RIFT VALLEY FEVER VIRUS IN MICE.
II. ADSORPTION AND MULTIPLICATION OF VIRUS

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THE preceding paper deals with the general characteristics of the infection of mice with Rift Valley Fever virus. Titration methods were evaluated, the distribution of virus in the body of infected mice determined, and clinical and pathological findings recorded. It was suggested that the mouse infected with R.V.F. virus might be used as a convenient laboratory model for the man or monkey infected with yellow fever. A quantitative study of the adsorption and growth of virus in the mouse is reported here.

MATERIALS AND METHODS

Virus and mice and routine titrations.—As described in Paper I.

Carcass titrations.—Mice were killed with ether, and heavily contaminated parts and virus inactivating fluids were removed. Skin (including paws, pinnae and snout), the gut (from lower end of oesophagus to lower end of rectum), gall bladder and bladder urine were all removed. Mice so treated were placed in pH 7·4 buffered 0·5 per cent bovine plasma albumin (500 ml. for three mice) in a sterile blendor of the Waring type. The blendor, in a refrigerator, was run slowly for 2 min. and fast for 3 min., to give a fairly homogeneous suspension which was never more than very slightly warm. Preliminary experiments had shown that virus in normal saline was markedly inactivated in the blendor, but remained stable when bovine plasma albumin was present. The suspension, after light centrifugation, was then titrated as routinely, with the addition of penicillin and streptomycin to each dilution before inoculation.

Growth curves.—Mice from whom growth curves were constructed were inoculated intracerebrally, unless the volume of inoculum was too large to be given by this route, in which case it was given intravenously. It was shown in the preceding paper that the response to inoculated virus was the same, whether it was given by the intracerebral or by the intravenous route.

Unless otherwise stated six mice were inoculated with each $10^{0.5}$-fold dilution in growth curve titrations.

RESULTS

Blood virus growth curves after various sized inocula.—Various dilutions of stock infective sera were inoculated intracerebrally, each dilution to a group of six mice. The size of the inocula ranged from $10^{0.7}$–$10^{7.3}$ LD_{50}, and the three largest were made from the same infective serum. At intervals after the inoculation the mice in each group were bled from their tails, and the pooled blood titrated. Growth curves were thus obtained (Fig. 1), and it is seen that the rate of increase in titre was proportional to the inoculum size, being greater for larger inocula. Whatever the inoculum, however, approximately the same final titre was reached. Mice began to sicken and die 1–2 hr. after peak titres were attained, and death occurred within the next 2 hr.

On several occasions growth curves were found to be similar when constructed from bleeds from the same single mouse throughout the experiment.

Two-step growth curves.—A more detailed determination was made of blood virus growth curves following large inocula. In these experiments $10^{6\cdot0}$–$10^{7\cdot5}$ LD_{50} were inoculated intracerebrally, in the form of tenfold diluted stock infective serum. The six inoculated mice were bled from the tail at closely-spaced time intervals, and growth curves again constructed from the pooled blood titres

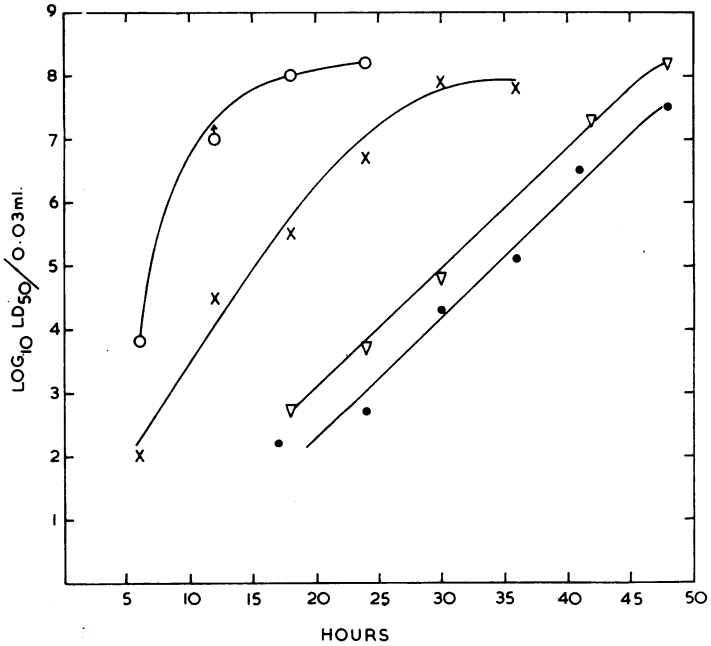

FIG. 1.—Growth curves for virus in the blood of mice following the inoculation of various doses of R.V.F. virus. Each curve was constructed from repeated samples, each one pooled, of the blood of six inoculated mice. The inocula given to each mouse were $10^{7\cdot3}$ LD_{50} (circles), $10^{4\cdot5}$ LD_{50} (crosses), $10^{1\cdot5}$ LD_{50} (triangles) and $10^{0\cdot7}$ LD_{50} (dots). Three mice only were inoculated with each $10^{0\cdot5}$ fold dilution in the titrations, except for the experiment with the smallest inoculum, where six mice were used.

(Fig. 2). In each experiment the initial rise in titre began after 7 or 8 hr., continued exponentially until the 10th hr., then remained steady or fell slightly for another hour before the second exponential rise to peak titre. Mice sickened and died after from 24 to 30 hr.

One-step growth curves.—Two more growth curves were constructed in the same way, following very large inocula. In these experiments three mice were inoculated intravenously by the tail vein, and $10^{8\cdot5}$ and $10^{8\cdot3}$ LD_{50} administered. The blood titre began to rise at 4 or 5 hr. (Fig. 3), and increased exponentially with no shelf or sag to a peak in as little as 7 or 8 hr. Mice sickened and died as the peak titre was reached, or very shortly afterwards. In one of these curves peak titres were high, and in the other peak titres were as high as occurred following intracerebral inoculation of the same material. It was repeatedly found in subsequent experiments that maximal peak titres could be produced following

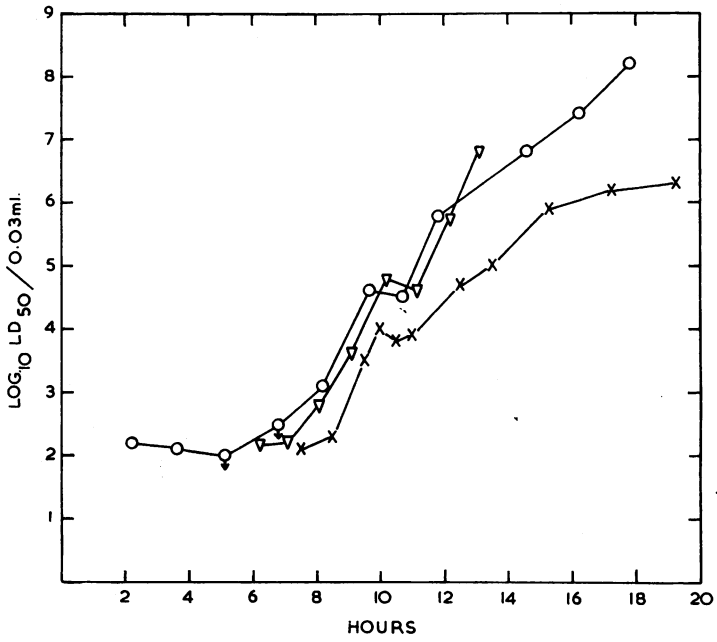

FIG. 2.—Growth curves for virus in the blood of mice following large inocula ($10^{6.0}$–$10^{7.5}$ LD_{50} per mouse). The points on each curve were obtained from repeated samples, each one pooled, of the blood of six mice which received the same inoculum.

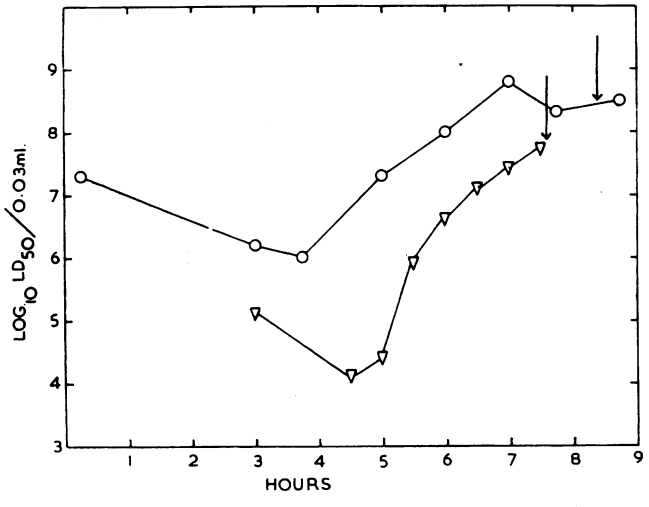

FIG. 3.—Growth curves for virus in the blood of mice following very large inocula. $10^{8.5}$ LD_{50} (circles) and $10^{8.3}$ LD_{50}/(triangles) were inoculated intravenously to three mice, and each curve was obtained from repeated samples, each one pooled, of the blood of these mice. The times of onset of sickness and death are indicated by arrows above the growth curves.

intravenous inocula. Thus, the susceptible cells which produce the virus found in blood are susceptible to intravenously inoculated virus.

Adsorption experiments.—The pattern of virus adsorption was studied in a similar way by titrating pooled tail blood from groups of mice at intervals after inoculations of various sizes. Very similar curves were obtained on many occasions, and the results of three of these experiments are given in Fig. 4.

The blood titre falls rapidly for the first hour, then less rapidly, although the fall seems to continue during the period of the experiments. The curves in Fig. 4 are drawn by eye through the determined points, and in each case, the titre has fallen by $10^{0 \cdot 6}$ within about 30 min. This means, on a log scale, that 75 per cent of the inoculum has disappeared from the blood within 30 min.

In two of these experiments inocula were made from infective sera of low titre ($10^{7 \cdot 0}$–$10^{8 \cdot 0}$). It is shown in Paper IV of this series, that such sera contain

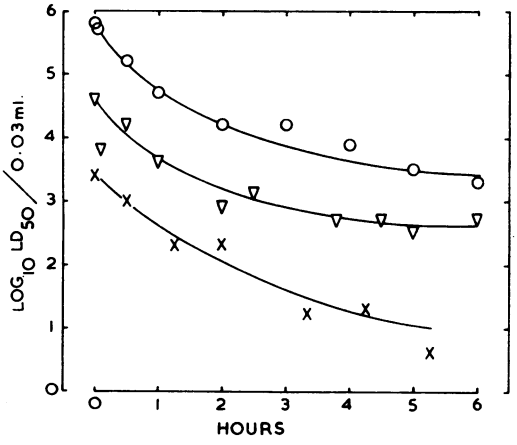

FIG. 4.—Adsorption curves for virus from the blood of mice following various sized inocula. Each mouse given $10^{7 \cdot 6}$ LD_{50} (circles), $10^{6 \cdot 4}$ LD_{50} (triangles) and $10^{5 \cdot 2}$ LD_{50} (crosses), and the theoretical immediate blood titre (calculated from the blood volume) is shown in each case. Points on each curve were obtained from repeated samples, each one pooled, of the blood of 4–6 inoculated mice.

" incomplete " virus, and the third adsorption experiment was performed with a high titre serum ($10^{9 \cdot 0}$) which probably contained little or no " incomplete " virus. In the curve obtained (triangles in Fig. 4) there is a suggestion of a steady plateau after the 4th hr., but the initial part of the curve is similar to that in the other experiments. The shapes of these growth curves, therefore, do not reflect any appreciable difference between " incomplete " and " complete " virus.

It was thought that perhaps it might, for purely circulatory reasons, take a long time for all virus particles to reach susceptible cells. If, however, virus encountered susceptible cells earlier, adsorption would perhaps be accelerated. About $10^{7 \cdot 0}$ LD_{50} of virus contained in 0·35 ml. of diluent was therefore injected very slowly (over the course of 4 min.) into the portal vein of an anaesthetised mouse, and the same dose into the tail vein of another mouse. Heart bloods were titrated after 10 min., and the titres were found to be the same ($10^{5 \cdot 9}$ and $10^{6 \cdot 1}$) in each. Thus, if liver cells adsorb virus from circulating blood, the rate

of adsorption is not markedly increased when inoculated virus enters the hepatic before the systemic circulation.

Virus stability at 37·5°.—Any apparent steadiness of the final titre in growth curve experiments could possibly represent an increase in blood titre masked by thermal inactivation of virus at the body temperature of mice. The progressive fall in titre which occurred after the second or third hour in adsorption experiments might also be due to thermal inactivation. Stock infective serum was therefore incubated at 37·5° and samples removed and titrated at intervals. The titre remained steady for many hours (Table), showing that thermal inactivation is not an important factor in interpreting the growth curves reported here. This stability at 37·5° was later confirmed, and it was shown that there was a 10-fold fall in titre by 24 hr., a 10,000-fold fall by 48 hr., and that no infective virus was demonstrable after five days. Antibiotics were added to the serum before incubation, and bacteriological cultures made at the time of each titration were sterile.

TABLE—*Virus Stability in Mouse Serum at* 37·5°

	Hours.					
	0.	1.	2.	3¼.	4.	5.
Titre	6·3	6·0	6·4	6·4	6·3	6·0

Adsorption and growth curves obtained from carcass titrations.—It was thought possible that the observed changes in blood titre in the adsorption and growth curves described above might partly be due to blood-tissue exchanges of virus in the body of the mouse. Growth curves were therefore constructed from the titration of entire carcasses.

A preliminary experiment was performed to see whether virus was inactivated in suspension of normal mouse carcass. After standing at room temperature for 15 min., the titres for virus in a normal carcass suspension and the same amount in standard diluent were found to be $10^{3\cdot9}$ and $10^{3\cdot5}$ respectively. Normal carcass emulsion thus does not inactivate virus.

Two experiments were then done, in which adsorption and growth curves were obtained from both blood and carcass titrations. Many mice were inoculated intracerebrally with $10^{7\cdot3}$ and $10^{7\cdot5}$ LD_{50} of virus, and at fixed time intervals three mice were sacrificed and their pooled blood and pooled carcasses titrated. These titrations were made using serial tenfold dilutions of infective material. In the curves obtained (Fig. 5), the average virus content per mouse carcass as directly determined is plotted, together with the average virus content per mouse blood volume (Kaliss and Pressman, 1950) calculated from the blood titration. The curves are, if anything, more irregular than when successive blood samples are taken from the same set of mice throughout the experiment, but it can be seen that carcass and blood titrations give similar curves. It is concluded that adsorption and growth curves for blood virus give a true picture of the behaviour of virus in the entire mouse. In both experiments, the carcass titre begins to increase at about the same time as the blood titre, showing that this increase is not detectable anywhere in the body very long before it is detectable in the blood.

It would appear from the growth curve that the residual virus content of the carcass after adsorption is greater than that of the blood. This, however, is not the case in the adsorption curve, and it could not be confirmed in subsequent

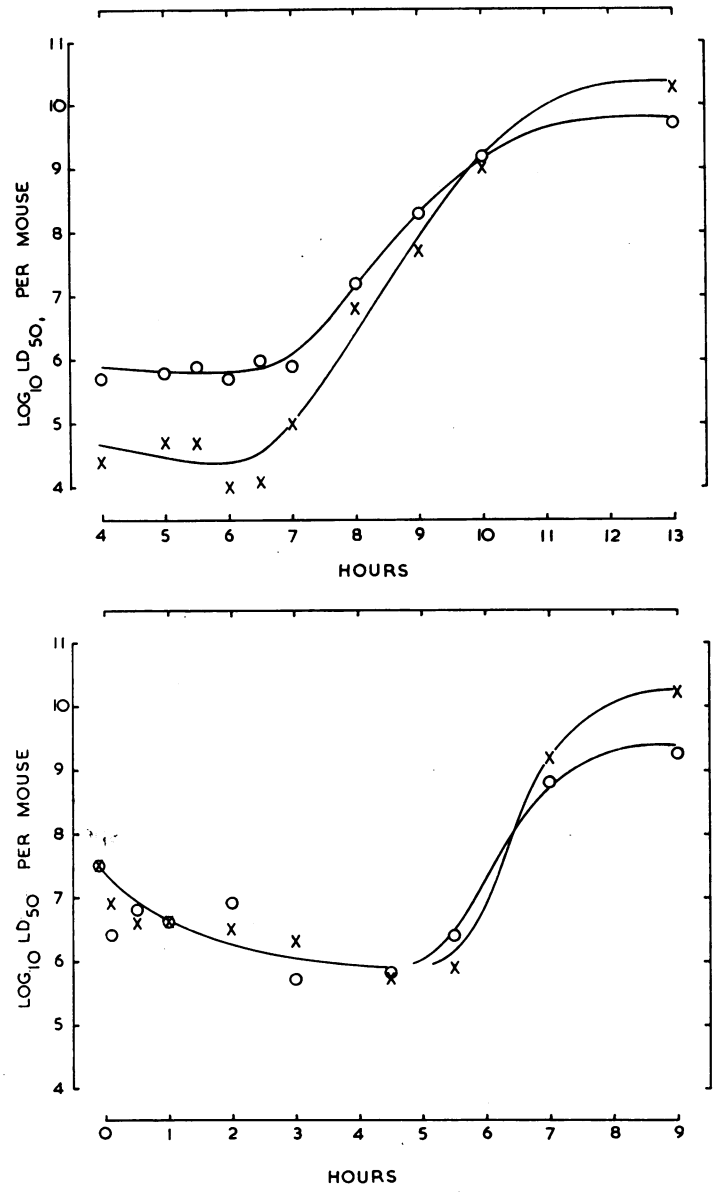

FIG. 5.—Adsorption and growth curves for virus in the carcasses of mice (circles) and in their blood (crosses). At each time interval three inoculated mice were sacrificed and their pooled blood and pooled carcasses titrated. The total virus content per mouse was determined directly in the carcass titrations, and the content per mouse blood volume was calculated from the blood titrations. $10^{7.3}$ and $10^{7.5}$ LD_{50} were inoculated per mouse, and in the adsorption experiment which terminated at 9 hr., the theoretical immediate virus content per mouse ($10^{7.5}$ LD_{50}) is shown.

experiments. The blood and tissue points on the adsorption curve are not regular enough to draw conclusions about the virus content of blood and tissues during adsorption. It can be seen in both the growth curve and the adsorption curve, however, that the whole blood usually contains about as much virus as the carcass.

DISCUSSION

These adsorption and growth curves may be interpreted as follows. After inoculation into the blood stream virus is adsorbed by susceptible cells, and the blood titre falls. It was shown in Paper I that an intracerebral inoculation is, in effect, a small intravenous one. Almost all of this adsorption takes place during the first hour, and any subsequent adsorption is, by comparison, very limited in extent. It is shown in the carcass experiments (and in Paper III of this series), that virus which has disappeared from the blood is no longer detectable even when the entire carcass is titrated. Within from 5 to 7 hr., virus which has undergone one multiplication cycle in cells is being released into the blood, raising the titre above the residual level. This increase in titre occurs exponentially, and in the experiments in Fig. 2, where the inoculum did not infect all available cells in the first place, the newly released virus is again taken up by uninfected cells. At 10–11 hr., this rate of uptake equals or even slightly exceeds the rate of release, so that the reproducible shelf or sag in the curve appears. When all susceptible cells have taken up virus, released virus can accumulate in the blood to give the final exponential rise in titre. With very large inocula (Fig. 3), which were themselves large enough to infect all susceptible cells, virus released after the first multiplication cycle can increase uninterruptedly in the blood, and the peak titre is reached after the first cycle of growth, by 10 hr. The size of a saturating inoculum, itself just large enough to infect all susceptible cells, is determined in Paper III.

Are such interpretations justified in analysing these curves? In the first place, the phenomenon, with virus disappearance and subsequent increase, has been shown to occur for other viruses on much the same time scale.* Secondly, the shapes of the growth curves in Fig. 2 and 3, which are most simply explained in these terms, are also reproducible. Thirdly, the virus disappearance and subsequent increase is detectable when the entire carcass is titrated (Fig. 5), and is thus not to be explained by any purely blood-tissue exchanges of virus. An attempt to reproduce the shelf in the growth curve (Fig. 2) with carcass titrations has not been made, because they are probably less accurate than blood titrations, and contributions to successive points on the growth curve are made by different mice, so that mouse variation becomes important.

Henle (1949) showed that when influenza virus grows in the chick embryo an increase in infectivity is detectable in the allantoic membrane a few hours before it is reflected in the allantoic fluid. For R.V.F. virus, since the growth curve for the mouse carcasses is similar to that for mouse blood, one can assume that almost as soon as virus increases in the tissues the increase is detectable in the blood, *i.e.*, virus is liberated into the blood stream soon after it is produced.

Although growth curves have been constructed for a number of viruses, discrete cycles of multiplication have been demonstrated only for a few, including bacterio-

* Stepwise increases in titre have been described for poliomyelitis virus in tissue culture, however, and in this *in vitro* system each cycle appears to last for ten days (Scherer and Syverton, 1952).

phage (Delbruck and Luria, 1942 ; Ellis and Delbruck, 1939), influenza virus (Henle, Henle and Rosenberg, 1947 ; Henle and Rosenberg, 1949 ; Cairns, 1952), pneumonia virus of mice (Ginsberg and Horsfall, 1951), Newcastle disease virus (Gordon, Birkeland and Dodd, 1952), Western Equine Encephalitis virus (Dulbecco and Vogt, 1954), and Theiler's GDVII virus (Sanders, 1953). Usually some special method has been needed to demonstrate these multiplication cycles. Where it is technically possible the medium surrounding infected cells can be removed and replaced, so that the total virus release is measured during fixed time intervals with no residual titre to confuse the picture. Methods have also been developed for preventing the re-uptake of virus after its release from the first cycle of growth in cells.

It seems that cycles of multiplication are visible in the straightforward R.V.F. virus growth curves obtained here. They are thus demonstrable in the intact virus-infected mammal. Ginsberg and Horsfall (1951) and Gordon *et al.* (1952) constructed such curves for pneumonia virus of mice in mice and for Newcastle disease virus in eggs. Von Magnus (1951) published growth curves for influenza virus in the allantoic cavity, in which a shelf or sag occurs repeatedly at 10 hr., although it is not commented on in the text. The very rapid growth rate of R.V.F. virus, together with the fact that blood from the same individuals can be titrated during the course of an experiment, may partly explain why the cyclic pattern was so clearly seen here.

Adsorption takes place fairly rapidly, and 75 per cent of inoculated virus had disappeared within about 30 min. Similar rates of adsorption have been reported for other viruses. Henle *et al.* (1947) showed that about 70 per cent of inoculated infective influenza virus was no longer detectable in the allantoic fluid after from 30 to 60 min. Dulbecco and Vogt (1954), in a precise *in vitro* experiment, showed that 80–90 per cent of Western Equine Encephalitis virus is adsorbed by a monolayer of susceptible cells within about 30 min. Gordon *et al.* (1952) reported that 87 per cent of inoculated Newcastle disease virus had disappeared from the allantoic fluid in as short a time as 10 min.

The titre remaining in the blood after adsorption is completed is related to the size of the inoculum (Fig. 1, 2 and 3). There is more virus left over when more is administered. A study of this phenomenon is made in the next paper of this series.

It can be seen from the growth curves in Fig. 2 and 3, that there is an increase in titre much earlier with large inocula than with smaller ones. The increase may occur after as short a time as 4 to 5 hr., or as long as 7 to 8 hr., after inoculation. A similar effect has been reported for other viruses. Liu and Henle (1951) showed that when the LEE strain of influenza B virus grows in the allantoic cavity, and growth curves are constructed from allantoic fluid samples, the " constant period " between inoculation and increase in titre could be reduced from 8–10 to 4 hr. by giving larger inocula. One conceivable explanation of the results obtained with R.V.F. virus is that because the rate of increase in titre is greater with larger inocula (Fig. 1), the curve will begin to rise above the admittedly higher residual titre at an earlier stage. Alternatively it is possible that with high multiplicities of infection (where many particles infect each cell) virus begins to be released from cells earlier, as has been shown for Western Equine Encephalitis virus *in vitro* (Dulbecco and Vogt, 1954). The " average liberation time ", or time between inoculation and the appearance of 50 per cent of the first cycle

yield (Fazekas de St. Groth and Cairns, 1952), is also shorter when large inocula are given. The average liberation time as judged from the one-step growth curves (Fig. 3) is about $6\frac{1}{2}$ hr., and considerably shorter than the $9\frac{3}{4}$ hr. in the two-step growth curves (Fig. 2). This is not easy to explain except by again suggesting that the intracellular latent period is shorter when a very large inoculum is given (Dulbecco and Vogt, 1954).

When large inocula were given mice sickened and died as peak blood titres were reached, or very shortly afterwards (Fig. 3). With smaller inocula sickness and death occurred 1–4 hr. after peak titres were attained. Thus, peak blood titres occur before mice become sick (except with extremely large inocula; see Paper III), and death occurs before thermal inactivation could affect these titres.

The experiments in Fig. 1 showed that the peak virus titre was approximately the same whatever the size of the inoculum. The peak titres vary to a greater extent in the growth curves in Fig. 2 and 3, and an analysis of these variations is made in Paper IV of this series. It will be shown that although peak titres are uniformly high following any inoculum consisting of " complete " virus, titres are more irregular when " incomplete " virus is present.

SUMMARY

Adsorption and growth curves are constructed for blood titres in mice following the inoculation of various doses of Rift Valley Fever virus. After inoculation the amount of virus in the blood decreases, almost all this decrease occurring in the first hour. The virus titre then decreases very slightly until a rise is detected from 5 to 9 hr. after inoculation, depending on the amount of virus administered. The length of the average liberation period, between inoculation and the appearance of half the first cycle yield, is shorter with large inocula. The rate of increase in titre is more rapid with large inocula.

Adsorption and growth curves constructed from carcass titrations confirm the general picture of virus adsorption and multiplication, and show in addition that virus is liberated into the blood almost as soon as it is produced in the mouse.

When large inocula are given there is a shelf or sag in the growth curve at 10 hr., which recurs in separate experiments. With very large inocula, however, the rise in the growth curve is uninterrupted, reaching a peak by 10 hr. These features are explained as due to cycles of multiplication in susceptible cells of the mouse.

As long as " incomplete " virus is not present peak blood titres are much the same, whatever the inoculum size, and whether intracerebral or intravenous inoculations are given. Mice sicken and die during the first few hours after these peak titres are reached, except when extremely large inocula are given.

Virus in mouse serum is stable for many hours at 37·5°.

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