THE TITRATION OF VIRUSES IN BABY MICE

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(With 7 Figures in the Text)

CONTENTS

INTRODUCTION

The influence of the age of the host on its susceptibility to viruses is now well recognized: the subject has been well reviewed by Sigel (1952). The best known example is probably the embryo chick, which is susceptible to a large number of viruses which cannot be made to infect adult fowls. In general, the susceptibility decreases with increasing age, although there are exceptions.

In spite of the uncertainty which surrounds the mechanism underlying it, considerable use has been made of the increased susceptibility of young animals in the study of viruses. As early as 1904 Maggiora & Valenti (1904) observed that young geese were more susceptible than older birds to the virus of 'fowl-pest' administered subcutaneously. More recently, baby mice have been extensively used in the study of a number of viruses, especially yellow fever virus (Theiler, 1930), the Coxsackie group and the arthropod-borne encephalitis viruses. Kilbourne & Horsfall (1951*a*, *b*) have recommended the use of the newborn mouse for the isolation of *Herpes simplex* virus from human sources. Young rabbits, rats, hamsters and guinea-pigs have also been used for various purposes.

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For all but the crudest qualitative work with viruses an experimental host can only be considered fully satisfactory if it is possible to use it for reliable titrations *in vivo* of the particular virus being studied. It is the purpose of this paper to draw attention to some of the special difficulties which arise when very young animals are used for virus titrations *in vivo*.

Although the conclusions are based entirely on work done with one virus, *H. simplex*, and with one host, the newborn mouse, there is no reason to suppose that the difficulties encountered are peculiar to this virus or to this host.

There is always one fundamental assumption which must be made if the results of any biological assay are to mean anything at all: this is that over a certain range of dosage some regular relationship exists between the dose of the agent administered and the response of the test animals. It was because the results of a preliminary series of titrations of four strains of *H. simplex* in baby mice suggested that no regular relationship existed between the dose of virus administered and the proportion of animals dying that the investigations described in this paper were put in hand. As further results became available it became clear that a relationship of some kind did in fact exist, but that it was far more irregular than the numbers of animals and the dosage intervals used might reasonably have led one to expect.

An attempt has been made to analyse the results in such a way as to reveal the principal causes of this irregularity and to suggest methods by which the data may be reduced to a form to which the accepted mathematical treatments may legitimately be applied.

METHODS AND MATERIALS

(*a*) *Virus strains*

Four strains of *H. simplex* virus were used: GOS/CA and F/CA were strains adapted to the chick embryo by the chorioallantoic route of inoculation, F/R was a strain adapted to the rabbit cornea, and F/SM was a strain adapted to the new born mouse by the intraperitoneal route of inoculation. GOS/CA* came originally from a case of Kaposi's varicelliform eruption. The three F strains were all derived originally from a sample of vesicle fluid from a young woman suffering from a vesicular eruption of the face.

(*b*) *Virus suspensions*

As a preliminary to an investigation of the differences between the four strains of virus, large pools were prepared and stored in 0·5 or 1 ml. quantities in sealed neutral glass ampoules in a solid carbon dioxide cabinet until required. The suspending medium used was sterile skim milk (Speck, Jawetz & Coleman, 1951) to which was added penicillin 1000 i.u./ml. and streptomycin 200 μg/ml. All dilutions are expressed in terms of the original tissue (w/v).

(*c*) *Egg titrations*

Titrations of all four pools were carried out by the pock counting method, using 11-day chick embryos. The method used was essentially that of Beveridge & Burnet (1946), except that extra weight was not given to 'good membranes' by

* Kindly supplied by Dr J. A. Dudgeon of St George's Hospital, London.

counting them twice in calculating the result. The egg titrations were carried out in parallel with the mouse titrations and, in general, on the same sets of dilutions. The results of these titrations showed that the irregularities observed in the results of the mouse titrations were not the result of variation between individual ampoules of the pools, irregular dispersion of the infective particles, errors in dilution or loss of infectivity on storage.

(*d*) *Mice*

All the mice used belonged to the inbred line of Glaxo champagne mice maintained in the Department of Pathology, Cambridge, by strict brother–sister mating (Laboratory Animals Bureau, 1953). The mice were inoculated when 24–48 hr. old. Litters of four or less and litters of which more than one mouse died during the first 24 hr. of life were not used for titrations.

(*e*) *The titrations in mice*

As the breeding colony was not large enough to ensure that all the litters needed for one titration were available on the same day, the litters had to be used as they became available. This unfortunately introduced two potential sources of error, that due to day-to-day and seasonal variations in the susceptibility of the mice (Trevan, 1927) and that due to possible loss of titre of the dilutions prepared from the concentrated extracts when more than a day or so elapsed between their preparation and their inoculation. The results of the egg titrations suggested that in fact, there was no appreciable loss of titre when dilutions were stored for up to 10 days, even if thawed and re-frozen once during the time of storage. The small size of the breeding colony also made it impossible to pool and redistribute the babies at random among the various mothers in the manner adopted by some other workers, for example Melnick & Ledinko (1950). However, it does not seem probable that these factors could have accounted for the irregularities observed in the results of the mouse titrations.

The plan adopted was to examine the pregnant does daily until at least two litters had been born during the previous 24 hr. and several more appeared likely to be born during the next few days. The following day an ampoule of the pool to be tested was opened and serial ten-fold dilutions prepared in sterile skim milk with added antibiotics. The two or more litters ready for inoculation were then injected and the unused dilutions stored at $-20°$ C. until litters became available: in no case were dilutions stored for more than 10 days; if not used during this time they were discarded. A control egg titration on the same set of dilutions was usually carried out after most of the dilutions had been inoculated into mice.

(*f*) *Injection of the mice*

The mice were inoculated intraperitoneally at the age of 24–48 hr. The injections were made with a 1 ml. tuberculin syringe carrying a $\frac{1}{4}$ in., 27-gauge needle. The needle was introduced obliquely through the left lower quadrant of the abdominal wall and the inoculum delivered high up in the peritoneal cavity, ventral to the

stomach. The volume of the inoculum was 0·05 ml. There was always a little regurgitation of fluid; but if the needle was sharp and the injection made slowly the volume regurgitated was usually small and apparently fairly constant. Variation in dose due to regurgitation was certainly insufficient to account for the irregularities observed in the results of the titrations.

(*g*) *Observation of the inoculated mice*

After inoculation the litters were examined daily, the surviving mice counted, sick mice noted and any dead bodies removed for histological examination. For reasons discussed below, mice dying during the first 48 hr. after inoculation were excluded from the calculations. The total duration of the observation period was limited to 10 days as soon as it was realized that all but a negligible proportion of the deaths occurring outside this period were non-specific.

(*h*) *Histological examination of the dead mice*

All dead bodies recovered during the period 2–10 days after inoculation, except those showing obvious signs of putrefaction, were examined histologically. None of the four strains produced paralysis after intraperitoneal inoculation, although the original unadapted virus from which the three F lines had been derived did so. All four strains, however, produced small cord lesions in a few of the inoculated mice.

(*i*) *Controls*

Three groups of control litters were observed in parallel with the titrations. One group consisted of nine litters which received no inoculum at all: the second group comprised twenty litters inoculated with sterile skim milk with added antibiotics: the third group comprised eleven litters inoculated with 20 % baby mouse extract during the course of a series of normal mouse to normal mouse passages. In addition, a number of litters inoculated with 10 % horse-serum broth as controls for some earlier experiments are included for comparison. The behaviour of the control litters is summarized in Table 1.

Table 1. *Control litters*

Inoculum	Overall mortality 0–10 days	Mice dead in first 48 hr. after inoculation	Mice dead 2–10 days after inoculation including mice eaten	Mice dead 2–10 days after inoculation excluding mice eaten
Nil	9/60 = 15 %	7/60 = 12 %	2/53 = 4 %	1/52 = 2 %
Milk	59/151 = 39 %	8/151 = 5 %	51/143 = 36 %	5/97 = 5 %
Mouse extract (20 %)	38/74 = 51 %	16/74 = 22 %	22/58 = 38 %	1/37 = 3 %
Broth	23/47 = 50 %	7/47 = 15 %	15/40 = 38 %	—

RESULTS

The results of the replicate titrations of the four pools and the behaviour of the milk and the mouse-extract controls are shown graphically in Fig. 1. Mice dying during the first 48 hr. after inoculation have been excluded. The numbers in

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parentheses at the head of each column represent the numbers of mice in each dosage group and the total height of the columns indicates the percentage mortality during the period 2–10 days after inoculation. It will be seen that although there appears to be some regular relationship between dose and mortality in the results of the F/R and GOS/CA titrations, no such relationship is apparent in the results

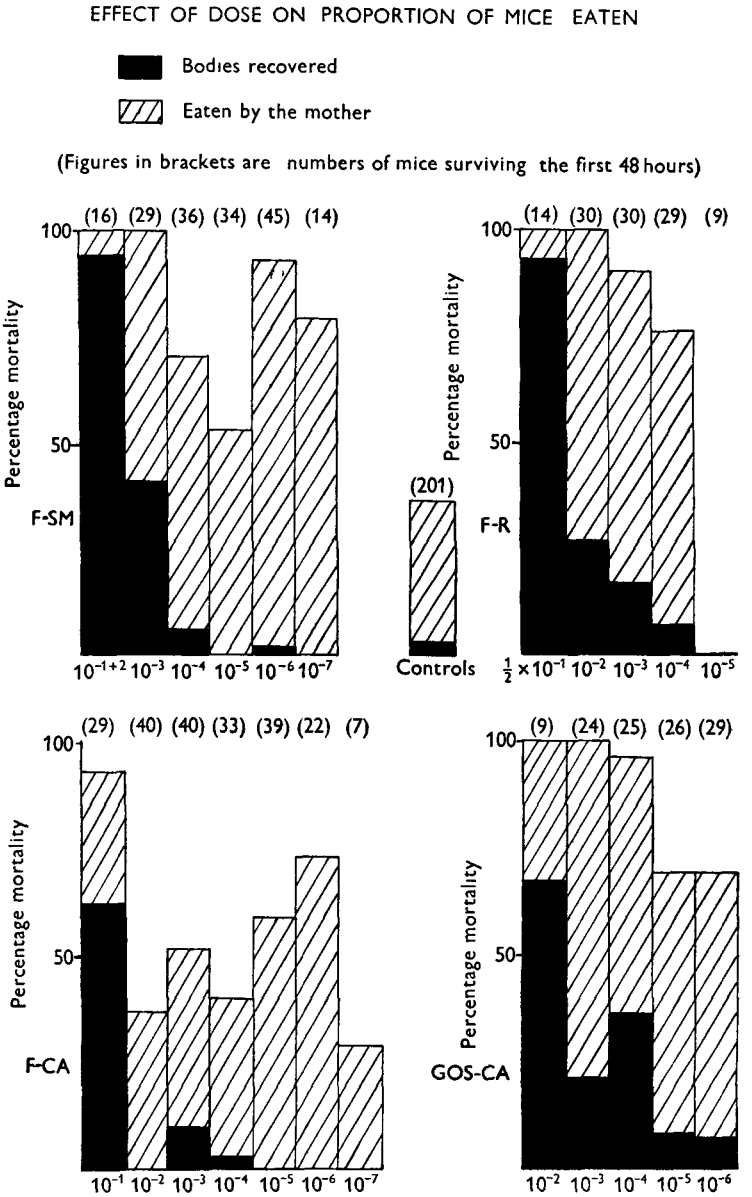

Fig. 1. A graphic representation of the pooled results of replicate titrations of four strains of virus. Mice dying during the first 48 hr. have been omitted. The heights of the solid black columns represent the numbers of dead bodies recovered as percentages of the mice surviving the first 48 hours.

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of the titrations of the other two strains, although more mice were used for titrating these two strains than for F/R and GOS/CA. It is also clear that, except at the larger doses, most of the dead mice were eaten by the mothers.

Smaller numbers of mice were inoculated with the highest and lowest dilutions tested than with the intermediate dilutions in order to make the best use of the available litters. As pointed out by Bliss (1935), each animal inoculated with a dose which produces a response in about half the animals inoculated, yields as much information as ten animals inoculated with a dose which produces a response in 1% or 99%.

Histological examination of dead mice was carried out whenever possible; however, many of the bodies recovered failed to give histological preparations which could be interpreted with any confidence. This was mainly due to advanced post-mortem change, which often occurs very rapidly because of the tendency of the mothers to push dead bodies down to the bottom of the nest where they are 'incubated' by the mother and the other babies. Of those mice whose bodies were recovered and which yielded satisfactory histological preparations only three failed to show specific lesions out of fifty-eight examined. This suggests that if a mouse dies and is not eaten by the mother it is likely to have been infected. One is not justified of course, in assuming that those mice eaten by the mother were *not* infected.

ANALYSIS OF RESULTS

(a) *Period of observation*

In seeking for causes of the irregularity in the relationship between dosage and mortality, the first factor considered was the duration of the observation period. It was felt that the omission of mice dying during the first 48 hr. might be in part responsible. However, as shown in Table 2, the inoculated litters showed a mortality in the first 48 hr. which was certainly no greater than that of the controls:

Table 2. *Deaths during the first* 48 *hr. after inoculation*

Group of litters	Mice dying during the first 48 hr. after inoculation	
Controls		
Uninoculated	7/60 = 12%	M. 38/332 = 11·5%
Broth	7/47 = 15%	
Milk	8/151 = 5%	
Mouse extract	16/74 = 22%	
Inoculated groups		
F/SM-passage*	21/326 = 7%	M. 78/1003 = 7·8%
F/SM-titration	28/202 = 14%	
F/R-titration	3/114 = 3%	
F/CA-titration	13/235 = 6%	
GOS/CA-titration	13/126 = 10%	

* The total (326) includes mice from some additional passage litters not included in Table 3.

indeed all but the F/SM-titration group show mortalities during this period which are lower than the mean for the controls and lower than the uninoculated control group. The F/SM-passage group of litters included in this table constitutes those

litters used during the twenty-one intraperitoneal passages of the original F virus in mice; the pool of F/SM was prepared from the mice of the 21st passage. The failure of the inoculated mice, especially those of the F/SM-passage group, to show a higher mortality than the controls during the 48 hr. following inoculation suggests that no appreciable number of specific deaths occurs during this period even with large doses of virus.

It was less easy to be certain that specific deaths were not occurring after the expiry of the 10-day observation period, more especially as most of the babies dying between the tenth and fifteenth day were eaten or badly mutilated by the mothers. Many of the litters were, in fact, observed for longer than 10 days and the inclusion of deaths occurring after 10 days does nothing to improve the relationship between dose and response. In addition, no mouse killed and examined more than 10 days after inoculation has shown histological evidence of infection.

(*b*) *Non-specific deaths*

Table 1 shows that even the uninoculated controls showed a mortality of 15% (9/60). One would scarcely regard a stock of adult animals of which one in seven was likely to die during an observation period of 10 days, in the absence of any experimental treatment, as suitable for a biological assay. However, mortalities of this order are probably inevitable if very young animals are to be used. If mice dying during the first 48 hr. and mice eaten by the mother are omitted, the mortality falls to 2%, but this is still not negligible.

The inoculated controls show much greater mortalities, although deaths in the first 48 hr. are not increased, except possibly in the mouse-extract group (22%). In addition, it will be noted that the extra deaths in the inoculated groups almost all comprise mice eaten by the mothers. Separate records of mice eaten and of bodies recovered were not kept for the broth group: however, most of the fifteen mice which died during the period 2–10 days after inoculation were eaten by the mother.

The explanation of the increased total mortality among the inoculated controls as compared with the uninoculated controls is obscure. It might be due to one or more of the following factors: disturbance of the mother and babies at the time of inoculation (subsequent disturbance was the same for both inoculated and uninoculated groups), the volume of the inocula, trauma at the time of inoculation or toxicity of the inocula. The fact that nearly all the extra deaths represent mice eaten by the mothers would perhaps argue in favour of the first of these. It is well known, especially with rabbits, that disturbance of the nest during the first few days of life is liable to make the mother destroy her young. However, if this be the correct explanation one would have to postulate a latent period of at least 48 hr. before the mother started to eat her babies in order to account for the fact that the inoculated groups do not show an increased mortality during the first 48 hr. after inoculation. Another difficulty is that the extra deaths continue to occur right up to the end of the observation period of 10 days: if the disturbance at the time of inoculation is sufficient to exert an effect on the mother for as

long as this, it is surprising that she does not more often destroy all her babies. Only three out of thirty-six inoculated control litters showed a mortality of 100%.

Much the same objections exist to accepting the other factors as explanations of the increased mortality. The volume of the inoculum might well be expected to have some effect, corresponding as it does to an intraperitoneal injection of about $2\frac{1}{2}$ l. in a 70 k. man. However, if this factor were responsible for the extra deaths one would surely expect some increase in the mortality during the first 48 hr. Death due to mechanical trauma at the time of injection would be unlikely to be delayed beyond the first 2 days, unless it were due to infection following the introduction of bacteria from outside or injury to a hollow viscus. None of the mice inoculated with the control inocula, killed and examined histologically, have shown evidence of generalized bacterial infection, although many of them would inevitably have died later. Toxicity of the inocula is harder to exclude. There are, however, a number of reasons for rejecting it as the main explanation of the extra deaths. The first of these is that, as shown in column 4 of Table 1, the mortality in the period 2–10 days after inoculation is practically the same for all three of the inoculated control groups, although the inocula are different. The second point is that it is difficult to see why the proportion of mice eaten by the mothers is so much greater among the inoculated controls (90%) than among the uninoculated (50 %). The third reason is that the deaths are distributed more or less uniformly throughout the observation period, in contrast to what one would expect and to what is observed when the inoculum actually contains a lethal agent (see Fig. 2). The fact that the mortality in different litters varies from nil to 100% would suggest an improbably large variation in the susceptibility of the different litters in view of the fact that the mice all belonged to a closely inbred strain. A final point, which is perhaps significant, is that the mice which received the control inocula showed no constant histological abnormality.

A high non-specific mortality does not necessarily invalidate the results of a biological assay, but it does introduce two possible sources of error. The first is that any estimate of the number of deaths to be regarded as non-specific is subject to errors of random sampling. The second is that, in general, the only way of estimating the number of non-specific deaths among the test animals is by reference to uninfected controls. This implies an assumption that the non-specific mortality is unaffected by the specific mortality. It is clear from Fig. 2 that this assumption is not justified in the present case. Mice of the infected F/SM–P group which, in the absence of infection, would have died on days 8–11 did not do so because they had died earlier, presumably killed by the virus.

These mice would have died anyway, and for most purposes it probably does not matter very much whether they were killed by the virus or not. However, such a variation in the proportion of non-specific deaths will affect the shape of the dose-response curve and any attempt to assess the capacity of the viruses to kill mice must surely treat them as specific. The relationship between the numbers of specific and non-specific deaths cannot very well be determined experimentally, and one has to be content with the knowledge that a relationship of some kind

exists and that probably the number of non-specific deaths decreases as the number of specific deaths increases.

It will be noted from Fig. 1 that with two of the strains tested (F/SM and GOS/CA) even the smallest doses used resulted in a total mortality greater than that of the controls. This could, of course, mean that even these doses were large

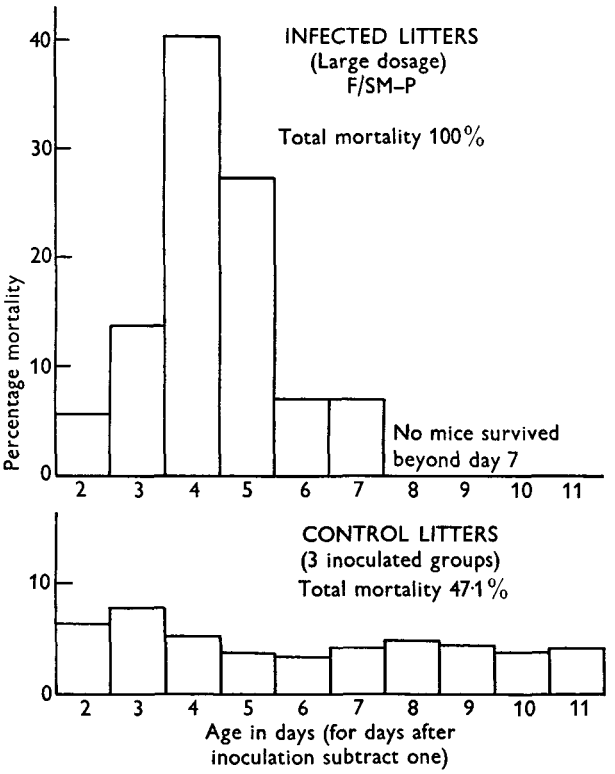

Fig. 2. The distribution of deaths by days after inoculation. Upper graph: a group of litters inoculated with large doses of virus and showing a total mortality of 100%. Lower graph: the three inoculated control groups; total mortality 47·1%.

enough to result in the death of some of the mice which would otherwise have survived or it could be due to errors of random sampling and differences between litters.

(*c*) *Cannibalism*

The high incidence of maternal cannibalism has already been emphasized. It remains to decide whether the eating of babies by the mothers can be held responsible for the irregularities observed in the results of the replicate titrations. Many of those who have used very young mice for titrations of viruses, for example Lennette & Koprowski (1944) and Melnick & Ledinko (1950), entirely omit from their calculations those mice which are eaten, basing their result on the number of mice which survive and the numbers which die without being eaten by the mothers. Litters of which most of the mice are eaten are regarded as 'unsatisfactory' and

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ignored altogether. Workers with the Coxsackie group of viruses, such as Dalldorf, Sickles, Plager & Gifford (1949) and Melnick & Ledinko (1950), kill all mice which show paralysis and treat their deaths as specific. However, the end-point which they calculate can scarcely be regarded as an LD_{50}; more especially as Melnick himself (1950) has shown that mice with paralysis due to these agents may recover.

The omission of unsatisfactory litters can only be condoned if absolutely inflexible and unambiguous criteria for their recognition are layed down in advance. Judging each case on its merits introduces a danger of intolerable personal bias whereby the experimenter will tend to get those results which he expects.

The omission of individual mice eaten by the mothers could only be justified on theoretical grounds if it could be shown that the mice eaten represented a random sample of the litter: that is to say, if the proportion which would have survived if they had not been eaten is the same as the proportion which actually survive of those which are not eaten. The assumption that the mice eaten represent a random sample of the litter in the sense defined above would appear to rest on little more than wishful thinking, and, as shown below, is almost certainly false.

Probably the most direct way of testing the assumption that the eaten mice are a random sample of the litter would be to inoculate a large number of litters with a dose of virus which produced an overall mortality of, say, 60%. Separate mortalities would then be calculated from those litters in which no cannibalism occurred and from those in which it did. If the two mortalities were about the same the assumption could probably be regarded as justified. However, formal proof would still be wanting as there would be no evidence to show that the litters were randomly distributed between the two groups in respect of all characteristics except the presence or absence of cannibalism. Unfortunately the data do not include a group of litters, inoculated with the same dose of the same virus, which contains enough litters in which none of the babies were eaten for the mortality among that group to be computed with any certainty. However, such figures as are available strongly suggest that the mortality among litters in which no cannibalism occurs is always substantially lower than the mortality among those litters receiving the same inoculum in which cannibalism does occur; except when the dose of virus is large and the mortality in both groups approaches 100%

The available data also show that the proportion of babies eaten is correlated with a number of other factors in such a way as to make it seem most improbable that the eaten babies are in fact random samples of the litters.

The most striking of these correlations is that between cannibalism and dose of virus. It is clear from Fig. 1 that far fewer mice are eaten when the dose of virus is large than when it is smaller or nil (the controls). Even when two doses produce mortalities of 100%, the litters inoculated with the larger dose show a smaller proportion of babies eaten.

In order to try and elucidate this remarkable and unexpected finding the data have been analysed to see whether any of a number of other factors were correlated with the proportion of mice eaten.

So as to provide a reasonably large number of litters, some of the litters used for the twenty-one passages of the 'F' strain in mice and, for some purposes only,

a miscellaneous group of litters (designated group D) have been considered with the litters used in the actual titrations. The group D litters had all been inoculated with intermediate passage virus of one or other of the four strains, usually in large dosage. Table 3 gives some details of the various groups of litters.

Table 3. *Details of the six groups of inoculated litters*

Group	No. of litters	No. of mice	Average size of litters	No. of litter days	Overall mortality (%)	Percentage of bodies recovered
A. F/SM-passage†	19	127	6·7	72 (39)*	100	76/127 = 60 %
B. F/SM-titration	27	204	7·5	204 (67)	84	30/172 = 17 %
C. F/R-titration	16	114	7·1	54 (18)	86	30/96 = 31 %
F/CA-titration	32	248	7·8	313 (89)	55	23/129 = 18 %
GOS/CA-titration	16	126	7·9	129 (48)	85	25/99 = 25 %
D. Miscellaneous (all inoculated with *Herpes simplex*)	56	437	7·8	263 (112)	81	125/354 = 35 %

* Days on which deaths occurred in brackets.

† Some of the early litters in which eaten mice were not separately recorded and litters of which mice were killed for passage or histological examination are not included.

Since it seemed unlikely that the dose directly influenced the proportion of mice eaten, it seemed probable that the dose exerted its effect in some indirect way. The first correlation tested was between the proportion of mice eaten and the number dying during successive 24 hr. periods. Fig. 3 shows a reasonably good correlation of the type that would be expected if the mother tended to eat all her sick babies but was unable to do so if more than two died on the same day.

Unfortunately what may be termed 'the appetite hypothesis' cannot readily explain the observed correlation. If the figures on which Fig. 3 is based are examined, it is found that a mother tends either to eat all her babies which die on a particular day or none: only exceptionally does she eat some and leave the rest. One mother managed to eat eight babies in 24 hr. Observation suggests that mothers seldom, if ever, eat babies which are already dead.

Fig. 4 shows graphically that there is some correlation between mortality and cannibalism. However, the correlation is not very striking and is probably merely an expression of the fact that both are correlated with the dose of virus.

Apparent correlations between age and cannibalism and between time after inoculation and cannibalism are, as shown in Fig. 5, merely expressions of the numbers of mice dying on different days. The effects of age and of time after inoculation cannot, of course, be distinguished because all the mice were inoculated at the same age.

Investigation of the relationship between litter size and cannibalism reveals that the two are poorly correlated, although there is a suggestion that litters of 10 or more show a smaller proportion of bodies recovered than do smaller litters (see Fig. 6). This figure also shows that there is no necessary correlation between mortality and cannibalism.

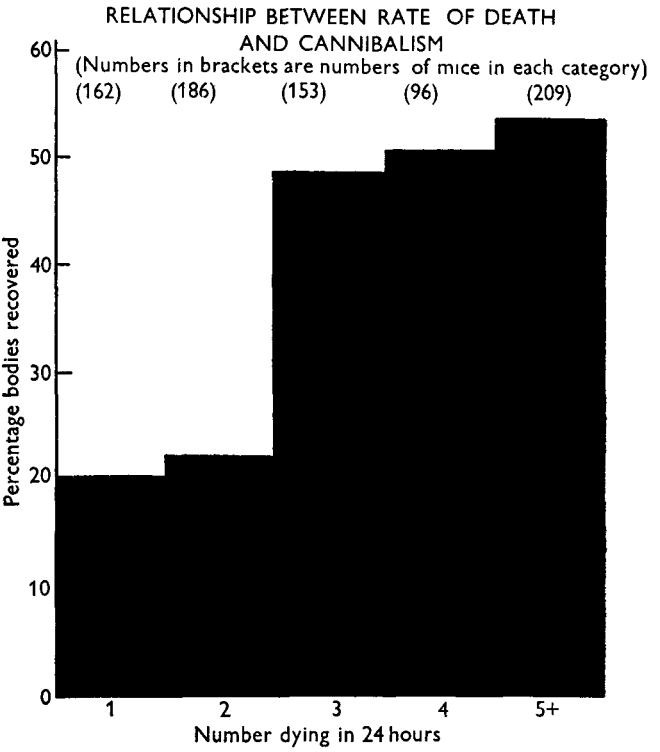

Fig. 3. The relationship between the numbers of mice dying in 24 hr. and the proportion of bodies recovered, showing that on days when one mouse dies only 20 % of the bodies are recovered whereas on days when five or more die over 50 % are recovered.

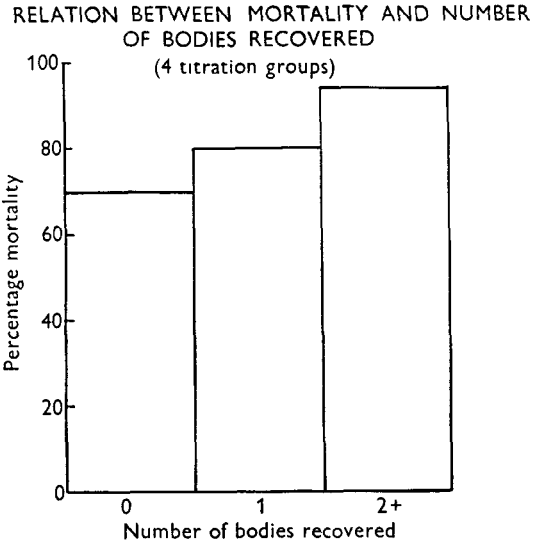

Fig. 4. The relationship between mortality and the number of bodies recovered.

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Litter size and dose showed no evidence of correlation.

It would probably be difficult to design an experiment specifically to show whether the proportion of babies eaten depended on the strain of virus inoculated. However, quite by chance, the present data do provide an opportunity of investigating this point. Table 4 shows that three of the titration groups of litters are essentially the same in respect of all those factors shown above to influence the

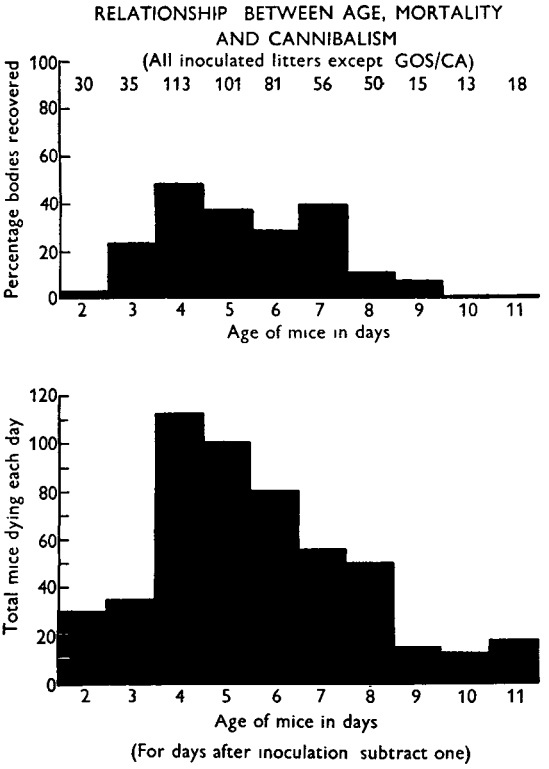

Fig. 5. Upper graph: the relationship between age of mice and the proportion of bodies recovered. Lower graph: the relationship between age of mice and the numbers of mice dying each day.

Table 4. *The influence of strain of virus on the proportion of bodies recovered*

Strain of virus	Overall mortality (%)	Litters showing 100% death %	Mean no. per litter	Litter-days with no deaths* (%)	Bodies recovered† (%)
F/SM‡	84	63	7·5	67	17
F/R‡	86	69	7·1	67	31
GOS/CA‡	85	63	7·9	63	25

* Detailed analysis of numbers of litter-days with different numbers of deaths indicates that there is no significant difference between the three groups. ($\chi^2 = 7{\cdot}58$; 6 D.F.; P = approx. 0·3.)

† Differences probably significant ($\chi^2 = 8{\cdot}18$; 2 D.F.; $P = 0{\cdot}016$) (see text).

‡ Groups of litters used for titration.

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incidence of cannibalism; nevertheless, the proportion of bodies recovered is different, and the difference is probably significant ($P = 0{\cdot}016$).

It is clear that the proportion of babies eaten by the mothers is related to the dose of virus and probably to the strain inoculated. While this does not prove that the mice eaten are not a random sample of the litter, it surely argues against it.

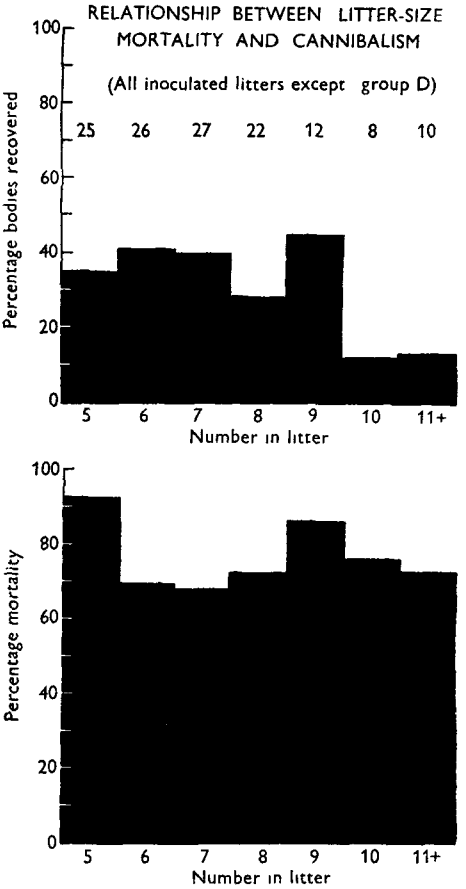

Fig. 6. Upper graph: relationship between litter-size and the proportion of bodies recovered. *Lower graph: the relationship between litter-size and mortality.*

Instead the data suggest that a mother is more likely to eat a baby which would otherwise have survived than one which would have died from the infection. Figs. 1 and 7 show that the correlation between dose and mortality is at least as good if the eaten mice are treated as *survivors*, as it is if they are omitted, that is treated as a random sample of the litter. If the eaten mice are treated as specific deaths the correlation is very poor, especially with F/SM and F/CA.

(*d*) *The influence of the mother*

The top row of Table 5 shows the mortality observed among the seven litters inoculated with F/CA virus at a dilution of 10^{-3}. The litter of one was originally larger; all but one of the mice died during the first 48 hr. after inoculation and have

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been omitted. The overall mortality is close to 50% (actually 52%), but the individual litters show mortalities ranging from 0 to 100%. The bottom row of Table 5 shows the probability of observing the mortalities shown in the top row if each litter represented a random sample from a population with a mortality of 50%. It will be seen that three of the values of P are less than 0·1. Mr T. H. Hollings-

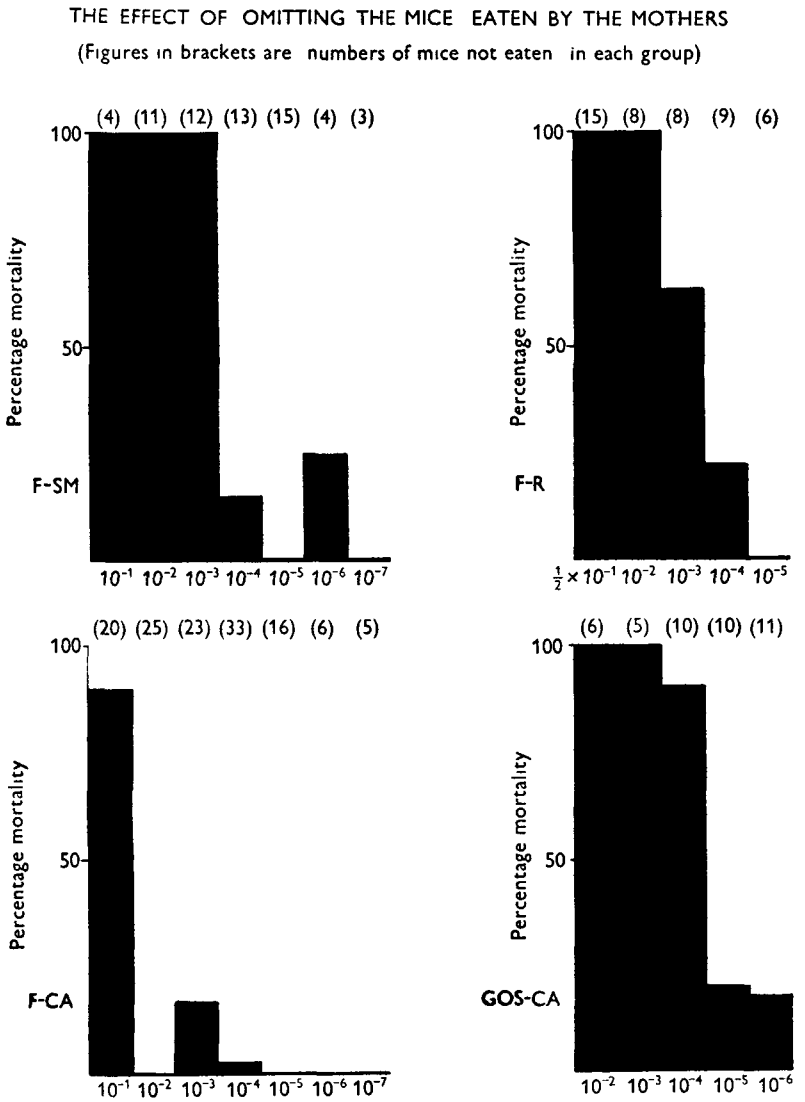

Fig. 7. The relationship between dose of virus and mortality, if mice failing to survive the first 48 hr. after inoculation and mice eaten by the mothers are omitted.

Table 5. *Litters inoculated with F/CA at a concentration of* 10^{-3}

Observed mortality ...	5/7	5/5	6/9	1/1	4/8	0/5	0/5	Total 21/40
(%)	71	100	67	100	50	0	0	52
P	0·16	0·03	0·16	0·5	0·27	0·03	0·03	—

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worth of the Department of Human Ecology, University of Cambridge, has kindly investigated the probability of observing a sample of seven litters as variable as, or more variable than, these if each litter represented a random sample of a uniform population. The conclusion which he reaches is that $P < 0{\cdot}0214$ (and probably $< 0{\cdot}01$).

This is barely significant, but as all the other dosage groups show a similar wide variation in mortality between the litters, except where the dose is large and all, or nearly all, the litters show mortalities of 100 %, it is clear that errors of random sampling cannot wholly account for the observed differences between litters receiving the same inoculum. It is also unlikely that the differences were genetically determined, since all the mice used belonged to the same in-bred strain. In seeking for alternative explanations of the observed differences between litters, undoubtedly the most important factor to be considered is the behaviour of the mothers. Individuals of even the most highly in-bred strains of animals show minor temperamental variations, and it would be surprising if the mothers did not vary in their response to disturbance of their litters; some eating their young, some abandoning them and some remaining unperturbed. Even such things as the quality of the nest which the mother builds and maintains for her babies must appreciably influence their chances of survival.

If it be conceded that, at least when closely in-bred lines of mice are used, differences between litters are mainly a reflexion of differences between mothers, the obvious implication is surely that the litter and not the individual mouse must be regarded as the unit of titration. In order to achieve the sort of accuracy obtainable with ten adult animals per dose one must use not ten baby mice but ten litters.

DISCUSSION

The foregoing analysis of the results of the replicate titrations of the pools of the four virus strains has shown that titrations in very young animals are beset by difficulties which are not encountered when older animals are used and has gone some way towards elucidating the causes of these difficulties.

These special difficulties are the unavoidably high non-specific mortality, the eating of babies by the mothers and the differences which exist between the response of different litters subjected to the same treatment, or even to no treatment at all (the uninoculated controls).

From the purely practical point of view it is sufficient to take note of these difficulties and exercise caution in the interpretation of the results, especially where only a small number of litters has been used. Only further experience can decide whether mice eaten by the mother should be omitted from the calculations or treated as survivors. There are theoretical objections to both courses and that one should be adopted which can be shown to give the most regular relationship between dosage and mortality and the most regularly reproducible titres. Non-specific deaths among the inoculated groups must be estimated from the mortality among the controls, since no better method is available. The effects of variation between the mothers can only be countered by the use of a sufficient number of

litters. The effects of maternal variation could be still further reduced if it were possible to devise some method of marking the babies in a way that was sufficiently permanent but which did not injure them or render them unacceptable to the mother (Fisher, 1947).

From the theoretical aspect, the most interesting implication of these results is the light which they throw on the question of maternal cannibalism. The correlations between cannibalism and dose of virus and between cannibalism and the strain of virus were quite unexpected. It is commonly believed that a mother is more likely to eat those of her babies which are weakly or sick. However, the results presented here suggest that the mothers are *less* likely to eat mice due to die from the effects of infection by the virus than mice which would otherwise have survived. This implies either that the mother somehow recognizes and avoids eating babies dying from an infection or that it is never the sickly babies which are the most likely to be eaten. The apparently higher incidence of cannibalism in the largest litters of ten or more (see Fig. 6) perhaps suggests that one factor determining the incidence of cannibalism is maternal shortage of some essential food constituent, such as amino-acids or minerals, which is likely to be most severe in those mothers which have borne the largest litters. Claims to have reduced cannibalism by supplementing the mothers diet with such things as cheese and bacon rind are perhaps explicable on these lines.

Whatever other factors may be involved in determining the incidence of cannibalism, disturbance of the mother and her newborn young undoubtedly plays a predominant role, although it is not clear how the disturbance produces cannibalism or even whether it acts primarily on the mother or on the babies.

It is not improbable that the variation between mothers and the tendency of the mothers to eat their babies differ in different strains of mice. Anyone contemplating using very young mice for *in vivo* titrations would be wise to test a number of different strains with a view to selecting that strain showing the smallest incidence of cannibalism and the least variation between litters.

SUMMARY

This paper describes efforts to determine the LD_{50} for mice, 24–48 hr. old, of pools of four strains of *Herpes simplex* virus, using the intraperitoneal route of inoculation.

The results have been analysed in an attempt to account for the very irregular relationship between dose of virus and mortality which was observed.

It is clear that, in addition to those difficulties which beset the interpretation of the results of any biological assay, titrations in very young animals raise difficulties of their own. The principal sources of difficulty are the high and uncertain non-specific mortality, the tendency of the mothers to eat their babies and individual variation between the litters. The latter, it is suggested, is mainly a reflexion of differences between the mothers, at least when a closely inbred strain of test animals is used.

Interesting and unexpected correlations were observed between the dose of

virus and the proportion of mice eaten by the mothers and between the strain of virus and the proportion of mice eaten by the mothers.

It is emphasized that the results of titrations in very young animals must be interpreted with the greatest care and that in carrying out titrations the litter rather than the individual baby mouse should be regarded as the unit. Mice eaten by the mothers are probably best ignored altogether, although there are grave theoretical objections to doing this.

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REFERENCES

Beveridge, W. I. B. & Burnet, F. M. (1946). The cultivation of viruses and rickettsiae in the chick embryo. *Spec. Rep. Ser., med. Res. Coun., Lond.*, no. 256.

Bliss, C. I. (1935). The calculation of the dosage mortality curve. *Ann. appl. Biol.* **22**, 134–67.

Dalldorf, G., Sickles, G. M., Plager, H. & Gifford, R. (1949). A virus recovered from the feces of 'poliomyelitis' patients pathogenic for suckling mice. *J. exp. Med.* **89**, 567–81.

Fisher, R. A. (1947). *The Design of Experiments*, 4th ed. pp. 210 *et seq.* Edinburgh.

Kilbourne, E. D. & Horsfall, F. L. (1951*a*). Primary *Herpes simplex* virus infection of the adult. *Arch. intern. Med.* **88**, 495–502.

Kilborne, E. D. & Horsfall, F. L. (1951*b*). Studies of *Herpes simplex* virus in newborn mice. *J. Immunol.* **67**, 321–9.

Laboratory Animals Bureau (1953). *Catalogue of Uniform Strains of Laboratory Animals maintained in Great Britain.* Serial No. 776*a*. Medical Research Council Laboratories, London, N.W. 3.

Lennette, E. H. & Koprowski, H. (1944). Influence of age on the susceptibility of mice to infection with certain neurotropic viruses. *J. Immunol.* **49**, 175–91.

Maggiora, A. & Valenti, G. L. Ueber den Virus des exsudativen Typhus bei Huhnern. *Z. Hyg. InfektKr.* **48**, 280–326.

Melnick, J. L. (1950). Studies on the Coxsackie viruses: properties, immunological aspects and distribution in nature. *Bull. N.Y. Acad. Med.* **26**, 342–56.

Melnick, J. L. & Ledinko, N. (1950). Immunological reactions of the Coxsackie viruses. I. The neutralization test: technic and application. *J. exp. Med.* **92**, 463–82.

Sigel, M. M. (1952). The influence of age on susceptibility to virus infections with particular reference to laboratory animals. *Ann. Rev. Microbiol.* **6**, 247–80.

Speck, R. S., Jawetz, E. & Coleman, V. R. (1951). Studies on *Herpes simplex* virus. I. The stability and preservation of egg-adapted *Herpes simplex* virus. *J. Bact.* **61**, 253–8.

Theiler, M. (1930). Studies on the action of yellow fever virus in mice. *Ann. trop. Med. Parasitol.* **24**, 249–72.

Trevan, J. W. (1927). The error of determination of toxicity. *Proc. Roy. Soc.* B, **101**, 483–514.

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