mental group, the average weight prior to injection was 286 g. At the end of the injection period, the weight had decreased to 276 g, and continued to fall to 274 g during the subsequent 24-hour period of continued nitrogen excretion. In the control group, on the contrary, the weight remained constant at 283 g.

For contrast, the data are presented in Fig. 1 for this and a similar experiment using purified growth hormone (GH) 0.5 mg intraperitoneally twice daily. On this graph the opposing effects of ACTH and GH on body weight and urinary nitrogen excretion are apparent.

Summary. ACTH causes an increase in urinary nitrogen excretion with proportionate loss of body weight in the normal rat. The effect of subcutaneous ACTH on nitrogen excretion is manifest on the second day and persists 24 hours after injection is stopped.

15387

Growth of Japanese B Encephalitic Virus in the Yolk of the Developing Egg.

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In an endeavor to produce a chick-embryo vaccine against the Nakayama strain* of Japanese B encephalitic virus, experiments were undertaken to determine how to develop a more potent virus in the egg. Haagen and Crodel[1] reported the cultivation of this virus on the chorio-allantoic membranes of the developing chick, while Smith and Lennette[2] later reported growth on the membranes of 10- to 13-day-old embryonated eggs. The titers in mice seldom were above 10^{-3}. Sabin[3] and his associates in the investigation of vaccine production considered the use of chick-embryo tissues as unsuitable because of the low potency obtained after inoculation either into the yolk or the allantoic fluid. Since they did not mention the titers obtained nor give any details of the egg work, it was thought of interest to investigate further what potency could be developed in the egg by either the allantoic or the yolk sac routes.

Allantoic route. Ten- to 11-day-old embryonated eggs were inoculated with 0.2 cc of a 10% mouse brain suspension through the air sac membrane into the allantoic fluid. To determine the most suitable incubation period for optimum virus growth, eggs were opened in 24, 48, 72, and 96 hours, respectively, after inoculation. Twenty percent suspensions of the embryos and chorio-allantoic membranes in buffered saline were prepared by grinding in a small Waring blendor for 2 minutes. After reduction to a 10% suspension, each material was titrated intracerebrally in mice in 0.03 cc amounts and the 50% endpoint obtained according to the method of Reed and Muench.[4] With the exception of a low titer ($10^{-3.74}$) in 24 hours, the other endpoints remained fairly constant—$10^{-6.2}$ to $10^{-6.5}$ and $10^{-6.74}$. Most of the embryos were alive, except on the fourth day.

Live embryos taken on the third day of incubation were then used for passage material, 16 serial passages being made in this

* The Nakayama strain was kindly sent from the Department of Virus Research at Lederle Laboratories.

1 Haagen, E., and Crodel, B., *Zentralbl. f. Bakt.*, 1938, **142**, 269.

2 Smith, M. G., and Lennette, E. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 323.

3 Sabin, A. B., assisted by Duffy, C. E., Warren, J., Ward, R., Peck, J. L., Jr., and Ruchman, I., *J. Am. Med. Assn.*, 1943, **122**, 477.

4 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
The Virus Concentrations Obtained by Varying the Age of the Eggs and Keeping the Inoculum Constant Using (1) Embryos and Membranes and (2) Yolk Sacs.

Age of eggs in days	Amt of virus	Titers in mice 50% endpoint of virus (log. exponent)
	cc	
7	0.2	7.50
		6.50
8	"	7.00
		6.50
9	"	7.24
		5.74
10	"	6.50
		5.24

manner, using 0.2 cc of a 10% suspension of embryo and chorio-allantoic membranes in buffered saline. The embryos and membranes were tested for potency in mice at different intervals through the 13th passage. The titers were again quite constant, varying from $10^{-6.50}$ to $10^{-6.74}$, with only one jump to $10^{-7.24}$ on the fifth passage and a drop to $10^{-6.0}$ on the 13th.

At no time was the potency equal to that of the mouse brains used in the initial inoculum ($10^{-8.33}$). Repeated egg passages failed to increase the virulence and the lethal effect was quite inconstant. Occasionally embryos would die on the third or fourth days, but more often all remained alive. The eggs were not incubated longer because of the development of feathers and the greater danger of an allergic reaction from the older embryonic tissues if they were used for vaccine production.

Yolk sac route. It was then decided to inoculate younger eggs directly into the yolk after the method of Cox[5] for Rocky Mountain spotted fever and epidemic typhus rickettsiae. The first trial inoculations, using 0.2 cc of a 20% suspension of mouse brain virus into 8-day-old eggs, resulted in 100% mortality of the embryos within 48 hours of incubation. This lethal effect was noticed after repeated inoculations into eggs not only after direct injection of virulent mouse brain but after serial passage. Bacterial cultures were made on all tissues removed and as only bacteria-free material was used for egg inoculations, the high mortality was apparently due to the virus.

Since death of the embryo occurred regularly by this method of yolk inoculation, the lethal effect could now be used for various experiments as well as for titrating the virus in eggs instead of mice, as heretofore. In fact, this method proved to be more delicate for determining the potency of the virus than did the intracerebral titration in mice. A suspension of the embryos and membranes of the fifth yolk passage in eggs had a titer in eggs of $10^{-7.66}$ and only $10^{-6.74}$ in mice. On another occasion the tissues were lethal in eggs to $10^{-7.24}$. However, the usual titer of the mouse brain virus was $10^{-8.5}$ or better.

Experiments to determine the highest virus concentration in eggs by varying different factors:

(a) *Keeping a constant amount of inoculum and varying the age of the egg.* 0.2 cc of 10% mouse brain virus (50% endpoint $10^{-8.5}$) in buffered saline was inoculated into the yolk of 7-, 8-, 9-, and 10-day-old eggs, respectively. There were 6 eggs of each age. All of the embryos were dead within 48 hours, except those initially 10 days old, which died after 72 hours incubation. The embryos, chorio-allantoic membranes, and yolk sacs were removed from each set of eggs. The first 2 substances were ground together and the other separately to a 20% suspension in buffered saline, diluted to 10% and titrated in tenfold dilutions in 11 g mice, using dextrose-saline and 10% normal rabbit serum as diluent. This holds true for the experiments given in Tables I, II, III, and IV.

As seen in Table I, there was not much difference in the titers of virus in the embryos and membranes from the 7-, 8-, or 9-day-old eggs, but there was decidedly less virus present in the yolk sacs. The tissues of the 10-day-old embryos were the least potent.

(b) *Varying the amount of mouse brain inoculum and keeping the age of the egg constant.* Eight-day-old eggs were inoculated into the yolk with varying amounts of 10% mouse brain virus, allowing 4 eggs to each dilution. All of the embryos, regardless of

[5] Cox, H. R., *Pub. Health Rep.*, 1939, **53**, 2241.

TABLE II.
The Virus Concentrations Obtained by Varying the Inoculum and Keeping the Age of the Egg Constant.

Age of egg	Amt of 10% mouse brain inoculum into yolk	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
	cc	
8 days	0.05	6.74
	0.10	6.74
	0.20	7.00
	0.30	6.74
	0.50	7.24
	0.50	7.50
	1.00	7.0
	1.00	7.54

the amount given, were dead within 48 hours. The results of the titrations of the combined embryos and membranes are given in Table II.

The titers of the virus in the embryos and membranes increased as the amount of the inoculum increased. The highest titer was $10^{-7.5}$ while the original mouse brain inoculum was $10^{-8.5}$.

(c) *Keeping the amount of mouse brain virus and the age of the egg constant but varying the incubation period.* Eight-day-old eggs were inoculated via the yolk sac route with 0.2 cc of high titer mouse brain virus. Two of them were removed, respectively, at different time intervals, and the amount of virus present in the embryos and membranes was determined by titration in mice as shown in Table III.

The embryos were alive through 36 hours of incubation but all those remaining died within the 48 hours. The highest titer was obtained in the embryos and membranes removed after 36 hours of incubation when they were still active. There was a tenfold drop in titer in the dead embryos.

(d) *To determine any increase in potency of the virus by serial passage in eggs.* Serial passages of 20% suspensions of embryos and membranes diluted in buffered saline were made by the yolk sac method in 8-day-old eggs. All embryos died in 48 hours and the tissues were tested for potency by intracerebral inoculation of mice weighing 10 to 11 g. The results are given in Table IV.

The titers in mice of the different egg passages remained fairly constant, although there was an increase for the ninth passage in this experiment. Usually the first passage showed the most virus, the titer depending on the strength of the original mouse brain virus.

(e) *Effect of temperature on the egg cultures.* Five different groups of 10-day-old eggs were inoculated via the allantoic fluid on different occasions and represented first, second, and third egg passages. One-half of each group was incubated at 95°F and the other half at 98°F. The embryos and chorioallantoic membranes were harvested on the third day of incubation and then tested for potency in mice. Invariably the highest titers occurred in the tissues incubated at 95°F.

Several groups of 8-day-old eggs were inoculated via the yolk sac method and harvested after the death of the embryos in two days. Part of these eggs had been incubated at 95°F and the remainder at 98°F. Once again the titers in mice ran slightly higher for those embryos placed at the lower temperature.

TABLE III.
The Virus Concentrations Obtained by Keeping the Inoculum and the Age of the Egg Constant but Varying the Incubation Period After Inoculation.

Hr of incubation	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
6	4.00
12	3.50
18	6.50
24	7.50
36	8.24
48	7.24

TABLE IV.
The Virus Concentrations Obtained After Serial Passage in Eggs.

No. of passages in eggs	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
1	7.24
2	6.50
3	7.24
4	7.00
5	6.74
6	6.74
9	7.50

For this reason all eggs used in these experiments with the Japanese B virus were placed at 95°F.

(f) *Use of rabbit serum in the diluent.* The results of these experiments had all been obtained when the chick embryos were ground in buffered saline. To determine if the addition of inactivated normal rabbit serum to the diluent would produce more potent virus, the following experiment was undertaken:

Eight-day-old eggs were inoculated into the yolk with 0.2 cc, 0.5 cc, and 1.0 cc, respectively, of a 10% mouse brain suspension of virus ground in dextrose-saline containing 50% normal rabbit serum. All of the embryos died within 48 hours. The harvested embryos and membranes gave titers in mice of $10^{-7.5}$, $10^{-7.74}$, $10^{-7.0}$, respectively, in the order of the doses given above. The inoculation of 0.5 cc of virus yielded the most potent product. However, while the titers obtained were fairly high, equal values had likewise resulted when the diluent was only buffered saline. Therefore, any slight increase in potency by addition of rabbit serum would not offset the disadvantage of a possible carrying over of another foreign protein into any prospective vaccine.

Discussion. Since this work was submitted for publication, Warren and Hough[6] have reported on the successful use of a vaccine against the Japanese B virus prepared from infected chick embryos. They were able to obtain slightly higher titers of the virus by inoculation into the allantoic fluid than shown here. They likewise found that the potency increased after serial passage and that the embryos died after 4 days incubation.

[6] Warren, J., and Hough, R. G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 109.

Perhaps their higher values may have been influenced by the addition of normal chicken serum to the inoculum and the lethal effect may have been due to chance inoculation of the yolk when using the younger (7-8-day-old) eggs for the allantoic route.

In the present work serum had not been added to any inoculum, except for one experiment, in order to avoid that complicating factor. Likewise when inoculating into the allantoic fluid, 10-11-day-old eggs had been employed because the surface area of the chorio-allantoic membranes was greater than in the younger eggs and there was less chance of injecting into the yolk. However, the lethal effect was both irregular and prolonged when using the allantoic route in the older eggs, so that it was a satisfaction to find that the yolk sac method in 7-8-day-old eggs gave quicker and more clear-cut results.

Summary. From the results of these experiments to develop a more potent virus in the egg, it is concluded that the greatest amount of virus of the Japanese Nakayama strain of encephalitis would probably be obtained in chick embryos by using 7- or 8-day-old embryonated eggs inoculated into the yolk with 0.5 cc amounts of mouse brain virus of a potency of $10^{-8.5}$ or better, and incubating 36 hours at 95° to 96°F. The use of normal rabbit serum in the diluent might raise the potency slightly, but would be contra-indicated if there was a possibility of carrying over another foreign protein into any prospective vaccine. Because of the constant lethal effect, inoculation of the egg yolk is of value for titration of this virus and for use in other quantitative experiments. At no time, however, did the titrations of egg tissue reach as high endpoints as those obtained with the mouse brain virus.