sucklin mouse brains in saline. The mice were bled from the heart 10–14 days after the las injection, the serum was separated and stored at – 20° C.

The serum which was employed for most experiments was capable of fixin complement in the presence of antigen prepared by the method of Casals from blue tongue-infected suckling mouse brains (Table 6). Such acetone and ether-extracte

Table 6. *Complement fixation with mouse immune serum and antigen prepared by Casals's method from blue-tongue-infected suckling mouse brains*

	Serum dilutions									
Antigen	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Nil
1/2	4	4	4	4	4	4	4	0	0	0
1/4	4	4	4	4	4	4	4	0	0	0
1/8	4	4	4	4	4	4	3	0	0	0
1/16	4	4	4	4	4	4	3	0	0	0
1/32	4	4	4	4	4	3	0	0	0	0
1/64	4	4	4	4	2	tr.	0	0	0	0
1/128	1	4	4	2	0	0	0	0	0	0
1/256	4	4	2	0	0	0	0	0	0	0
Nil	0	0	0	0	0	0	0	0	0	0
MEF*	tr.	0	0	0	0	0	—	—	—	—

4 = complete fixation, 0 = no fixation (complete haemolysis).
* Antigen prepared from brains of suckling mice infected with M.E.F. strain of poliomyelitis virus used as control.

antigens have given consistent results in complement-fixation tests. Positive results have, however, also been obtained with crude saline extracts of blue-tongue-infected suckling mouse brains, or with crude saline emulsions extracted with ether. These results, recorded in Table 7, show further that complement-fixing antigen is present in the supernatant fluid of extracts after centrifugation at speeds known to cause sedimentation of the blue-tongue virus. This 'soluble' antigen is apparently as effective as the virus itself in complement-fixation tests.

With such a mouse immune serum, complement-fixation tests were carried out with antigens prepared from eggs infected with blue-tongue virus and then incubated at 38·5° or 34° C. Five of ten blue-tongue-infected eggs incubated at 34° C. died on the 3rd day. The pooled embryos of the five dead eggs were used for titration and preparation of complement-fixing antigen. Two of ten similarly infected eggs incubated at 38·5° C. died on the 3rd day. Embryos from the two dead eggs and three living ones were pooled for titration and preparation of complement-fixing antigen. The titrations of the virus content were carried out in eggs incubated at 34° C. Antigens were prepared from the two groups of embryos by acetone and ether extraction. The results recorded in Table 8 show that eggs incubated at 38·5° C. contained insufficient complement-fixing antigen to be detectable, whereas good complement fixation was obtained with antigen from eggs incubated at 34° C. The titres of infectivity for eggs (log LD_{50}) of the suspensions from which the antigens were prepared, were 1·8 and 5·4 respectively.

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Table 7. *Complement-fixation tests with mouse immune serum and extracts of blue-tongue-infected suckling mouse brains*

Antigen	Antigen dilutions 1/2	1/4	1/8	1/16	1/32	1/64	Serum control	Saline control
10% crude saline extract.	4	4	4	3	0	0	0	0
10% crude saline extract, control without serum.	1	1	tr.	tr.	1	0	—	—
Crude emulsion, ether extracted.	4	4	4	0	0	0	0	—
Crude emulsion, ether extracted, control without serum.	tr.	0	0	0	0	0	—	—
Ether extracted, spun 30,000, deposit.	4	4	3	0	0	0	0	—
Ether extracted, spun 30,000, deposit. Control without serum.	tr.	0	0	1	tr.	0	—	—
Ether extracted, spun 30,000, supernatant fluid.	4	4	4	0	0	0	0	—
Ether extracted, spun 30,000 S.N.F., control without serum.	0	0	0	0	0	0	—	—
Control mouse brain antigen (acetone and ether extracted).	4	4	4	4	4	1	0	—
Control 10% crude saline extract normal brains (6 to 7-day mice).	tr.	0	0	0	0	0	—	—

Dilution of serum used 1/20, 4= complete fixation.

Table 8. *Complement-fixation tests with mouse immune serum and antigens prepared by acetone and ether extraction method*

Antigens from	Serum dilutions 1/2	1/4	1/8	1/16	1/32	1/64	Nil
34° C. egg	4	4	4	3	1	0	0
38·5° C. egg	3	0	0	0	0	0	0
Normal egg	2	0	0	0	0	0	0
Mouse brain antigen	4	4	4	4	4	4	0
Nil	0	0	0	0	0	0	0

We have also attempted to demonstrate by complement fixation the presence of specific antigen in brains of adult mice infected with blue-tongue virus. We have failed, however, to obtain significant fixation in excess of that which occurs with similarly prepared 'antigens' from normal adult mouse brains.

DISCUSSION

Whilst our results do not allow us to offer an explanation of the apparent insusceptibility of adult mice to blue-tongue virus, they do show that this insusceptibility does not depend on an inability of the virus to multiply in adult mouse brains. In this respect our results are similar to those reported by Cairns (1951) on the multiplication of influenza viruses in the brains of mice.

We are also unable to explain why blue-tongue virus multiplies more readily in eggs incubated at 34° C. than in eggs at 38° C. Titration results suggest the occurrence of interference and therefore the presence, under certain as yet not

https://doi.org/10.1017/S0022172400027364 Published online by Cambridge University Press

recognized conditions, of incomplete or non-infective virus. We have been unable to demonstrate, with the means at our disposal, the formation of such incomplete virus. There certainly seems to be no more serologically identifiable antigen, even in eggs incubated at 38° C., than can be expected from the amount of infective virus present.

The extreme susceptibility of blue-tongue virus to temperature of incubation, and its behaviour in mice make it an important one for studies on the multiplication of animal viruses. For this reason our results are published. They also draw attention to the complement-fixation test, particularly with antigens prepared from suckling mouse brains, and to the multiplication of virus in adult mouse brains. These observations may be of practical significance in the diagnosis of the disease, and in the preparation of vaccines.

SUMMARY

The multiplication rate of blue-tongue virus in suckling and adult mouse brains has been determined. The first cycle of virus multiplication appears to take 8–12 hr. both in fully susceptible sucklings and in adults which suffer an inapparent infection with this virus.

Antigens suitable for complement-fixation tests have been made from suckling mouse brains and from eggs infected with blue-tongue virus.

A potent immune serum has been obtained from mice immunized by repeated intraperitoneal injections of blue-tongue virus in suckling mouse brain.

Attempts, with negative results, have been made to demonstrate interference with virus multiplication by virus from eggs incubated at 38·5° C. and by virus from adult mouse brains.

We are grateful to Dr R. Alexander for his helpful and critical interest in this work and to Mr G. S. Turner and Dr G. Selzer for their able and willing assistance.

The Virus Research Unit is financed by the South African Council for Scientific and Industrial Research and by grants from the Nkana-Kitwe and Chingola Poliomyelitis Research Funds.

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(*MS. received for publication* 16. XI. 53)

https://doi.org/10.1017/S0022172400027364 Published online by Cambridge University Press