STUDIES ON THE PSITTACOSIS-LYMPHOGRANULOMA GROUP

1. The Pattern of Multiplication of Meningopneumonitis Virus in the Allantois of the Chick Embryo*

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The psittacosis-lymphogranuloma group of viruses are characterized by a number of properties which set them off from most viral agents. Among these properties are, (1) size of the agents, (2) complex developmental cycle, (3) susceptibility to sulfonamides and antibiotics, (4) possible extracellular phases of growth, and (5) presence of a large quantity of common antigen(s) which makes it difficult and often impossible to differentiate between infections caused by the various members of this group by means of serologic tests.

These properties have been subjected to extensive studies in the past. Nevertheless, two basic questions still remain unanswered: (*a*) In view of the first four properties listed above, should these agents be classified as true viruses, or perhaps as forms intermediate between viruses and higher microbes? (*b*) Relative to the property listed under (5), does there exist in these agents a specific antigenic fraction which may make it possible to distinguish between infections with any of these agents on the basis of complement fixation, agglutination, or precipitation reactions?

The first question is of more than taxonomic importance for the answers may bear on at least two highly significant problems: (1) Evolution in the microbial world and (2) mechanisms of the action of certain substances (antibiotics) on obligate parasites. The second question is of great importance for practical reasons because at present there are no satisfactory procedures for differential diagnosis of infections by members of the psittacosis-lymphogranuloma group except for virus isolation.

Previously published reports on the growth of viruses in the psittacosis-lymphogranuloma group concerned primarily the morphologic changes in the growth cycles of these agents, with little emphasis on the development of

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infectivity. Recent contributions to the understanding of multiplication of viruses, especially of bacteriophage (1) and influenza virus (2), suggested a new approach to this problem. It was thought that a study of the pattern of multiplication would help answer the first question as to the position of the psittacosis-lymphogranuloma group in the microbial world; *i.e.*, do any or all phases of the growth cycle conform more to the phenomena observed in the multiplication of influenza and bacteriophage or to those of bacteria? This paper concerns primarily the methods and procedures employed in the study of the dynamics of reproduction and the general pattern of multiplication observed with meningopneumonitis virus in the allantois of the chick embryo.

Methods and Materials

The Virus.—The virus used throughout these studies has been the Cal 10 strain of meningopneumonitis (MP) obtained through the courtesy of Dr. Sam C. Wong, Lederle Laboratories, Pearl River, New York. This strain has been well adapted to growth in the allantoic cavity of the developing chick embryo. Freshly harvested virus or virus stored at −70°C. in the CO_2 box was used at all times.

Developmental Cycle.—The developmental cycle of MP was studied in the allantois of the chick embryo. 8½ day old embryos were inoculated with 0.2 ml. of appropriately diluted virus by the allantoic route and were incubated at 36°C. At certain intervals following inoculation, 4 living embryos were selected at random and the allantoic fluids were harvested, pooled, and titrated for infectivity in mice and/or chick embryos. Following their removal from these same embryos, the pooled allantoic membranes were washed in 5 to 10 changes of a mixture of infusion broth and buffered water (0.02 M phosphate buffer),[1] ground in a mortar with sterile alundum (90 mesh), and the 20 per cent suspensions which were prepared in the broth-buffered water mixture were titrated for infectivity.

The Titrations for Infectivity.—Serial tenfold dilutions of the materials to be studied were prepared in a broth-buffered water mixture and determinations for infectivity were performed in mice and/or chick embryos.

Titration in Mice.—Albino Swiss mice of the NIH strain weighing 12 to 15 gm. were inoculated by the intracerebral route with 0.03 ml. of the various dilutions used—5 mice were used per dilution. The criterion for infectivity was death of mice; the 50 per cent end-point (LD_{50}) was determined by the method of Reed and Muench. (3)

Titration in Chick Embryos.—8½ day old chick embryos were inoculated by the allantoic route with 0.2 ml. of the various dilutions used—6 embryos were used per dilution. After 6 days' incubation at 36°C., smears were prepared from the allantoic fluid of each living embryo and allowed to air dry overnight prior to staining by the method of Macchiavello. The criterion for infectivity was the presence of elementary bodies; the 50 per cent end-point (ID_{50}) was determined by the method of Reed and Muench.

Analysis of Experimental Methods

Titration Procedures.—A comparison of the 2 methods of titration described and a third method—based on the mortality of chick embryos—is shown in Table I. Mor-

[1] Buffered water—70 ml. Na_2HPO_4 (0.02 M) to 30 ml. KH_2PO_4 (0.02 M). The broth-buffered water mixture was prepared with brain heart infusion broth (Difco)—1 part broth + 2 parts of buffered water.

tality of embryos was not a satisfactory criterion for the determination of infectivity end-points because it gave the lowest end-points and rather irregular results.

Although the presence of elementary bodies in allantoic fluids is a fairly satisfactory criterion for determination of infectivity end-points, this procedure has several drawbacks: (*a*) It is a tedious method for large scale experiments; (*b*) there is the inherent difficulty of evaluating ± readings; one wonders about the significance of a very few particles resembling elementary bodies; they may actually be artefacts. (*c*) The procedure may give irregular results; negative smears in low dilutions, and occasionally some dilutions between those giving positive smears may be negative.

TABLE I

Comparison of Infectivity End-Points

Material	Dilution	Results in embryos				Results in mice Dead/inoculated
		No. inoculated	No. dead in <48 hrs.	No. dead in >48 hrs.	EB* in live eggs	
A (4/25)						
	10^{-4}	7	0	3	4+, 4+, 2+, ±	5/5
	10^{-5}	6	1	0	4+, 3+, 3+, 3+	4/5
	10^{-6}	6	1	0	3+, 3+, 3+, −, −	0/5
	10^{-7}	6	0	0	4+, 3+, 3+, 2+, −	0/5
	10^{-8}	6	0	1	2+, −, −, −	
	10^{-9}	6	0	0	?, −, −, −, −	
M-1 (4/25)						
	10^{-4}	6	0	4	4+, −	5/5
	10^{-5}	6	0	3	3+, 3+, ±	5/5
	10^{-6}	6	0	1	4+, 4+, 2+, −, −	5/5
	10^{-7}	6	0	0	4+, 2+, 2+, 2+, −	1/4
	10^{-8}	6	1	0	3+, 2+, 2+, 1+, −	0/5
	10^{-9}	6	0	0	2+, 1+, −, −, −	

* Elementary bodies.

The reason for the discrepancies with chick embryo titrations is not known. It is conceivable that technical errors such as failure to introduce virus into the allantoic cavity of every embryo in a large series of eggs, as well as such fundamental factors as autointerference, may be responsible for some of the irregularities.

The infection and death of mice inoculated intracerebrally appear to be a highly satisfactory method for determination of infectivity end-points: (*a*) the procedure is simple, (*b*) the results are quite uniform, (*c*) thus far this method has given the sharpest end-points.

Therefore, titration in mice has been selected as the method of choice for most of our experiments. Chick embryo titrations have been used when minute amounts of virus were present in preparations to be studied, since this method was more sensitive—the ID_{50} in chick embryos being about 1 to 2 logs higher than the LD_{50} in mice.

Sampling Materials for Titration.—Preliminary investigations into the methods of sampling the materials for titration included studies of three separate techniques. (1) At each interval 4 living embryos were selected at random from a previously inoculated group and the allantoic fluids were pooled and titrated for infectivity. (2) In the second method the harvested allantoic fluids from the 4 embryos were titrated individually. (3) In the third method a small amount of allantoic fluid was removed from several embryos and these same embryos were used at each interval of the growth cycle.

In Fig. 1 a comparison is made of growth curves resulting with the three techniques. There was little difference noted with methods 1 and 2; that is, the average titer of 4 separate preparations approximated the values resulting when the 4 preparations were pooled. The third method showed a more drastic decrease of virus infectivity. However, the use of this technique—repeated harvests from the same eggs—resulted

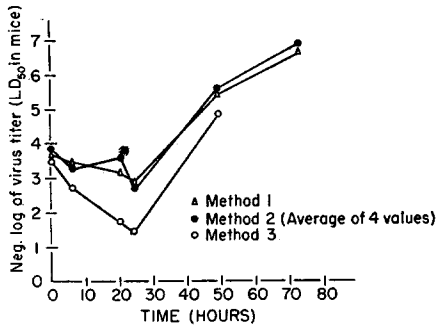

FIG. 1. Comparison of sampling methods. Changes in the amount of virus in the allantoic fluid.

in damage to the chick embryo. By 20 hours most of the original group of embryos had died and the allantoic fluids of those still living often contained red blood cells. The undue vascular and tissue trauma made this method undesirable for the study of virus growth. It should be noted that even with these undesirable side effects the method showed the same developmental pattern; a decrease of infectivity during the first 24 hours, and a subsequent increase between 24 and 48 hours.

In the experiments to be described the simplest method was employed, namely, pooling the allantoic fluids before titration.

Titration of Allantoic Membranes.—The presence of superficial virus from the allantoic fluid necessitated the washing of the allantoic membranes before their titration. Investigations on the efficiency of the washing procedure indicated that the infectious titer recorded for a given membrane suspension is a measure of the infecting, and not superficially adsorbed virus (Table II). In such studies the virus present in the membrane washings was concentrated by centrifuging these washings at 13,000 R.P.M. for 60 minutes and the resuspended sediment was titrated in chick embryos. It was noted that after 5 washings the small amount of superficial virus could not affect the infectious titer of the membrane suspension had it been allowed to remain with the membranes.

Although the results in Table II indicate typical titrations with membranes harvested at only 1 and 4 hours following inoculation of virus, it should be noted that after 4 hours less active virus is present in allantoic fluid and therefore a smaller percentage of superficial virus is found in membrane suspensions. It would seem that an experimental error in end-points due to superficial virus is insignificant.

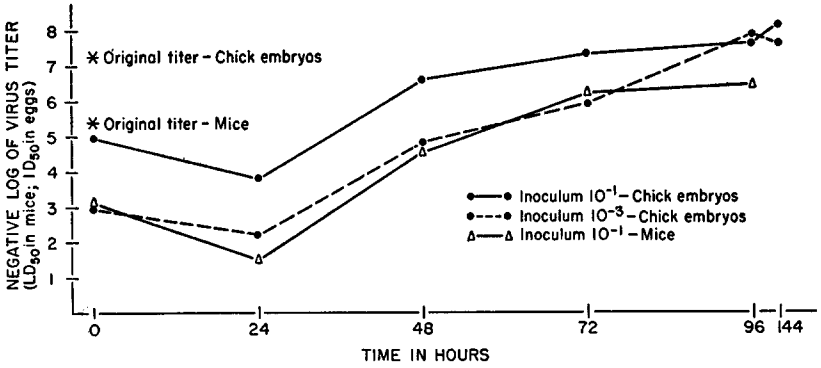

FIG. 2. Changes in amount of virus in allantoic fluids as determined by titration in mice and chick embryos.

TABLE II

Comparison between Virus Adsorbed to Allantoic Membranes and Superficial Virus

Harvest time	Titer membrane*	Titer 5th washing	Infectivity in washing
hrs.			*per cent*
1	5.41	4.00	4
4 A‡	4.00	2.00	1
4 B‡	3.00	<Undiluted	<0.1

* ID_{50} in chick embryos.

‡ Different experiments.

EXPERIMENTAL

Changes of Infectivity in Allantoic Fluids

The preliminary investigations on the development of infectivity in allantoic fluids were undertaken to: (1) Determine the general pattern of development. (2) Compare titrations in mice and chick embryos. (3) Observe the effects on the developmental pattern of varying concentrations of virus.

Fig. 2. shows the changes in infectivity in chick embryos that had received virus inocula of 10^{-1} and 10^{-3}. Allantoic fluids were harvested at daily intervals and titrated for infectivity as described. (Figures at zero time, in this experiment only, were calculated from the known original titers of the virus and the dilution factors involved. In all other experiments the materials were harvested immediately following inoculation and titrated.)

1. All curves showed a decrease of virus during the first 24 hours, with the appearance of new virus between 24 and 48 hours; the maximum titer being attained between 72 and 96 hours following inoculation.

2. Comparative titrations in mice and chick embryos on allantoic fluids harvested from embryos receiving a 10^{-1} virus inoculum showed that the procedure in embryos was more sensitive, yielding titers 1 to 2 logs higher than the mouse titrations throughout the entire cycle; otherwise, the curves were comparable.

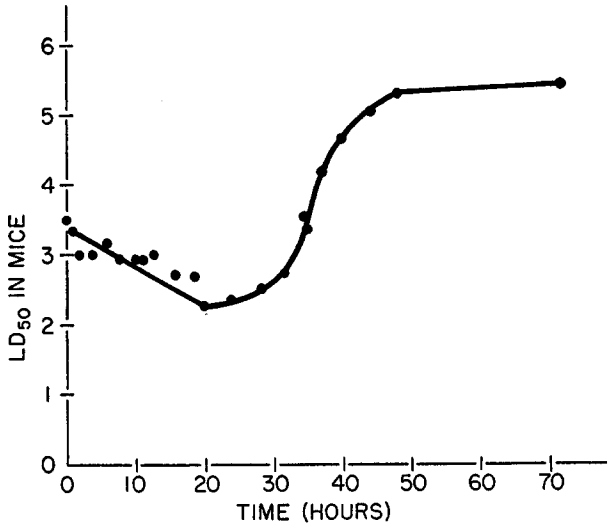

FIG. 3. Changes in amount of virus in allantoic fluids titrated in mice. Average values o six growth curves. Inoculum 10^{-2}

3. When the allantoic fluids from embryos receiving different inocula (10^{-1} and 10^{-3}) were compared by titration in chick embryos it was observed that the time required for the maximum infectivity to develop increased as the amount of virus inoculated was decreased, although the final amount of virus developing was the same. The maximum titer seemed to be lower only when minute amounts of virus, as a 10^{-7} dilution, were employed.

It can be seen that the important period for the study of the first cycle of virus growth is that between 0 and 48 hours and Fig. 3 shows the changes in the infectious titer of allantoic fluids at close intervals during the first 48 hours following inoculation with a 10^{-2} dilution of virus. This curve is actually the geometric mean of six growth curves.

A drop in the infectious titer was observed during the first 19 to 24 hours. Between 24 and 36 hours there was a rapid appearance of virus, and by 48 hours the infectious titer was about 100 times that present at zero time.

There are two possible explanations for the pattern observed with allantoic

fluids during the early stage of the growth cycle of MP virus. The slow decline in the amount of virus may reflect either (1) prolonged adsorption of virus onto the allantoic membrane, or (2) inactivation of the virus due to the temperature of incubation. In an attempt to answer this question the following experiment was effected.

Parallel Tests in Vivo and in Vitro

The volume of allantoic fluid present in $8\frac{1}{2}$ day old embryos was found to be approximately 3.8 ml. This volume of normal allantoic fluid *in vitro* was seeded with 0.2 ml. of the virus inoculum used for chick embryos, and incubated at 36°C. The dilution of virus in the *in vitro* preparation corresponds to that present in the chick embryo. Such *in vitro* experiments accompanied

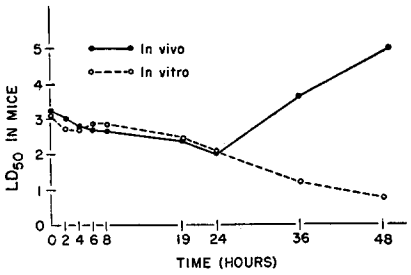

FIG. 4. Changes in amount of virus in allantoic fluid. *In vivo vs. in vitro.*

several *in vivo* experiments. Titrations of the amount of surviving virus *in vitro* were performed as was described for *in vivo* experiments. Fig. 4 shows the observed results.

It was found that the slope of inactivation of the virus *in vitro* paralleled the slope of virus reduction *in vivo*. A similar *in vitro* culture which had been refrigerated (4–6°C.) showed no decrease of virus activity in 24 hours.

The negligible difference between the two lines indicated that the amount of virus which is adsorbed to the allantoic membrane could not be determined by measuring the decrease of infectivity in the allantoic fluid.

By 48 hours new virus had appeared in the chick embryos, whereas the *in vitro* cultures showed a continued drop in titer. Thus the disappearance of infectivity from the allantoic fluid during the early stages of growth seemed to be due to the degradation of non-adsorbed virus during incubation at 36°C.

The Site of Virus Development

An implication that one might draw from titers resulting between 24 and 48 hours in the *in vitro* experiment (Fig. 4) is that the virus does not multiply in allantoic fluid; an association with host tissue seems to be necessary. However this interpretation is open to the criticism inherent in a situation in which one

studies materials out of their ecological niche. To overcome such criticism the following experiment was performed.

Allantoic fluids were harvested from chick embryos 20 hours following inoculation of virus—before the occurrence of a rise in titer—and this material was reinoculated by the allantoic route into a second series of normal embryos (0.4 ml./embryo).

Allantoic fluids were removed from a portion of the second series of embryos immediately following their inoculation and titrated for infectivity in mice. This represented the 20 hour titration. The remaining embryos of this series were incubated for 16 hours at which time fluids were harvested and titrated. This preparation contained virus which had been incubated in allantoic fluids *in vivo* for 36 hours. The titer of the 36 hour fluid was found to be 1 log lower than that of the 20 hour preparation indicating that no growth had taken place in the allantoic fluids; in fact, only continued degradation of virus occurred. These results would seem to demonstrate that the increase in the amount of infectious virus in the allantoic fluids after 24 hours is due to release of new virus from infected tissue.

The Pattern of Growth in the Allantoic Membrane

The observations thus far described have dealt with the appearance of infectivity in the allantoic fluid but the growth of the virus presumably occurs within the cells of the allantoic membrane. A study of the mechanisms of reproduction would entail investigations on the pattern of development in the host tissue.

Membranes were removed from embryos at various intervals following inoculation of virus, washed to remove superficial virus, and 20 per cent suspensions of these membrane pools were titrated in mice. Allantoic fluids were also titrated.

Fig. 5 shows the observed results.

In allantoic fluids the usual degradation of virus and subsequent increase after 24 hours were observed. The allantoic membranes showed an increase of virus infectivity during the first few hours following inoculation, after which there was a decrease of infectivity. A steep increase in the amount of virus in allantoic membranes occurred between 19 and 36 hours. This increase occurred before any observed increase of infectivity in the allantoic fluids, the latter presumably due to virus released from the cells of the allantoic membranes. Once new virus appears in the membranes their titer is higher than, or at least equal to, that in allantoic fluids.

This general pattern has appeared consistently and it seems that the maximum level of infectivity attained during the early hours in the allantoic membrane was related to the amount of virus inoculated, the higher the concentration of virus, the higher the level attained. This has been shown in experiments

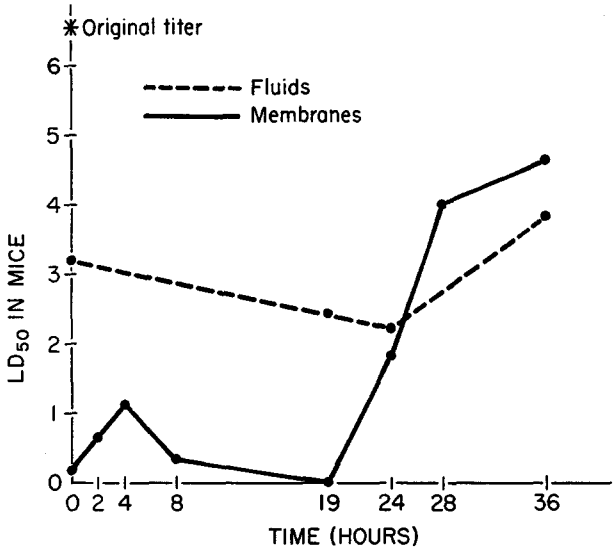

FIG. 5. Comparison of infectivity in fluids and membranes.

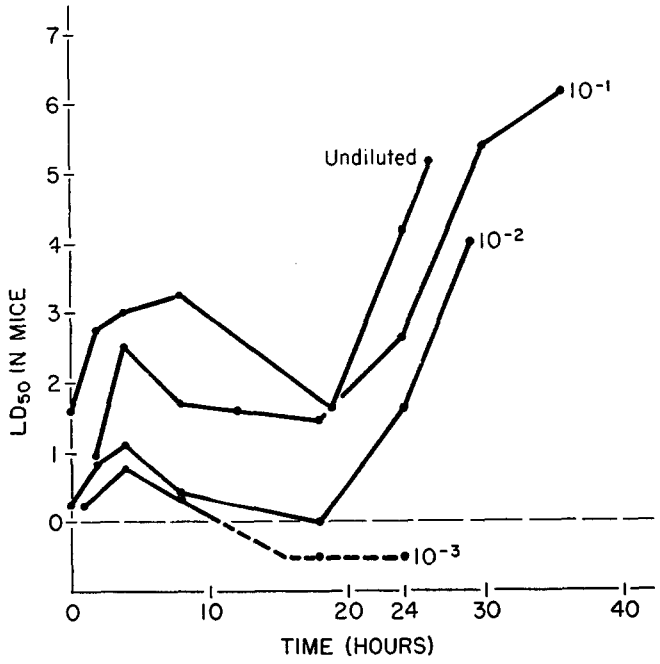

FIG. 6. Influence of dilution of virus inoculum on the amount of virus in allantoic membranes at various stages of the growth cycle.

in which virus was inoculated undiluted, and diluted 10^{-1}, 10^{-2}, and 10^{-3} (Fig. 6).

The increased infectivity in allantoic membranes during the early hours of the growth cycle corresponded to about 1 per cent of the infectivity of allantoic fluids. Results of tissue culture experiments to be reported in the next paper of this series (6) have suggested that this increase was due to adsorption of virus and not to immediate reproduction of virus in the allantoic membranes.

The decrease of infectivity after the virus had been adsorbed to the allantoic membranes occurred consistently (Fig. 6), a low level being attained at about 20 hours. With the dilutions employed, the time of appearance of new virus and the rates of formation were similar. By 30 to 36 hours there had been about a 4 log increase of virus.

DISCUSSION

Following inoculation into the allantoic cavity of the chick embryo, MP virus may follow one of two paths:

(*a*) The majority of the virus remains in the allantoic fluid and is inactivated due to the temperature of incubation. The results show that thermal inactivation follows a slope representing a first order reaction as has been described for TMV (4) and feline pneumonitis (5).

(*b*) A small percentage of the virus is adsorbed to the cells of the allantoic membrane during the first few hours following inoculation, initiating the infectious cycle.

Studies on the changes in infectivity in allantoic fluid—both *in vivo* and *in vitro*—as well as the observation that the rise of infectivity in allantoic membranes precedes that occurring in allantoic fluids strongly suggest that multiplication of this agent proceeds only in intimate association with host tissue.

The increase in infectivity in allantoic membranes following immediate initial adsorption may represent either a continued adsorption of virus or adsorption accompanied by immediate virus growth. Experiments not presented in this report suggest that the first alternative is probably the correct one (6).

Once this initial increase has been effected a rather marked decrease of infectivity occurs in the allantoic membrane suspensions—about 90 per cent in many cases. One might explain such a decrease by postulating: (*a*) A release of virus from the cells of the allantoic membrane or (*b*) The development of a non-infectious entity within the cells of the host.

Preliminary observations (6) based on experiments in different systems such as tissue culture seem to support the second alternative, but more extensive studies are being conducted at present to elucidate the involved mechanism.

Bedson (7) and Bedson and Bland (8) have described for psittacosis growth cycles in mouse spleens a decrease of infectivity for mice as large morphologic forms appeared within the host cells. Rake and Jones (9) have noted a decrease of infectivity during the growth cycle of lymphogranuloma venereum in the yolk sacs of chick embryos but have indicated that this decrease is due

to a liberation of virus from yolk sac cells. It is interesting to note that in the work of Rake and Jones the infectivity decrease was accompanied by the development of large bodies within the yolk sac cells. However, the cause-effect relationship of such an observation is not clear, especially since these workers have postulated that the loss of infectivity is due to a release of virus from host cells.

With influenza virus in allantoic membranes Henle (2) has suggested that after adsorption to host cells the seed virus which participates in the propagation becomes altered in such a way that it no longer can be demonstrated by infectivity titrations. In the study of bacterial host-virus systems (10, 11) there has been observed a disappearance of infectious virus in the host cell during the early stages of the infectious cycle. This has been accounted for by postulating a disruption of the virus particle within the host cell. It would be quite premature to compare the observations in MP studies with the rather clear-cut results obtained in those with bacteriophage systems but this possibility should not be overlooked.

A decrease of infectivity might also result from the clumping of virus particles which would tend to reduce the number of infectious units. However, it seems unlikely that this could account for the entire decrease observed. Another alternative which is available would suggest that the virus particle after entering the cell combines with a host constituent in such a manner as to render it non-infectious.

New virus begins to appear in the allantoic membranes approximately 22 hours following the inoculation of seed virus by the allantoic route. When the virus inocula contained sufficient amounts of the infectious agent to permit titration of allantoic membranes, new virus appeared quite consistently after this time interval. Furthermore, the rate of formation of new virus appeared to be constant regardless of the dilution employed. This has been observed not only in the experiments here recorded, but in other unpublished ones as well. The exponential nature of the increase is difficult to interpret; the possibility that binary fission is involved in the late stages of growth must still be investigated. Morphologic investigations are now in progress in this laboratory and an attempt is being made to correlate the findings with the developmental pattern of infectivity.

The growth cycle of MP herein described is not unlike that which has been observed for other members of the psittacosis group. Thus Bedson (7) studying psittacosis in mouse spleens has reported approximately the same time sequence for the disappearance and reappearance of virus infectivity in host tissue. Bland and Canti (12) noticed the sudden appearance of intracellular bodies in living tissue between 18 and 24 hours; at a given hour none were present, the next very many. The appearance of infectivity in MP growth cycles in allantoic membranes seems to follow the same pattern.

Hamre, Rake, and Rake (13) noted in their work with feline pneumonitis in

the allantois of the chick embryo a decrease of infectivity in the allantoic fluids between 0 and 24 hours with a subsequent increase thereafter. Allantoic membranes showed increased infectivity between 24 and 48 hours following inoculation and the titers remained higher than allantoic fluids thereafter.

A developmental pattern somewhat similar to that of MP in allantoic membranes was described by Rake and Jones (9) for lymphogranuloma venereum in yolk sacs and has already been discussed.

SUMMARY

Because of the peculiar properties of the psittacosis-lymphogranuloma group of viruses, the pattern of multiplication in the allantois of the chick embryo of one of their number, meningopneumonitis virus, was studied. This was done by determination of the changes in its infectivity for mice and chick embryos.

Titration of infectivity in embryos proved to be a more sensitive procedure than titration in mice; the latter procedure however, had the advantage of greater simplicity and gave more clear-cut results. The mouse titration method was used in most of the experiments.

Following inoculation of virus into the allantois, there was a slow decrease in infectivity in the allantoic fluids followed by an increase due to appearance of new virus between 24 and 48 hours.

The slope of declining infectivity in the allantoic fluids *in ovo* was similar if not identical with the slope of decreasing infectivity in allantoic fluids *in vitro* caused by thermal degradation of virus.

Multiplication of the virus in allantoic membranes was characterized by the following pattern: (*a*) Increase in infectivity in the first few hours (exact duration of increase depended on concentration of virus in inoculum) due to adsorption of virus. (*b*) Decrease in infectivity up to about 20 to 24 hours. (*c*) Increase in infectivity due to appearance of the new generation of virus.

The growth curve of meningopneumonitis is analyzed and the pattern of growth is discussed in the light of the present concepts of viral multiplication.

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