TABLE I. Effect of Hypophysectomy and Administration of ACTH and Insulin to Hypophysectomized Rats on Phagocytic Activity of Animals Fed Diets Containing 3'-Me-DAB.

Wk on diet	Hypophysectomized rats: 3'-Me-DAB	Hypophysectomized rats: 3'-Me-DAB, insulin and ACTH*
	Phagocytic index—k	
1	.014 (2)†	.006 (2)
2	.007 (2)	.016 (2)
3	.008 (2)	.013 (2)
4	.007 (2)	.014 (2)
5	.005 (1)	.016 (2)
6	.007 (2)	.033 (2)
7	.010 (1)	.031 (1)

* Daily subcut. injections of .5 unit of ACTH and .0075 unit of insulin.
† No. of animals is shown in parentheses.

ceiving carcinogenic diets and elimination of the carcinogenic potency of azo dyes by splenectomy(9) or injections of trypan blue (8), thorotrast or iron oxide(7), suggest an essential role of the RES in hepatocarcinogenesis. Further work will be necessary to establish and clarify the function of the RES in this complex process.

Summary. An early stimulation in the clearance of colloidal carbon by the reticuloendothelial system was noted in rats fed 3'-methyl-4-dimethylaminozobenzene or DL-ethionine. No stimulation was observed in animals receiving the basal diet or 4'-methyl-4-dimethylaminozobenzene. Elimination of the carcinogenic potency of the azo dye by hypophysectomy and of ethionine by methionine abolished the stimulation of phagocytic activity by these carcinogens. Administration of ACTH and insulin restored phagocytic activity in hypophysectomized rats fed diets containing 3'-methyl-4-dimethylaminozobenzene.

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Received June 6, 1961. P.S.E.B.M., 1961, v108.

Effect of Age, Temperature and Inoculum Size on Non-Infective Vaccinia Virus Yields in Eggs.* (26897)

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Ratios of 2 biological properties of viruses, such as the ratio of infectivity to hemagglutination units, have been used frequently in studies of the growth characteristics of some viruses. With such technics von Magnus(1) and others(2,3) have demonstrated the appearance of incomplete influenza virus in certain growth situations wherein the proportion

* This work was supported by U.S.P.H.S. grant.
† U.S.P.H.S. Predoctoral Fellow.

of infective virus particles was small compared to the total number of particles as indirectly measured by hemagglutinin titrations. Obviously, a direct total particle count would be preferable but this is a very difficult procedure with influenza viruses. In recent years, methods have been devised which allow rapid and accurate counts to be made of vaccinia virus particles in crude suspensions by electron microscopy so that particle/infectivity ratio experiments with vaccinia virus are feasible(4). Initial application of ratio studies with vaccinia virus was concerned with ratio changes during growth and adaptation of vaccinia virus in eggs, rabbits and guinea pigs(5). Briefly, these experiments demonstrated low ratios of particles to infectious units in serial passages in the chorioallantoic membrane of the embryonated egg which is naturally susceptible to vaccinia virus. However, during adaptive passages in rabbits and guinea pigs high ratios occurred which subsequently dropped as adaptation was accomplished. Thus, during adaptation of vaccinia virus in these animals incomplete virus might be said to have been produced comparable to incomplete influenza virus as described by von Magnus.

The present study is part of a continuing effort to determine the factors which influence the ratio of vaccinia virus particles to virus infective units and is concerned primarily with attempts to vary this ratio in a naturally susceptible host, the chorioallantoic membrane of the embryonated egg, by allowing vaccinia virus growth to occur under suboptimal conditions. Such conditions included: Incubation of virus infected eggs at below normal temperatures; inoculations of eggs younger and older than the accepted optimal age and passage of virus in high concentrations.

Materials and methods. Virus. Vaccinia virus was obtained from the New York State Board of Health as a calf lymph suspension. It has been subsequently maintained in this laboratory by more than 225 serial passages on the chorioallantoic membrane of 12-day-old embryonated eggs.

Infectivity titrations and virus passage experiments. All growth studies were carried out by inoculation of 0.2 ml virus suspension onto the dropped chorioallantoic membranes of embryonated eggs. Membranes were dropped as described previously(6). At time of harvest eggs were cut open, membranes removed, and extracted to 10% of wet weight. All titrations were made in 12-day-old embryonated eggs as previously described(7) except when specifically indicated. Virus infectious units are expressed in terms of pock-forming units/ml. (PkFU's/ml). All eggs were incubated at the temperature stated ± 1.0°C.

Virus particle counts. This procedure has been described in detail(4,5). In brief, suspensions to be counted were diluted in buffered saline and sonic vibrated at 9 KC for 3 minutes in volumes of 25-50 ml. After sonication the suspension was placed in specially designed centrifugation cells‡ containing an agar block. The cell was placed in a Servall-Sharp‡ counting rotor and centrifuged at 10,000 rpm for 30 min. During this procedure the virus particles were sedimented out of suspension onto the agar block surface. Following this run the block was removed and flooded with collodion which was allowed to dry into a film. The film was stripped off, picked up on an electron microscope grid and examined. Calculation of the number of virus particles was made by the following formula:

$$N = \frac{n\,M^2\,D}{0.97\,(h-t)\,A}$$

N—No. of particles/ml
n —No. of particles per micrograph
M—magnification
D—dilution
h —height of cell
t —thickness of agar
A —area of micrograph

Results. The initial experiment was an attempt to produce relatively large amounts of non-infective vaccinia virus by making serial passages with high virus concentrations since this procedure uniformly results in incomplete influenza virus(1). Table I gives the results and it is apparent that no significant

‡ Manufactured by Ivan Sorvall, Inc., Norwalk, Conn.

TABLE I. Yields of Infective and Non-Infective Vaccinia Virus after Passage with High and Low Virus Content of Inocula.

	Inoculum					
	1.2×10^{7}*			1.2×10^{4}*		
Virus passage	Virus particle count	PkFU's/ml	Particles: PkFU's/ml ratio	Virus particle count	PkFU's/ml	Particles: PkFU's/ml ratio
1	42×10^{8}	7×10^{8}	6:1	72×10^{8}	9×10^{8}	8:1
2	102×10^{8}	17×10^{8}	6:1	119×10^{8}	17×10^{8}	7:1
3	161×10^{8}	23×10^{8}	7:1	130×10^{8}	26×10^{8}	5:1
6	80×10^{8}	10×10^{8}	8:1	91×10^{8}	13×10^{8}	7:1

* PkFU's/ml = Pock-forming units/ml.

decrease in amount of infective virus resulted. In fact, although a similar procedure with influenza virus results in a marked and progressive infective virus titer decrease in the first 3 passages, vaccinia virus consistently increased for 3 passages. Ratios of total to infective virus were comparable for both concentrated and diluted virus inocula.

Influence of age of eggs on growth of vaccinia virus. Previous experiments in this laboratory have indicated that the age of the chorioallantoic membrane is most important in obtaining maximum titers of vaccinia virus. Table II presents a typical titration in which a stock vaccinia suspension was titered in eggs 8, 12, and 15 days old demonstrating that at both a younger and older age membranes were less responsive to vaccinia virus. This resulted in *apparent* high particle/infectivity ratios of vaccinia virus but actually this was due to non-responsiveness of the membranes involved with consequent decrease in sensitivity to detect infective virus rather than an increase in non-infectious vaccinia.

In another experiment eggs of 8, 12, and 15 days of age were inoculated with vaccinia and the membranes removed and extracted at 48 and 72 hours after inoculation. Extracts were then titered for infective virus in 12-day-old eggs and total particle counts made. Results are given in Table III.

TABLE II. Titration of Stock Vaccinia Virus on Chorioallantoic Membranes of 8-, 12- and 15-Day-Old Eggs Incubated at 37°C.

Age	Virus particle count of stock virus	Infectivity titer of stock virus, PkFU's/ml	Ratio, particles: PkFU's/ml
8	$15. \times 10^{9}$	$.1 \times 10^{9}$	150:1
12	$15. \times 10^{9}$	2.2×10^{9}	7:1
15	$15. \times 10^{9}$	$.9 \times 10^{9}$	17:1

Here, in contrast to the previous experiment, it is clear that by deviating from the accepted age of 12 days conditions for vaccinia virus growth are less favorable. The total infective virus yield was about 6-7 times less in both younger and older eggs, but since there was a parallel decrease in total virus particle count the ratios were comparable in all 3 instances, although the single ratio of 10:1 is slightly high.

TABLE III. Growth of Vaccinia Virus in Chorioallantoic Membranes of 8-, 12- and 15-Day-Old Eggs Incubated at 37°C.

Age eggs (days)	Interval inoculation to harvest (hr)*	Virus particle count/ml $\times 10^{9}$	Infectious units/ml (PkFU's/ml) $\times 10^{9}$	Ratio, particles: PkFU's/ml
8	48	1.3	.19	7:1
	72	8.0	1.3	6:1
12	48	7.3	1.2	6:1
	72	57.0	7.0	8:1
15	48	1.4	.2	7:1
	72	11.2	1.1	10:1

* Inocula = 1.2×10^{4} PkFU's/ml vaccinia virus.

Effect of incubation temperature on yield of non-infective vaccinia virus. Since temperature of incubation may influence virus yield a series of growth curves of vaccinia virus were performed in 12-day-old eggs incubated at 26°, 30°, 37° and 41°C. None of the eggs incubated at 41°C survived 48 hours and were discarded from the experiment. Most of the eggs incubated at 26°C survived a maximum of 6 days. Results are presented in Table IV. It is apparent that eggs incubated at 26°C produced virus in an amount

TABLE IV. Yield of Non-Infective Vaccinia Virus in Egg Membranes Incubated at 26°, 30° and 37°C.

Egg incubation temp. (°C)	Interval inoculation to harvest (hr)	Virus particle count $\times 10^8$	PkFU's/ml $\times 10^8$	Ratio, particles: PkFU's/ml
26	48	.05	.003	17 :1
	72	4.6	.4	11.5:1
	96	5.6	.5	11.2:1
	120	21.0	1.3	16 :1
	144	27	2.7	10 :1
30	48	58	1.8	32 :1
	72	420	30.0	14 :1
37	48	50	10.	5 :1
	72	560	80.	7 :1

* Inocula for all eggs = 1.2×10^4 PkFU's/ml.

significantly less than at 37°C. With prolonged incubation the virus content increased so that, comparing virus yields at 26° and 37°C, the 3000-fold difference at 48 hours was reduced to a 200-fold difference at 72 hours. The titer of 2.7×10^8 at 6 days represents the maximum virus yield obtainable at 26°C; at this temperature all eggs were dead at 7 days. Embryo deaths occurred at 26°C from 96 hours on. Membranes were removed only from eggs containing living embryos. Of further interest was the comparison of gross and microscopic pathology of membranes incubated at 26° and 37°C. Intact membranes which were virus infected and incubated at 26° and 37°C and removed 3 days after inoculation are pictured in Fig. 1 & 2. All eggs received the same inoculum of 1.2×10^4 PkFU's/ml vaccinia virus. Careful examination of the 26°C membranes (Fig. 1) failed to reveal any gross lesions of any kind in contrast to the marked lesions found on the 37°C eggs (Fig. 2). Moreover, membranes from the 26°C eggs removed at 4, 5, and 6 days after inoculation were without gross lesions and were identical to the membrane pictured in Fig. 1. Histologic studies of the membranes from virus infected eggs incubated at 26°C (Fig. 4) demonstrated definite cellular infiltration in the choriollantoic membrane but with minimal cell necrosis as compared to marked necrosis found in membranes from eggs incubated at the higher temperature (Fig. 5). It is quite clear from this experiment that the amount of tissue reaction to virus growth may be reduced out of proportion to the reduction in virus multiplication. This is, of course, the situation found with cortisone-treated tissues infected with many bacterial and viral agents. Membranes from eggs incubated at 30°C showed gross and microscopic pathology intermediate between the other two temperatures.

Of primary interest was the increase in proportion of non-infective virus although degree of increase was not as great as had been hoped for and amounted to about a 2-fold increase at 26°C over the average for this type of tissue at 37°C. This increase is significant on the basis of past studies(5). Not included in Table IV are results of titrations of membranes incubated at 26°C but harvested 10, 16, 24, and 36 hours after inoculation, which showed infective vaccinia in titers of $3. \times 10^2$, $6. \times 10^2$, $300. \times 10^2$ and $2000. \times 10^2$. Ratios could not be calculated on these extracts since the particle concentration was too low for particle counting. These titers did indicate, however, that the virus extracted 10-24 hours and later after inoculation represented virus growth and was not residual virus from the original inoculum.

Discussion. The present experiments demonstrate that it is possible to increase the yield of non-infective vaccinia virus by allowing virus growth to occur under sub-optimal conditions, in this case in the chorioallantoic membranes of eggs incubated at well below normal temperatures. The amount of non-infective vaccinia was measured by the ratio of total particles present as compared to number of infectious units and, in the case of the membranes incubated at the lower temperatures, was found to be increased from an average ratio of 6:1 at 37°C to an average of 13:1 at 26°C. A previously reported average ratio in the chorioallantoic membrane at 48-72 hours at 37°C was 4:1(5) and other unpublished ratios for similar material range from 4:1 to 8:1. Thus, on the basis of the present experiments as well as past experience a ratio of 13:1 represents an increase of non-infective virus of not less than 60% based on the highest (8:1) ratio obtained at 37°C. The standard deviation of the particle count technique is about 10%

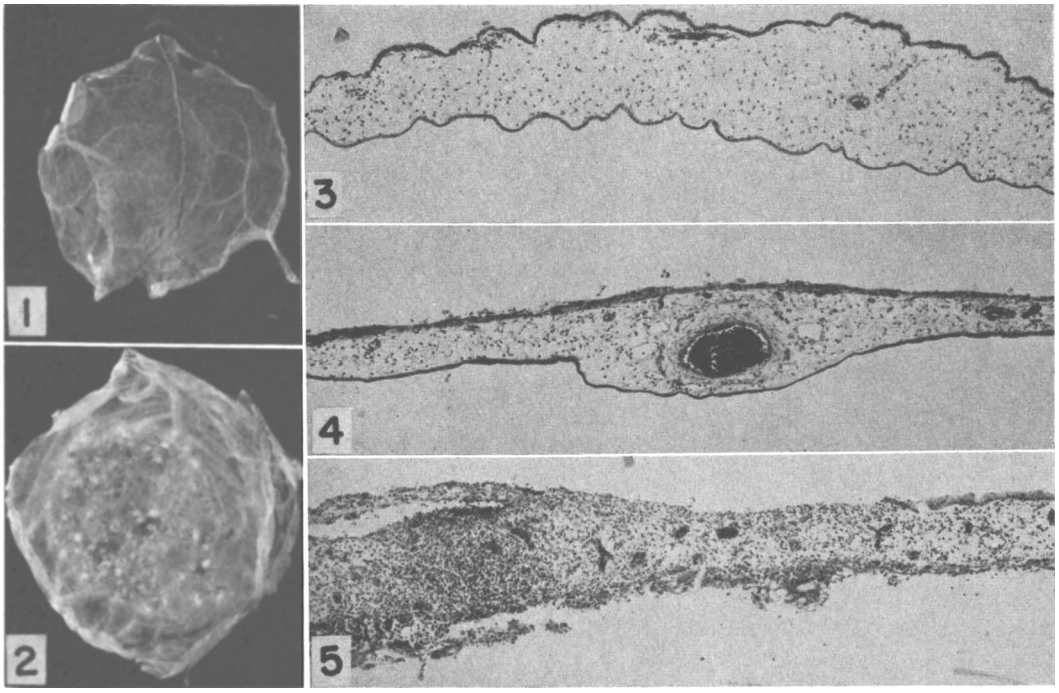

FIG. 1. Membrane inoculated with 1.2×10^4 PkFU's/ml of vaccinia virus and removed after 3-day incubation at 26°C. Extracts of this and similar membranes contained approximately 0.4×10^8 PkFU's/ml of virus.

FIG. 2. Membrane similar to that in Fig. 1 but incubated at 37°C for 3 days. Virus content about 200-fold greater than those membranes incubated at 26°C.

FIG. 3. Control section of chorioallantoic membrane inoculated with diluent and incubated at 26°C for 3 days. ×60.

FIG. 4. Section of membrane in Fig. 1. Definite increase in cell infiltration with absent or minimal cell necrosis. ×60.

FIG. 5. Section of membrane in Fig. 2. Marked cell infiltration and necrosis in all areas. ×60.

(4) and that of the infectivity titrations 16% (7).

During incubation at 26°C the virus content showed a steady increase over the 6-day period so that none of these determinations represented ratios obtained after peak virus growth was reached. A previous growth curve of vaccinia in chorioallantoic membranes incubated at 37°C and harvested 1 to 6 days after inoculation showed increased ratios beginning at the fourth day after inoculation and associated with both a fall in the infectivity titer and death of the embryos(5). In this latter instance it was presumed that virus inactivation had occurred leading to the increased ratios. Virus inactivation, as opposed to production of inactive or incomplete virus, is difficult to rule out of any experiments of this present type; however, it is apparent that the high ratios obtained at 26°C were found at the earliest interval at which particle counts were possible and showed no tendency to increase as might be expected on a time-temperature inactivation basis.

Few studies of virus growth at subnormal temperatures have been published. Gohd(8) demonstrated growth of influenza virus at 26°C but reduced in amount and delayed in appearance as in the present experiments. Wheeler and Canby(9) stated that herpes simplex virus in tissue culture failed to grow at 26°C and remained in a latent state; later elevation of incubation temperature resulted in a prompt acceleration of virus production. There is no question that vaccinia growth is both delayed and reduced at 26°C. The extent of the pathologic lesions both gross and microscopic was likewise reduced. However, the absolute amount of virus production was

considerable. It is surprising that the membrane in Fig. 1 represents a vaccinia virus content of 0.4×10^7 PkFU's/ml (extracted) when on the basis of gross pathology the membranes appeared entirely normal. The reduction in pathologic changes at the lower temperatures may be due to a reduced accumulation of virus per cell of the total number of cells infected.

The experiments of vaccinia growth in the 8- and 15-day-old chorioallantoic membrane suggest that a certain rather specific metabolic or physiologic state of these cells is required for maximal virus growth. The chorioallantoic membrane is in this optimal state for only a few days in the total embryonic life span. Some experiments were attempted whereby the 8- and 15-day eggs were incubated at sub-optimal temperatures but this resulted in a virus particle production too low to count. Unfortunately, particle count experiments are not possible (at present) when particle concentration is less than 1×10^7 particles/ml.

The mechanism of incomplete virus production is not known but with influenza virus it is produced by serial passage of virus in high concentrations. Since the nucleic acid content of incomplete influenza virus is low it has been theorized that the multiple cell infection strains the cells' ability to produce nucleic acid. No data are available as to whether cell incubation at low temperatures reduces or affects either cell or virus nucleic acid production.

Summary. A relative increase in non-infective or incomplete vaccinia virus occurs during virus growth in the chorioallantoic membrane of 12-day-old eggs incubated at 26°C and 30°C. Vaccinia growth in membranes of eggs 8 and 15 days of age is reduced but no increase in non-infective vaccinia was found. Serial passage of high concentrations of vaccinia virus in 12-day-old eggs likewise failed to increase the proportion of non-infective virus.

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Received June 19, 1961. P.S.E.B.M., 1961, v108.

Phosphorylase Activity in Denervated Skeletal Muscle.* (26898)

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The glycogen level of skeletal muscle decreases within 3 days following denervation (1) and returns to normal upon reinnervation(2). However, the changes in muscle phosphorylase activity, following denervation, are not in agreement. Enzyme activity in denervated muscle was reported to decrease slightly after several weeks and then decrease rapidly(3), or to decrease steadily from the day of denervation(4), or to increase up to 20 days after denervation(5). Furthermore, the increase in phosphorylase activity induced in normal skeletal muscle by epinephrine failed to occur in denervated muscle(5). The present study concerns an investigation of skeletal muscle phosphorylase activity at various periods after denervation,

* Aided by a grant from Muscular Dystrophy Assn. of America, and Sage and Sackett Research Funds of Dept. of Zoology, Cornell University.