RIFT VALLEY FEVER VIRUS IN MICE. IV. INCOMPLETE VIRUS; ITS PRODUCTION AND PROPERTIES

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EARLY in these studies it was discovered that mice died with different blood titres. In routine passages 10^{-1} dilutions of infective serum were inoculated, and although occasionally a serum pool was obtained which had a titre of $10^{8\cdot5}$ LD_{50}/0·03 ml. or greater, titres of $10^{7\cdot0}$–$10^{8\cdot0}$ were more common, and levels as low as $10^{6\cdot0}$ were encountered. Stock sera whose titre was found to be $10^{7\cdot0}$ or greater were selected for use in experiments, but mice inoculated with these sera in concentrated form might die with low or with high blood titres. As a result titration end-points were sometimes missed in growth curve experiments, because peak titres were unpredictable. This difficulty persisted during much of the work described in previous papers.

On one occasion a pooled blood titre of $10^{8\cdot8}$ occurred in mice sick after an inoculum of stock infective serum which had been diluted 10^{-4}, instead of 10^{-1} as was usual. It was thought possible that this uncommonly high titre occurred because of the relatively small amount of virus contained in the 10^{-4} diluted inoculum. It is known that when influenza virus grows in the allantoic cavity, the final infective titre depends on the amount of virus inoculated. Von Magnus (1951*a*) showed that when undiluted allantoic fluid was serially passaged, peak infective titres fell, whereas they were regularly high during serial passages of 10^{-6} diluted material. Peak infective titres were shown to be low because so-called "incomplete" virus had been produced. It was these and subsequent studies on "incomplete" influenza virus which prompted the investigations described in this paper.

MATERIALS AND METHODS

Virus.—As in Paper I. The standard diluent was pH 7·4 Sørensen-buffered 0·5 per cent bovine plasma albumin.

Mice.—As described in Paper I.

Routine titrations.—As described in Paper I. Organ titres expressed as $\log_{10}$ LD_{50}/0·03 ml. tissue.

Carcass titrations.—As described in Paper II.

Neutralisation tests.—As described in Paper I.

RESULTS

Serial passage of infective serum at various dilutions

It was thought that the serial passage of infective serum at various dilutions would show directly whether peak blood titres were low when there were large amounts of virus in the inoculum. Virus was serially passaged using undiluted,

10^{-1}, 10^{-4}, 10^{-6} and finally 10^{-8} diluted infective serum. At each passage pooled serum from 3–6 sick or recently dead mice was diluted to the appropriate extent and inoculated immediately or after storage up to 24 hr. at −20°. The titre was as a rule determined directly by titration, but it was occasionally very roughly calculated from the observed median death time (see below). Independent passages were sometimes made from a given serum either concurrently or after storage of the serum at −20°. It was shown in a preliminary experiment that the ability of high titre sera to produce high titres when inoculated undiluted and 10^{-2} diluted (see below) was unaffected by storage up to five weeks at −20°.

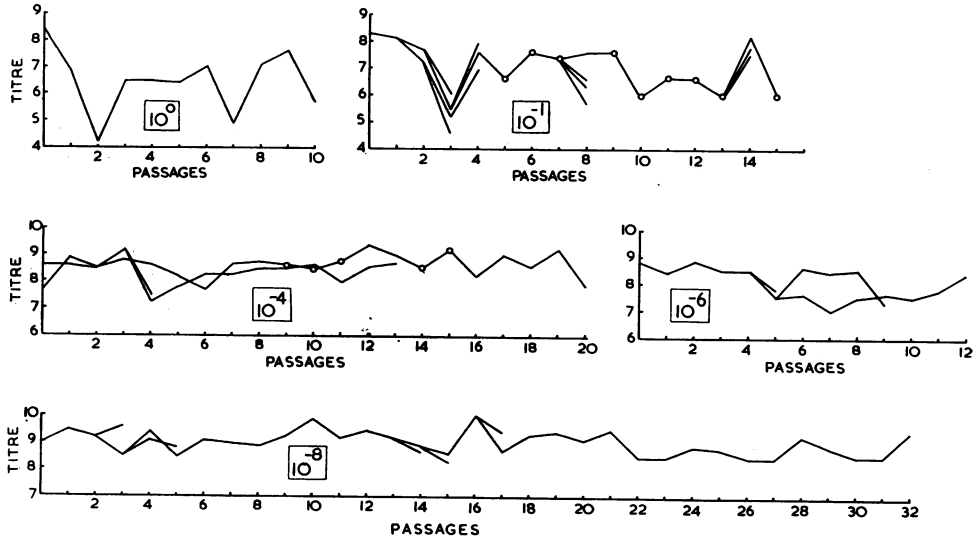

FIG. 1.—Continuous serial passages of R.V.F. virus infective serum at various dilutions. All inoculations were intracerebral. Each point on each passage line is the titre of pooled serum from 3–5 sick or dead mice which had been inoculated with the appropriate dilution of the preceding serum in the passage series. The serum titre was usually determined directly by titration, although at the points indicated by circles it was roughly calculated from the death-time in inoculated mice. The titre of passage No. 0 is in each case the titre of the serum used to start the series. Distinct passage lines, which sometimes originate from the same infective serum, are shown as distinct lines. Titres in $\log_{10}$ $LD_{50}/0{\cdot}03$ ml. serum. All titres shown on the same scale.

Results are shown graphically in Fig. 1. It can be seen that in the undiluted and 10^{-1} diluted serial passages titres fluctuate, and fall as low as $10^{4{\cdot}2}$, with recoveries which rarely reach the initial levels. Sera passaged at 10^{-4} and 10^{-6} in general maintained high titres, but even here there were falls to titres of less than $10^{8{\cdot}0}$, which could be produced on more than one occasion from the same serum. In the 10^{-8} passage series, however, titres never fell below $10^{8{\cdot}5}$, although 32 consecutive passages were made.

Often with the concentrated inocula (undiluted and 10^{-1} diluted passage series), a given serum, although itself of high titre, would repeatedly produce low titres in inoculated mice, on separate occasions. Again, a low titre serum might repeatedly produce high titres. There is therefore something about an infective serum which makes it give rise to low or high titres when inoculated in concentrated

form. Even two sera of the same infectivity titre may behave differently, one producing high and one producing low titres at the next passage. Clearly there is something in addition to the mere infectivity titre that plays a part in these phenomena.

Characterisation of sera by titres produced in individual mice.—All titres in the passage series were determined for serum pools, and it was thought that individual mouse titres might vary while the pooled titre, as measured on a log scale, reflected that of the highest contributor. A series of individual serum titrations were therefore made from mice when sick following inoculation with the appropriate dilutions of 10^{-1}, 10^{-4} and 10^{-8} passaged sera. The results (Table I) show that mice given the 10^{-1} serum passaged at 10^{-1} had low but variable titres; the titre of these when pooled would have been $10^{6\cdot4}$. Thus when peak titres following a given inoculum are low they are very variable in individual mice, as measured on a log scale. Since a random sample of these is taken when 3–6 mice are bled, some irregularity in the level of low titres is to be expected. Mice given one of the 10^{-4} sera at 10^{-4} had high titres, but there were low and variable titres with the other 10^{-4} serum tested. All except one of 34 mice given 10^{-8} serum passaged at 10^{-8}, however, had titres greater than $10^{8\cdot0}$, and only 7 of them were lower than $10^{8\cdot5}$. It is concluded, first, that sera with low titre-producing power may occur in the 10^{-4} passage series, and second, that the titres produced following 10^{-8} diluted inoculations of 10^{-8} passaged serum, are high in all individual mice.

TABLE I.—*Peak Blood Virus Titres in Individual Mice after Inoculation with the Appropriate Dilution of* 10^{-1}, 10^{-4}, *and* 10^{-8} *Passaged Sera.*

Inoculum.	Titre of serum inoculated.	Titres in individual mice.											
10^{-1} passaged serum diluted 10^{-1}	7·8	7·1	6·8	6·7	6·5	6·0	5·4	5·4	5·2	5·0	4·8	<4·0	—
10^{-4} passaged serum diluted 10^{-4}	8·8	8·6	8·6	8·6	8·4	8·4	7·8	7·8	7·8	7·8	—	—	—
	9·2	7·8	>7·0	>7·0	>7·0	6·5	6·0	5·6	—	—	—	—	—
10^{-8} passaged serum diluted 10^{-8}	8·6	9·5	9·0	9·0	8·7	8·6	8·5	8·5	8·3	8·2	8·0	—	—
	8·7	9·6	9·1	9·0	9·0	9·0	8·7	8·7	8·6	8·6	8·2	8·1	7·5
	9·5	9·5	9·5	9·1	9·1	9·1	8·7	8·6	8·6	8·5	8·5	8·5	8·4

The titres of the 34 mice inoculated with 10^{-8} diluted serum showed a variation of $10^{2\cdot1}$, or $10^{1\cdot6}$ if the lowest titre is not counted, and it was shown in Paper I that the experimental titration error was not usually greater than $10^{0\cdot5}$. The arithmetical variation in individual titres, therefore, is considerable even when high titre-producing sera are inoculated. The differences between individual titres presumably reflect differences in the ability of individual mice to support virus growth.

Characterisation of sera by the titres produced on inoculation at various dilutions.—It had been found on many occasions that a serum which produced low peak titres when inoculated in concentrated form would produce high titres when considerably diluted. For instance, in the undiluted intracerebral passage series (Fig. 1), two sera of titres $10^{6\cdot8}$ and $10^{4\cdot2}$, which produced as low or lower

titres when passaged undiluted, nevertheless produced titres of $10^{8\cdot5}$ and $10^{8\cdot4}$ when passaged at limiting ($10^{-5\cdot5}$ and $10^{-3\cdot5}$) dilutions.

Rather low titres occurred in the 10^{-4} passage series, and it was thought that any low titre-producing power of these sera might be more clearly demonstrated in the following way. Sera were diluted in serial tenfold steps, and each dilution inoculated intracerebrally into a box of 6 mice. Three to 6 mice from each box were bled when sick or recently dead, and their pooled blood titrated. For comparison, a few sera from the 10^{-8} passage series were dealt with similarly. Results are shown in Table II. It is seen that none of the 10^{-4} passaged sera tested produced high titres with all the dilutions inoculated. Each of the 10^{-8} passaged sera, however, gave high titres, whichever dilution was inoculated.

TABLE II.—*Titres Produced by Sera on Inoculation at Various Dilutions.*

Serum inoculated.	Dilution inoculated.								
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
10^{-4} passaged. Titre $10^{8\cdot5}$	7·1	7·5	5·8	7·6	8·8	7·9	—	—	—
10^{-4} ,, ,, $10^{8\cdot8}$	7·6	7·1	6·4	6·7	7·8	8·2	—	—	—
10^{-4} ,, ,, $10^{7\cdot8}$	8·4	6·8	6·7	8·5	9·0	8·9	—	—	—
10^{-8} ,, ,, $10^{9\cdot2}$	9·4	9·2	8·9	8·8	8·7	9·2	—	—	9·5
10^{-8} ,, ,, $10^{8\cdot9}$	9·6	9·5	8·4	9·0	9·4	9·4	8·2	8·6	8·4

Although two of the 10^{-4} passaged sera tested in this experiment were themselves of high titre ($10^{8\cdot5}$ and $10^{8\cdot8}$), they thus possessed low titre-producing power, as detected in this way. They produced titres as high as $10^{8\cdot5}$ and $10^{8\cdot8}$ when inoculated at 10^{-4} or 10^{-5}, but these were from serum pools, and some of the individual mice may nevertheless have had low titres (see above).

It is worthy of note that mice given 10^{-2} diluted low titre-producing sera had lower titres than those given undiluted sera.

Post-mortem incubation does not account for the low titre-producing power of sera.—In the serial passages mice were often bled when dead, and it was sometimes difficult to judge how recently they had died unless the corpse was still soft and warm. An experiment was therefore performed to see whether blood developed the capacity to produce low titres when it remained in the body for long after death. Blood from the tail of a mouse sick following a 10^{-4} diluted inoculum of high titre serum was obtained, titrated, and then inoculated at 10^{-1} to 6 mice, whose pooled blood was in turn titrated when they were sick. Tenfold diluted blood was inoculated because it was thought that this, the most concentrated inoculum readily obtainable from the sick mouse, would be the most sensitive test of any low titre-producing power. After the mouse had been dead 3 hr. blood was again obtained, titrated, and passaged at 10^{-1} and titrated. In another experiment blood was taken from a mouse sick following a 10^{-8} diluted inoculum of high titre serum, and treated in the same way. The mouse died $2\frac{1}{2}$ hr. later, and 5 hr. and 20 hr. after death blood was again taken and dealt with similarly. The results (Table III) show that prolonged post-mortem incubation at room temperature (22–26°) affects neither the blood titre, nor the ability of the blood to produce low titres.

Carcass titres are low when blood titres are low.—In the serial passage experiments blood or serum was titrated. On several other occasions livers, and once an entire carcass (see Paper II for methods), were titrated in sick mice whose

TABLE III.—*The Effect of Post-mortem Incubation on Blood Titres and on the Capacity of Blood to Produce Low Titres.*

	Blood titre.	Results of inoculation of each blood, tenfold diluted, into four mice.	
		Time at which half these mice were dead.	Titre of pooled blood from the four inoculated mice.
Sick mouse A	8·9	17 hr.	7·4
Mouse A dead 3 hr.	8·7	18 ,,	7·7
Sick mouse B	8·9	18 ,,	9·5
Mouse B dead 5 hr.	8·8	17 ,,	9·2
,, B ,, 20 hr.	8·5	21 ,,	8·7

blood titre was low. Table IV summarises these experiments, and it can be seen that liver titres and the carcass titre were also low. The high titres which occurred in mice sick from a dilute inoculum of high titre serum are shown in the Table for comparison. Thus, low titres are produced in the entire mouse, and not just in the blood, when concentrated inocula are passaged.

Table IV also shows that although the liver titre is low in low-titre-mice, it is often tenfold or even a hundredfold higher than the blood titre. This is not usually the case (see Paper I), and suggests, first, that virus is coming from the liver, and, second, that there may be some delay in its release when blood titres are low.

TABLE IV.—*Organ and Carcass Titres in Individual Mice in which Peak Blood Titres were Low.*

Inoculated blood.		Incubation period (hr.)	Blood titre.	Liver titre.	Carcass virus content.*
$\log_{10}$ titre.	Amount given.				
3·7	0·1 ml. I.V.	96	4·4	6·0†	—
5·9	,,	13½	3·6	4·5	—
5·9	,,	14	<3·0	3·7	—
5·9	,,	14	<3·0	4·2	—
6·1	,,	14	4·3	>5·0	—
6·8	,,	43	4·3	4·5	5·8 (6·0)
6·8	,,	46	3·2	4·1	—
7·2	0·03 ml. I.C. at 10^{-1}	22½	7·0	7·5	—
7·2	0·03 ml. I.C. at 10^{-1}	24½	5·2	7·5	—
8·2	0·03 ml. I.C. at 10^{-4}	33	8·4	8·2	10·0 (9·9)

For comparison pooled titres in two mice with high blood titres are shown at the bottom of the Table.

* Carcass virus content, directly determined, in $\log_{10}$ LD_{50}. Titres per whole mouse blood, calculated from the blood titration, are included in brackets.

† The brain titre in this mouse was found to be $10^{2·0}$.

Dose-incubation period relation of low titre sera

In Paper III of this series, the median death time in mice was shown to be proportional to the logarithm of the dose of virus inoculated. A high titre 10^{-4} passaged serum was used in that experiment, and it was frequently noticed that

low titre sera gave much later death-times when a given infective dose was inoculated. A more detailed study of the incubation periods (as represented by the death-times) of these low titre sera inocula was therefore made.

In these experiments two sera (of titres $10^{3\cdot2}$ and $10^{5\cdot2}$) from the undiluted intravenous passage series (see below), a serum of titre $10^{7\cdot4}$ from a 10^{-1} passage series, and a high titre serum ($10^{8\cdot1}$) from a 10^{-4} passage series, were studied. They were diluted in serial tenfold or $10^{0\cdot5}$-fold steps, and each dilution inoculated into 15–18 mice. Large inocula, of a volume greater than 0·03 ml., were given intravenously to a smaller number of mice. Death times were recorded and the median value calculated (Fig. 2). For the $10^{7\cdot4}$ titre serum the mean instead of

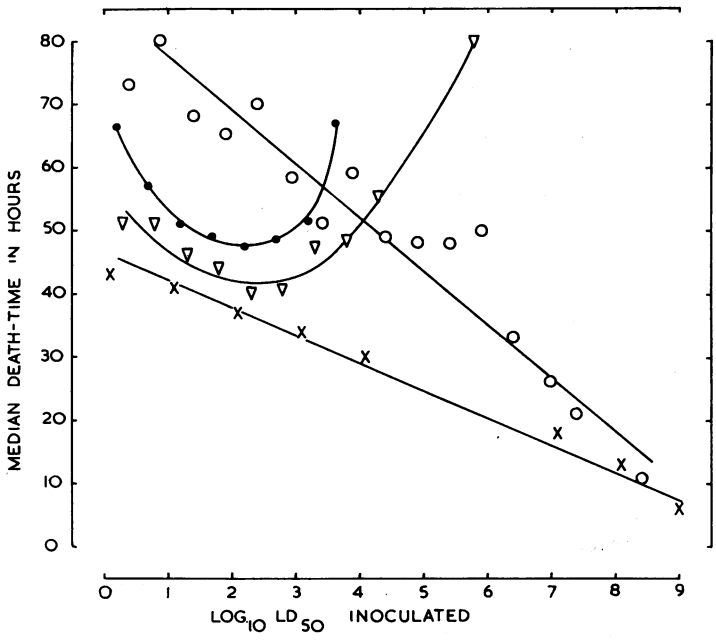

FIG. 2.—Relation between dose and incubation period, for sera of titres $10^{8\cdot1}$ (crosses), $10^{7\cdot4}$ (circles), $10^{5\cdot3}$ (triangles) and $10^{3\cdot2}$ (dots). Each serum dilution was inoculated into 10–15 mice, except in the case of the largest inocula, which were given to smaller numbers. Death-times were recorded, and the median death-time (mean death-time for the serum of titre $10^{7\cdot4}$) calculated.

the median death-time had been calculated and points are irregular, but the relationship is probably a straight line one. Large doses of this serum gave death-times as early as those produced by the high titre serum, but all smaller doses resulted in much later death times. This difference was apparent when as few as 1–10 LD_{50} were inoculated. Moreover, there was a much greater scatter in individual death-times following inoculations of this serum, and even if the occasional unusually late death was not counted, mice receiving a given inoculum died over the course of 20–50 hr. When, however, any dilution of the high titre serum was inoculated, and the occasional late death again not counted, mice were found to die within 10 hr. of one another. This high titre serum, from a

10^{-4} passage series, gave median death-times as short as did any serum encountered in the 10^{-8} passage series. Thus, the most rapidly lethal virus material obtainable was probably present in this serum.

The very low titre sera obtained in the undiluted intravenous passage series gave curves of a different shape. When small infective doses were inoculated these sera also gave a later median death-time as well as a greater scatter in individual death-times than did the same infective dose of the high titre serum. When larger amounts were inoculated, however, death-times were very much delayed. It was after large inocula of one of these low titre sera that mice died with even lower blood titres, others died after 6–12 days with virus in the brain but not in the liver, and a few survived and became immune. To see whether these much prolonged incubation periods could be shortened if enough infective virus was present in the inoculum, 0·1 ml. of one of these low titre sera was given intravenously to mice, together with an equal volume of high titre serum containing about $10^{9\cdot5}$ LD_{50}. Incubation periods were then as short as when the high titre serum was administered alone. Thus, although large doses of low titre sera may give prolonged incubation periods, the effect can be overcome if enough infective virus is inoculated. Perhaps this is why the dose-incubation period curve for the 10^{-1} passaged serum (Fig. 2) approaches the high titre serum curve as the infective dose becomes very large.

Demonstration of non-infective interfering agent in low titre-producing sera.

The results of the serial passage experiments show that when large inocula of low titre-producing serum are inoculated, something, presumably in the inoculated serum, prevents high peak titres being reached. The dose-incubation period experiments indicate that something in such serum also prolongs the incubation period. These effects were directly demonstrated in the following growth curve experiment. A 10^{-4} passaged high titre serum was diluted 10-fold and inoculated intracerebrally in 4 adult mice. Two hours later half the mice were given 0·2 ml. of a low titre-producing serum intravenously. The titre of this serum was $10^{5\cdot5}$, so that it was a relatively minute extra infective dose, and as low or lower titres were produced when it was inoculated undiluted. Growth curves were constructed for both groups of mice from the titration of pooled tail blood samples at fixed time intervals. Results (Fig. 3) show that the low titre serum markedly depressed the infective virus yield and in addition prolonged the incubation period, compared with the yield and incubation period when high titre serum alone was given. The fall in titre during the 22 hr. before mice began to die might be a result of thermal inactivation of virus at the body temperature of the mice. In Paper II it was shown that a similar 10-fold fall in titre occurred when infective serum was incubated at 37·5° for 24 hr.

To make sure that the titre of a high titre serum is not depressed when it is merely mixed with a low titre serum, another experiment was performed. A low titre serum when mixed with an equal quantity of standard diluent had a titre of $10^{5\cdot5}$. A high titre serum mixed similarly had a titre of $10^{8\cdot2}$. When the two sera were mixed in equal quantities the titre was $10^{8\cdot3}$, so it appears that low titre sera cannot by their very presence depress the titration end-point of high titre sera.

Low titre sera therefore contain something which both depresses the final

virus yield and prolongs the incubation period when very large quantities are inoculated into mice following relatively small quantities of high titre serum.

Blood virus growth curves were then constructed, using a low titre-producing serum by itself as inoculum. A certain 10^{-1} passaged serum had repeatedly produced titres of $10^{6\cdot0}$ or less on different occasions. Six mice were inoculated

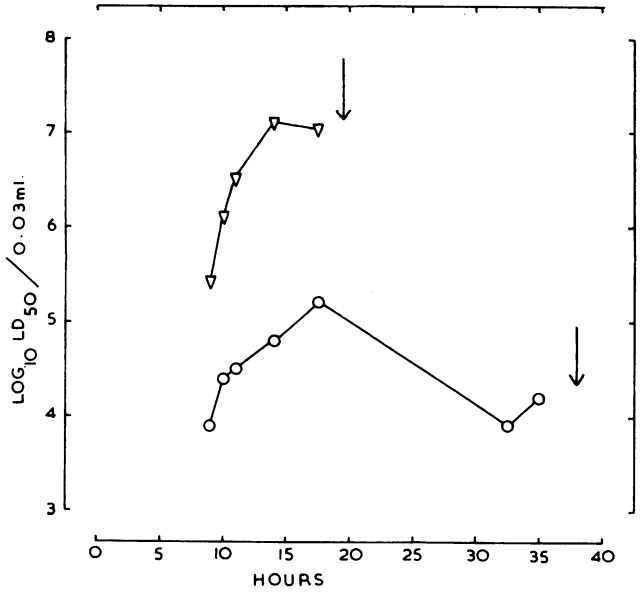

FIG. 3.—The depression of the peak titre and lengthening of the incubation period by large doses of low titre serum. Each growth is for virus in the pooled blood samples from two inoculated mice. One group received $10^{7\cdot0}$ LD_{50} of a high titre ($10^{8\cdot0}$) serum intracerebrally (triangles), and the other group (circles) an identical inoculum followed two hours later by 0·2 ml. of a low titre serum intravenously. Arrows above the growth curves indicate the time at which mice began to sicken and die.

with a 10^{-1} dilution of this serum, and a growth curve (Fig. 4) was constructed from repeated pooled samples of their tail blood. The first cycle of growth (see Paper II) is visible, but the titre did not continue to increase exponentially, and stayed at $10^{5\cdot5}$–$10^{6\cdot0}$ for about 20 hr., when the mice began to die. The shape of the curve is very similar to that obtained in the interference experiment (Fig. 3), suggesting that an interfering agent contained in the low titre-producing serum was again depressing peak titres and prolonging the incubation period. Another growth curve, where a low titre was produced following a 10^{-1} inoculum, is included in Fig. 4, together with the type of two-step growth curve where a peak titre of $10^{8\cdot5}$ is reached (see Paper II).

Antibody-binding power of low titre-producing sera diluted beyond their infectivity end-point

A high titre 10^{-4} passaged serum was diluted $10^{-5\cdot5}$ (to contain about $10^{3\cdot0}$ LD_{50}/0·03 ml.) and added to (*a*) the same volume of serial tenfold dilutions of the author's R.V.F. virus immune serum, each immune serum dilution having been

mixed 30 min. earlier with the same volume of buffered 0·5 per cent bovine plasma albumin and (*b*) the same volume of serial 10-fold dilutions of this immune serum, each serum dilution having been mixed 30 min. earlier with the same volume of a low titre serum diluted beyond its end-point. The low titre serum, of titre $10^{4\cdot2}$, was diluted $10^{-5\cdot0}$, and did not then kill mice when inoculated by itself. The contents of each tube were inoculated (0·03 ml.) intraperitoneally into five adult mice. Considered as an immune serum titration, the Reed-Muench end-point for (*a*) was $10^{3\cdot9}$ and for (*b*) $10^{3\cdot2}$.

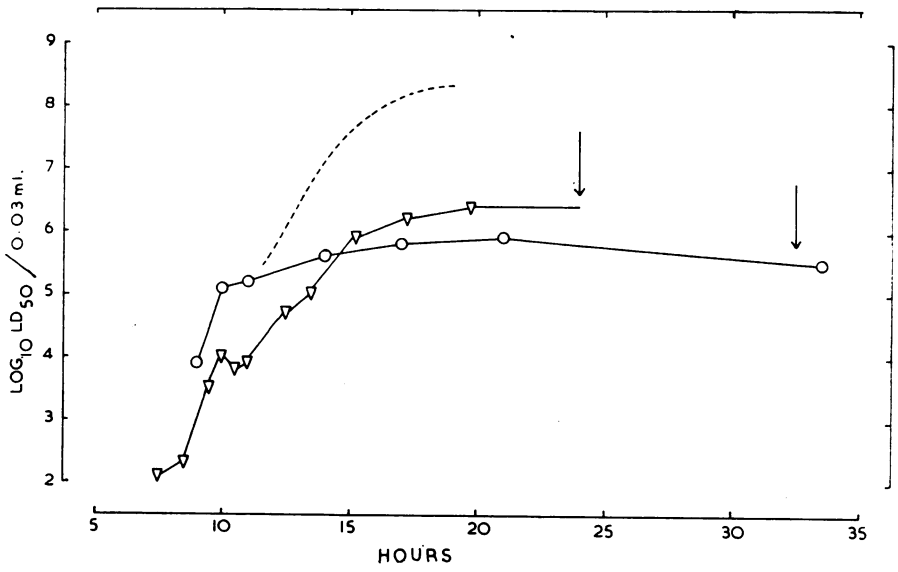

FIG. 4.—Blood virus growth curves in mice following inocula which produced low peak titres. The points on each curve represent the pooled blood samples from the same six mice, taken at fixed time intervals. Inoculations were $10^{6\cdot3}$ LD_{50} of serum of titre $10^{7\cdot3}$ (circles), and $10^{7\cdot3}$ LD_{50} of serum of titre $10^{8\cdot3}$ (triangles). The shape of the second part of the type of two-step growth curve when high titres are produced, is included as a dotted line. (See Paper II.)

To confirm that there was a real difference, another low titre serum (titre $10^{5\cdot4}$) was used, and the titrations were made more accurate by using serial $10^{0\cdot5}$-fold dilutions of immune serum, and inoculating the contents of each tube into 8 mice. Once again, mice given the diluted low titre serum alone did not die. The immune serum end-point for (*a*) was $10^{6\cdot1}$, and for (*b*) $10^{4\cdot8}$. It would appear, therefore, that the antibody titre of an immune serum is reduced when mixed with a low titre infective serum diluted beyond its end-point. Both the low titre sera used produced low titres when inoculated undiluted.

Serial passage of undiluted blood intravenously, the survival of mice, and the localisation of virus in the brain

Undiluted infective blood was passaged intravenously, to see whether, with the larger inocula which could thus be given, even greater falls in titre would occur. It was shown in Paper I of this series that the response to intravenously

and to intracerebrally inoculated R.V.F. virus was the same, the latter acting as a small intravenous inoculum. The blood used to start the series had previously had four 10^{-4} passages in mice, was of high titre and high titre producing. Twelve mice were inoculated intravenously with 0·1 ml. of this blood, and when they were sick or recently dead blood was taken from the thorax into a tuberculin syringe, of which the needle contained a trace of heparin. The blood was pooled, titrated, and inoculated intravenously in the mice of the next passage. Since a large number of mice were used to start the series, there was enough blood available at each passage to inoculate at least six mice. Any blood left over was centrifuged, and the serum stored at −20°. Successive titres (Fig. 5) showed a regular "switchback" pattern, and a similar succession of titres was obtained in independent passage lines. High titres were maintained for a few passages and then there were precipitous falls, followed by recoveries; this cycle of events recurred every 4–5 passages. On occasion mice given the sera which produced minimal titres failed to die or even become sick. Such surviving mice, when bled a month later, were found to possess neutralising antibodies to R.V.F. virus. Of 16 mice inoculated with serum 3 (Fig. 5) 5 survived and acquired antibodies.

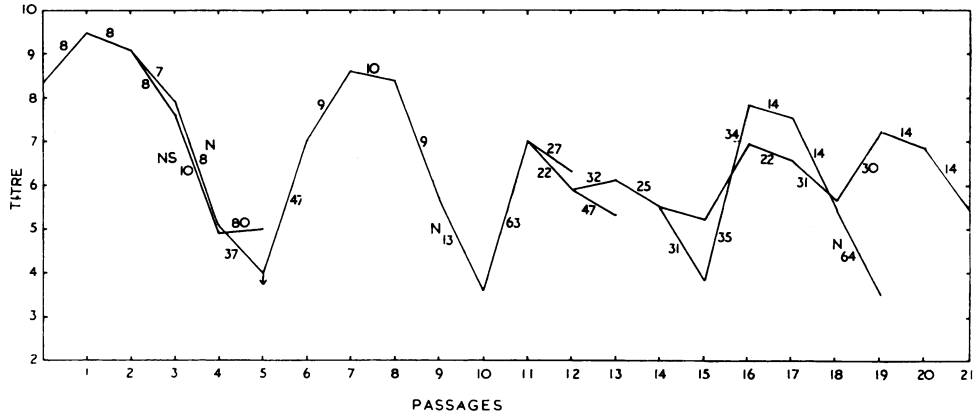

FIG. 5.—Serial passages using undiluted blood inoculated intravenously. Each point obtained by titrating pooled blood from at least 6 sick or dead mice, except in the case of one or two very low titre bloods where smaller numbers were bled, because the rest either died very late or survived (see text). The time in hours at which mice were bled is indicated against the line leading to the titre of the blood obtained. At least one mouse was dead, and the rest sick, at the time of bleeding. When any mice died late "neuro"-deaths or survived the fact is indicated by "N" or "S" respectively against the incubation period of those who were bled. Titres in $\log_{10}$ LD_{50}/0·03 ml. blood.

The phenomenon was noticed again when 2 mice survived and became immune during the course of another intravenous passage series using undiluted blood.

When mice were inoculated with certain sera of titre $10^{5\cdot0-6\cdot0}$, they died with even lower blood titres, and did so after lengthy incubation periods (see, for example, Fig. 2). In Paper III R.V.F. virus was shown to kill inoculated mice within 5 days, however small the inoculum, but after these low titre sera inoculations some mice died after as long as 6–12 days. Their seitz-filtered liver suspensions, however, failed to kill inoculated mice, and since a few of them became paralysed or sick the day before death occurred, seitz-filtered brain suspensions were also

tested, and found to kill mice in 2–3 days. Thenceforth livers and brains were tested, and it was discovered that each of 8 mice sick or dead 6–12 days after large inoculations of four different $10^{5\cdot0-6\cdot0}$ titre bloods, had virus in the brain but not in the liver. Such virus was neutralised by R.V.F. virus immune serum, and behaved in a viscerotropic way, killing adult mice 2–3 days after intraperitoneal inoculation and producing characteristic changes in the liver.

Thus, when some of the lower titre bloods in this passage series were given in large doses an occasional mouse survived and became immune, and, in those dying very late after a prolonged period of sickness, virus was present in the brain but not in the liver. Other equally low titre bloods occurring in the passage series did not give rise to these phenomena, which were restricted to the very low titre bloods which contained large amounts of interfering material, in that they produced lengthy incubation periods and even lower titres at the next passage. The phenomena did not occur, for instance, with blood of titre $10^{3\cdot0}$, and such blood produced higher titres at the next passage.

Unexpectedly short incubation periods following maximal doses of low titre-producing sera

On several occasions in undiluted intravenous serial passages incubation periods following inoculations of low titre-producing sera, far from being prolonged, were very short, even when compared with those following the same infective dose of high titre serum. These low titre sera contained large quantities of interfering material, for they not only gave rise to even lower titres in inoculated mice, but some produced late " neuro "-deaths as well. Death-times following large intravenous inoculations of two of these sera, compared with the shortest death-times ever encountered with similar infective inoculations of high titre serum, are included in Table V. The high titre serum is the one whose dose-incubation period relation is shown in Fig. 2.

TABLE V.—*Death-times in Mice Inoculated with Large Doses of Certain Low Titre Sera.*

	Inoculum.	Number mice inoculated.	Median death-time (hr.).	Shortest death-time in any individual mouse (hr.).
Low titre sera	$10^{6\cdot4}$ LD_{50} of $10^{5\cdot9}$ titre serum	16	17	11
	$10^{6\cdot2}$ LD_{50} of $10^{5\cdot7}$ „ „	7	13	9
High titre sera	$10^{7\cdot1}$ LD_{50} of $10^{8\cdot1}$ titre serum	9	18	15
	$10^{6\cdot2}$ LD_{50} of $10^{8\cdot1}$ „ „	—	20	—

Death-times for high titre, quick killing sera are shown for comparison. Exactly equivalent infective doses were not inoculated: in the first, the nearest largest dose inoculated is shown and in the second, the median value was read off on the high titre serum dose-incubation period curve.

DISCUSSION

It has been shown that when R.V.F. virus is passaged in concentrated form sera with low infectivity titres are produced, which on inoculation give prolonged incubation periods. It was demonstrated directly that this was due to an interfering agent which was present in low titre-producing sera. The effect

could not be accounted for by post-mortem incubation of blood. The phenomenon is thus similar to that described for influenza virus in the allantoic cavity (von Magnus, 1951*a*) and de-embryonated egg (Bernkopf, 1950). The infectivity titre of the allantoic fluid was shown to fall when large inocula were passaged, although the haemagglutinin titre was, initially, unchanged. It was concluded that so-called " incomplete " influenza virus particles had been produced, which haemagglutinated but which were not infective. It was later shown that such particles also combined with antibody, fixed complement, interfered with the growth of infective virus, were adsorbed on to and eluted from red blood cells, and were antigenic.

Incomplete R.V.F. virus can therefore be produced in the same way as incomplete influenza virus. It has been shown that a non-infective agent in low titre-producing R.V.F. sera will combine with antibody, and, moreover, that large doses of low titre-producing sera may be antigenic, if they are not lethal. It is concluded that incomplete R.V.F. virus not only depresses peak titres and prolongs incubation periods, but also combines with specific antibody and is antigenic.

The effect of incomplete influenza virus on the incubation period is not demonstrable in the chick embryo, but von Magnus (1951*b*) showed that low titre allantoic fluids (produced by undiluted serial passage) inoculated intranasally in mice resulted in longer incubation periods, and fewer deaths and lesions, than did high titre (10^{-6} serially passaged) virus.

The prolongation of the incubation period when the inoculum contains large quantities of incomplete virus might be attributed to interference, or the "blockage " of susceptible cells by incomplete virus particles. If, however, the majority of susceptible cells have supported virus growth by the time of death, any blocking effect must by then have been overcome, allowing infective particles to grow in cells. Moreover, the " blocking " effect would presumably disappear on dilution, yet low titre sera, even when diluted 10^{-7} (see Fig. 2) have longer incubation periods than do equivalent inocula of high titre sera. To explain this latter phenomenon it seems necessary to postulate that the virus particles in low titre serum differ in that they are either not capable of such rapid growth, or are not so rapidly lethal when they grow, as are the particles in high titre serum. This means that there may be degrees of " incompleteness ".

The unusual shape of the dose-incubation period curve of very low titre sera (Fig. 2) is difficult to account for. As larger amounts of the sera were inoculated the incubation period became progressively more prolonged. It was shown that the effect could be overcome if enough infective virus was present in the inoculum. Presumably larger inocula contained larger amounts of the agent which prolonged the incubation period, but not enough infective virus to overcome the effect.

The infectivity titre of a serum does not by itself give reliable information as to the incomplete virus content. For instance, in the 10^{-4} serial passages sera with titres as high as $10^{8.5}$ or $10^{8.8}$ (Table II) may produce low pooled titres when inoculated in concentrated form, and a low titre in an occasional mouse even when inoculated at 10^{-4} (Table I). Again, the 10^{-4} passaged serum in Fig. 2 gives much the same dose-incubation period curve as any 10^{-8} passaged serum. The 10^{-4} passaged serum studied in Paper III of this series, however, gave considerably longer incubation periods for given infective doses. One concludes that this latter serum, in spite of its higher titre ($10^{9.0}$), contained incomplete, or slow

growing, particles. The various passage series in Fig. 1 and 5 were started with different 10^{-4} passaged sera, which contained different quantities of incomplete virus. If the starting serum in the undiluted intracerebral series, for instance, contained more incomplete virus than in the undiluted intravenous series the more rapid fall in successive titres is explained. Incomplete virus is probably produced even in the 10^{-6} passage series (Fig. 1), for low titres occurred here as they did in the 10^{-4} series. If an infective serum produces high titres whatever the dilution inoculated, it probably contains little or no incomplete virus. Such serum is obtained when virus is serially passaged using a dilution factor of 10^{-8}. The $\log_{10}$ infectivity titre, therefore, is an unreliable guide to the incomplete virus content of a serum. Even if half the particles are incomplete the titre is only reduced by $10^{0 \cdot 3}$, and this is within the range of experimental titration error (see Paper I).

In the undiluted serial passages large doses of low titre sera frequently produced low titres, whereas the same infective dose of high titre sera (Table II) produced high titres. This is presumably because the non-infective, interfering particles in low titre sera contribute to the low titre-producing effect, as has also been postulated for incomplete influenza virus by von Magnus (1954). If the virus which is serially passaged in concentrated form is 100 per cent complete to start with it is logical to assume that incomplete virus is produced even at the first passage, although the $\log_{10}$ titre is not detectably lowered. On further passage the infective titre falls progressively (incomplete virus accumulates), because the large quantity of incomplete virus produced together with infective virus still ensures that a very large particle dose is inoculated. At some stage, however, either the total number of particles produced diminishes or, alternatively, many of the particles become too incomplete even to interfere. At this stage large inocula of low titre sera contain much less interfering material, so that titres rise again. Such a sequence of events explains the " switchback'" effect in the undiluted intravenous serial passages (Fig. 5), where there are slow falls in titre followed by relatively rapid recoveries. If interfering virus accumulated indefinitely such recoveries would, of course, be impossible. It follows that sera containing the largest amounts of interfering material are those which produce the lowest titres, *i.e.*, sera nos. 3, 4, 9, 14, and 18 in Fig. 5. It was these sera, moreover, which produced the late " neuro " deaths and survivals with immunity, additional evidence that maximum amounts of interfering material were present.

The interfering property of incomplete virus may account for the mice in the intravenous passage series which died after 7–12 days with virus in the brain but not in the liver. Visceral cells may have been occupied by incomplete particles, but if an infective particle somehow chanced to reach an extravascular site in the brain it would grow if it was potentially, or from the start, neurotropic. R.V.F. virus recovered from the brains of mice which died very late was viscerotropic, and probably viscerotropic R.V.F. virus is capable of slow growth in the brain, although it rarely gets a chance to do so because it kills the host too soon.

The occasional mice which survived and became immune after large doses of low titre serum probably did so as a result of the interfering and antigenic properties of the incomplete particles. It is assumed that infective virus could not have multiplied and itself produced antibodies without causing sickness or death. Either incomplete virus multiplied in cells to produce more incomplete virus, or, more probably, immunity was produced by the sheer antigenic bulk of

the inoculum. Each immune survivor had received a dose of infective virus which, on dilution, would kill hundreds of thousands of mice, but the enormous amounts of interfering material present prevented the growth of infective particles.

In the intravenous passage series (Fig. 5) mice sicken and die with blood titres as low as 10^3 to 10^5, that is to say, when virtually all the virus produced is incomplete. If death is a result of cell damage it seems that this damage is just as extensive when incomplete particles are produced. Perhaps, therefore, there is no very great difference in the behaviour of virus during most of its growth in the cell, whether complete or incomplete particles are yielded. This may mean that incomplete virus results from some defect operating fairly late in intracellular development.

Low titre sera sometimes gave unexpectedly short incubation periods when large amounts were inoculated (Table V). These low titre sera produced even lower titres at the next passage, and some produced "neuro" deaths as well. They therefore contained maximal amounts of interfering material and one would expect to see prolonged incubation periods when large doses were inoculated (Fig. 2). The fact that incubation periods were even shorter than warranted by the amount of infective virus administered is not easy to explain. Conceivably some sort of " multiplicity reactivation " took place, so that complete particles were produced in cells from the pooled fragments of incomplete ones.

Incomplete virus formation has been thoroughly recorded and studied only for influenza virus. The incomplete virus type of phenomenon, however, has been reported on occasions. For instance, yellow fever investigators have recorded that monkeys and galagos given small inocula usually attain higher blood titres than those given large inocula (Smithburn and Haddow, 1949; Smithburn, 1949).

It should not be forgotten how artificial the circumstances are when incomplete virus is produced. By means of a syringe or pipette virus is confronted with a situation it has never before encountered. Nature's inocula, whether by insect-bite or infective droplet, are usually much smaller, and it is not surprising that the highly efficient system of virus growth which has always followed such inocula should break down under unprecedented laboratory circumstances. Nevertheless, from an analysis of the growth disorders which follow these large inocula, some insight into the normal mechanism and requirements of virus growth may eventually be obtained.

SUMMARY

Low R.V.F.virus infectivity titres are produced when large inocula are serially passaged, and high titres when small inocula are passaged. This is explained by the production of an incomplete, non-infective form of virus.

The incomplete virus present in low titre-producing sera is shown to interfere with the visceral growth of infective virus, first, by prolonging the incubation period and second, by reducing the peak infectivity titre attained.

Very large quantities of incomplete virus may interfere with visceral multiplication to such an extent that mice survive, or if they do die, some of them do so as a result of virus growth in the brain, not in the liver. Since the surviving mice develop specific neutralising antibodies, it is concluded that incomplete virus is antigenically active.

Incomplete virus combines with specific neutralising antibody.

It is shown that, by itself, the $\log_{10}$ infectivity titre of a serum is an unreliable guide to the incomplete virus content. Little or no incomplete virus is produced when serum is serially passaged using a dilution factor of 10^{-8}.

The suggestion that a non-interfering type of incomplete particle is eventually produced during the serial passage of large inocula explains the regular reappearance of high titres.

An inoculum may be lethal even when almost all the virus yielded is incomplete.

REFERENCES

BERNKOPF, H.—(1950) *J. Immunol.*, **65**, 571.
SMITHBURN, K. C.—(1949) *Amer. J. trop. Med.*, **29**, 414.
Idem AND HADDOW, A. J.—(1949) *Ibid.*, **29**, 389.
VON MAGNUS, P.—(1951*a*) *Acta path. microbiol. scand.*, **28**, 278.—(1951*b*) *Ibid.*, **29**, 157.—(1954) ' Advances in Virus Research ' **2**, p 59. New York (Academic Press).