experiments in the past, such as those reported by Moore and Price(4), antagonistic or neutralizing effects were not observed due to the use of relatively large doses of the stimulating hormones. In our study of the vaginal epithelium, if only the 0.15 γ per day or larger doses of estrone had been employed no inhibitory or cooperative effects of testicular function would have been observed. If instead of graded doses, a single low level such as 0.05 γ had been employed, only the cooperative effects would have been observed in the target tissue.

Of particular interest is the apparent lack of estrogen-androgen antagonism in the studies with diethylstilbestrol. Although an explanation of the mechanism for hormone antagonism must await further experimental data, the interactions of the naturally occurring estrogens and androgens may possibly represent another instance of biological competition occurring between structurally related compounds similar to the competitive inhibition exhibited between the metabolite thiamine and its structural analogue pyrithiamine(22). One might hypothecate further that the lack of such competitive inhibition between diethylstilbestrol and testicular androgens might suggest that the androgens are not able to successfully compete with diethylstilbestrol for some particular intermediary substance, possibly an enzyme, whereas they can successfully compete for this substance with estrone or estradiol. This difference in action would also result if the metabolic pathways for the utilization of, or the stimulation by, the two types of estrogens were somewhat different so that the step at which androgens inhibit or block the steroid estrogens is not involved in diethylstilbestrol stimulation of these target organs.

Summary. 1. Studies of the effect of injections of steroid and non-steroid estrogenic substances on the mammary gland and vaginal mucosa of castrate and non-castrate male mice bearing subcutaneous vaginal grafts are reported. 2. It is suggested that antagonistic actions exist between the naturally-occurring estrogens (estrone, estradiol) and testicular androgens in regard to the mammary gland and vaginal mucosa. 3. Such antagonism did not seem to occur when the synthetic estrogenic substance diethylstilbestrol was employed.

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22. Woolley, D., *Physiol. Rev.*, 1947, v27, 308.

Propagation in Chick Embryos of Dengue Virus, Hawaiian Strain. II. Findings in Infected Eggs.* (18641)

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It has been reported(1) that propagation of the Hawaiian strain of dengue virus was facilitated by progressive adaptation of the virus to mice by means of intracerebral passages. After 101 such passages, the virus was transferred to chick embryos, and it has to date been carried through more than 90 passages in eggs without demonstrable changes in its pathogenicity for mice and without change in its limited host range. The sera of human beings and of monkeys convalescent from infection with the original, human Hawaiian strain react specifically with the egg-adapted virus. Of two human volunteers inoculated with it, only one had a mild febrile reaction, but both responded with production of neutralizing antibody against the mouse-adapted virus(1).

This paper will describe certain characteristics of the infection in eggs. Of main interest is the finding that the pronounced neurotropism, acquired by the virus as a result of passage through mice, has persisted in the

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1. Schlesinger, R. W., *Am. J. Hyg.*, 1950, v51, 248.

TABLE I. Effect of Age of Eggs at Time of Inoculation on Concentration of Virus After 7 Days' Incubation.

Inoculum	Age of embryos at inoculation (days)	Mortality rates among mice inoc with embryo dilution						Log LD_{50}*
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
M101-E88	4	5/5	5/5	5/5	4/4	2/5	0/5	4.8
	5	5/5	5/5	5/5	4/5	3/5	0/5	5.0
M101-E15	5	5/5	5/5	4/4	3/5	3/5	1/5	5.0
	7	5/5	5/5	5/5	2/5	0/5	0/5	3.8
	9	4/4	4/5	1/5	0/5	0/5	0/5	2.5

* Log LD_{50} expressed as reciprocal of highest dilution estimated to kill 50% of the mice.

infection of chick embryos.

Materials and methods. The materials and methods used in this work have been described in the preceding paper of this series(1). The virus samples used were from egg passages which will be referred to below as "M101-E (n)." Unless otherwise mentioned, the test materials were 50% suspensions of whole embryos prepared as described previously(1). Dilutions were made in saline containing 1/10 volume of heated (56°C for 30 minutes), Seitz-filtered normal rabbit serum and 500 units per ml of sodium penicillin G. The inoculum consisted of 0.1 ml which was deposited in the neighborhood of the embryo with a 1.5 inch needle which was inserted through the air sac ("peri-embryonic stab") (2). The infectivity of materials harvested from eggs was determined by intracerebral subinoculation in mice. 0.03 ml of the test sample in suitable concentration was the inoculum. All titers have been expressed in terms of LD_{50} per 0.03 g of tissue or ml of fluid.

Experimental. Factors affecting the yield of virus in chick embryos. A. Age of embryos at time of inoculation. When the age of embryos at the time of inoculation was varied, results such as shown in Table I were obtained. All of the embryos were harvested after seven days' secondary incubation. The yield of virus was comparable in eggs inoculated on the fourth or fifth day of incubation, but inoculation on the seventh or ninth day resulted in reduced titers. This indicated a trend similar to that shown for the mouse-adapted Hawaiian virus which multiplies to higher titers in baby than in adult mice(3). A single attempt was made to see if further reduction in age of chick embryos at the time of inoculation might further enhance the titer. Two-day-old embryos were inoculated through a hole in the vitelline membrane, according to the method used by Hamburger and Habel in experiments with influenza and mumps viruses (4). The yield of virus in the embryos surviving after 7 days' secondary incubation was not significantly different from that obtained in 5-day-old embryos.

B. Period of secondary incubation. When the present series in eggs was initiated, it was found(1) that serial passages at 5-day intervals led to progressive decrease in viral concentration so that no virus was detectable beyond the sixth passage. On the other hand, with egg-to-egg transfers done every 7 days, the series could be maintained satisfactorily. After the virus had been propagated in this manner through a number of passages, its rate of multiplication in eggs increased so that infective titers of embryos reached maximum after 5 days, and remained at the highest level through the seventh day. Embryos harvested later, *i.e.* after intervals of up to 12 days after inoculation, contained only slightly reduced amounts of virus. Thus, 4 days before normal hatching time, the titer remained at levels of 10^{-3} to 10^{-4}.

C. Temperature of incubation. Eggs inoculated with embryo suspension from passage M101-E52 were incubated for 7 days at 35 or 39°C. The infective titers of the embryos of

2. Fox, J. P., and Laemmert, H. W., *Am. J. Hyg.*, 1947, v46, 21.

3. Sabin, A. B., and Young, I., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 478.

4. Hamburger, V., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1948, v66, 608.

TABLE II. Comparative Susceptibility of Mice and Chick Embryos to Egg-Adapted Hawaiian Dengue Virus. (Passage M101-E29).

	Dilution of embryo suspension inoculated					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
No. of mice dead after inoculation of indicated dilution	5/5	5/5	5/5	5/5	1/5	0/5
Log LD_{50}/0.03 g of embryo tissue 7 days after inoculation of indicated dilution	4.0	3.2	3.2	3.6	2.9	n.t.

TABLE III. Distribution of Virus in Eggs Harvested 7 Days After Inoculation.

Inoculum egg passage No.	Log LD_{50} per .03 g or .03 ml of								
	Whole embryo	CNS	Skeletal muscle	Viscera	Amniotic membrane	Amniotic fluid	Chorio-allantoic membrane	Allantoic fluid	Yolk sac
M101-E9	4.6	5.5	—	—	1.7	.6?*	.5?	0†	1.0?
M101-E11	—	4.6	<2.6	—	—	—	—	—	—
M101-E13	—	5.4	2.3	—	—	—	—	—	—
M101-E23	—	4.2	2.2	—	—	—	—	—	—
M101-E49	—	5.3	3.6	2.7	1.8	.5?	.8?	.3?	1.5
M101-E85	—	5.4	2.5		1.3	.6?	.7?	.4?	1.2

*.6? Question marks indicate that endpoints are rough estimates in titrations in which only a few mice died after inoculaton of undiluted materials. In the case of the chorio-allantoic membranes, the dilution factor of the original suspension is inaccurate because of admixture of allantoic fluid.

† "0" indicates failure of undiluted fluid to produce paralysis or death in any of the mice inoculated.

— Not tested.

the two groups did not differ significantly.

D. Size of seed inoculum. Several experiments were carried out to determine the amount of virus which would result in optimal yields in eggs, and to compare the susceptibility to infection of embryos with that of mice. One such experiment is summarized in Table II. Ten-fold serial dilutions of embryo suspension M101-E29 were inoculated simultaneously into mice and into 5-day-old eggs. It was found that the infectivity of the virus for eggs was at least as high as for mice, in that the 10^{-5} dilution gave rise to infection in embryos as well as in one of the 5 mice inoculated. Here, as in other similar tests, dilute seed inocula resulted in reduced rather than in increased viral yield after 7 days. There was no indication that large inocula might produce the phenomenon of "auto-interference" similar to that described for influenza virus(5).

Distribution of virus in infected eggs. Eggs inoculated by periembryonic stab with material derived from different passages were harvested after 7 days' incubation and the virus contents of various fluids and organs were assayed. As can be seen in Table III, the highest concentration of virus was found in the central nervous system. Skeletal muscles and viscera contained 100 to 1,000 times less. Some viral multiplication may have taken place in the amniotic membranes and yolk sacs, since they contained more virus than amniotic or allantoic fluids or the chorio-allantoic membrane. The low degree of susceptibility of the chorio-allantoic membrane to infection was further illustrated by the following experiment. Embryo suspension from passage M101-E17 was inoculated on the dropped chorio-allantoic membrane of seven-day-old eggs, which were then incubated at 37°C. Three eggs each were harvested after 4, 5, 6 and 7 days, and their fluids and tissues assayed for virus. On the fourth day, virus was detectable only in blood. It appeared in the embryo on the fifth day, but the titer failed to rise above 10^{-2} in any of the specimens tested. All extra-embryonic fluids and membranes remained free of demonstrable

5. Henle, W., and Henle, G., *Am. J. Med. Sc.*, 1944, v207, 705.

virus throughout the experimental period. The low concentration of virus attained under these conditions may be related to the time elapsed before virus reached the embryo itself when inoculated by this route (negative on the fourth day after inoculation, *i.e.* 11th day of incubation).

Effect of infection with dengue virus on the chick embryo. A. Mortality and hatchability. As a rule, infected eggs were incubated at 35°C and at this temperature even un-inoculated embryos showed poor growth, and a high proportion died at hatching time. On several occasions, small numbers of infected and normal control eggs were incubated at higher temperatures until hatching time. Thus, in one group of 26 normal eggs incubated at 38°C, only 11 hatched. Those which failed to hatch survived until hatching time but died on the 23rd to 25th day of incubation. On the other hand, of 24 eggs inoculated on the eighth day of incubation with virus (M101-E17), 21 survived until hatching time; of these 13 hatched. While the existing conditions appeared to be generally unfavorable for hatching, the hatchability did not seem to be affected by infection with dengue virus. None of the hatched normal or infected chicks survived beyond the tenth day of life. The cause of their death has not been determined.

B. Pathological findings. The gross appearance of embryos harvested from infected eggs from 5 to 12 days after inoculation showed no consistent deviation from that of normal embryos of the same ages. All harvested embryos were weighed, and there was no difference between normal and infected groups. Semi-serial cross-sections of normal and infected 13-day-old embryos were stained with gallocyanin-eosin and compared carefully at each level. Because the central nervous system supported viral growth preferentially, this organ was studied with particular care. No pathological lesions were seen. There were large numbers of mitotic figures in the CNS and in other organs of infected as well as normal embryos, suggesting that infection did not interfere with their growth and development.

Persistence of infection after hatching. It had been found that dengue virus persisted in embryos at fairly high concentrations for at least 12 days after inoculation, *i.e.* up to 4 days before normal hatching time. In view of the inability of chick embryos to respond to infection with antibody formation, it was considered of interest to see how long after hatching virus remained demonstrable. Twelve one- or 2-day-old chicks hatched from eggs inoculated on the eighth day of incubation with embryo suspension M101-E17 were bled from the heart into a syringe containing heparin. Each blood sample was immediately inoculated intracerebrally into 5 mice. As shown in Table IV, seven or eight of the specimens contained virus. However, the number of mice which became paralyzed was so small, and their mean survival time so long, that the concentration of virus in each positive sample must have been near the limits of detectability. It has been reported previously(1), and since then confirmed in numerous titrations, that a consistent correlation exists between virus concentration and mean survival time.

Because of the presence of large amounts of virus in the CNS tissue of infected embryos, it was of interest to see whether it persisted there after hatching. A total of 19 chicks from groups inoculated with 3 different virus samples died or were sacrificed at various intervals of up to 10 days after hatching. Their brains and spinal cords were removed and ground up individually. Twenty percent suspensions were inoculated into groups of mice. The results are summarized in Table V. Of 19 chicks, 17 or 18 harbored dengue virus in their CNS. The incidence of infection in the individual groups of mice, and their mean survival time, indicated that the CNS of all chicks dead or sacrificed up to 7 days after hatching or up to 20 days after inoculation contained an estimated 10 to 1,000 LD_{50}, while those tested 8 to 10 days after hatching, *i.e.* 22 days after inoculation, contained less virus or none at all. Unfortunately, as has been mentioned, attempts to raise either normal or infected chicks beyond the tenth day have thus far been unsuccessful. Further efforts in this direction will be made.

TABLE IV. Presence of Virus in Blood of Chicks Hatched from Dengue-infected Eggs. Inoculum: Passage M101-E17 embryo suspension.

Chick No.	Age of embryo at inoculation	Day of hatching	Age of chick at time of bleeding (days)	Interval betw. inoculation and bleeding (days)	Result of intracerebral inoculation in mice	No. of positive samples
1	8	21	2	15	1/5* (14)†	2/3
2					3/5 (15)	
3					0/5	
4	8	22	1	15	0/5	2/4
5					1/5 (13)	
6					0/5	
7					3/5 (13)	
8	8	23	2	17	2/5 (13.5)	3 or 4/5
9					1/4 (12)	
10					0/4	
11					1?/4‡ (14)	
12					2/5 (13.5)	
				Total	13 or 14/58	7 or 8/12

* 1/5 No. of mice dying of dengue infection in enumerator, No. observed in denominator.
† Figures in parentheses indicate average time of death of paralyzed mice.
‡ One mouse died, cause of death not ascertained.

TABLE V. Presence of Virus in CNS of Chicks Hatched from Dengue-infected Eggs.

Inoculum	Age of embryos at time of inoc. (days)	Chick No.	Day of hatching	Age of chick at time of test (days)	Interval between inoc. and harvest of CNS (days)	Result of intracerebral inoculation in mice	No. of positive samples
M101-E14	9	1	25	4	20	10/10* (12)†	1/1
		13	23	0	15	6/6 (11.2)	
		8	23	2	17	5/5 (9.8)	
		10	23	2	17	5/5 (10.8)	
		11	23	2	17	5/5 (11.8)	
		12	23	2	17	5/5 (10.0)	
		9	23	5	20	5/5 (11.0)	
M101-E17§	8	4	22	6	20	5/5 (11.2)	13/13
		5	22	6	20	4/5 (11.8)	
		2	21	7	20	5/5 (11.8)	
		3	21	7	20	5/5 (10.8)	
		6	22	8	22	4/5 (13.0)	
		7	22	8	22	1/5 (13.0)	
		1	21	9	22	3/5 (12.7)	
M101-E51	9	521	21–22	9–10	22	0/5	3 or 4/5
		522	21–22	9–10	22	3/4 (14.3)	
		524	21–22	9–10	22	1?/3 (12.0)‡	
		525	21–22	9–10	22	4/5 (11.2)	
		526	21–22	9–10	22	4/4 (12.8)	
					Total	79–80/97	17 or 18/19

§ The chicks inoculated with M101-E17 are the same as those listed in Table IV, but are listed here according to the age at which their CNS was tested. For explanation of other symbols see Table IV.

It is of special interest that on 12 of the 13 chicks inoculated with material from passage M101-E17 tests were done on both, CNS and blood. Four of these (Nos. 8, 10, 11, and 12) were tested simultaneously, *i.e.* 2 days after hatching. The results indicated that the CNS contained probably about 100 times more virus than the blood. The brains and cords of the other chicks, tested 3 to 7 days later, were still more heavily infected than their blood had been on the second day. A pool of serum from chicks No. 521-527, obtained at the same time as the CNS of 5 of this group (Table V), failed to produce paralysis in 24

baby mice even though the CNS was positive in 3 or 4 of 5 tested.

Attempt to infect baby chicks. Only a single attempt was made to determine whether egg-adapted Hawaiian dengue virus could multiply in the CNS of hatched chicks. Seven one-day-old chicks were inoculated intracerebrally with embryo suspension M101-E18. The inoculum contained 10^3 mouse LD_{50}. All 7, as well as 2 un-inoculated chicks of the same age, died 6 or 7 days later. The brains and spinal cords of 3 inoculated chicks were removed immediately after death on the sixth day. Twenty percent suspensions were inoculated into mice. Only one of the 3 suspensions contained traces of virus, not enough to suggest that viral multiplication had taken place.

Discussion. The pronounced predilection of the egg-adapted Hawaiian dengue virus for nervous tissue of chick embryos is of special interest. As far as could be ascertained, Colorado tick fever virus is the only other virus for which similarly preferential growth in the CNS of chick embryos has been reported(6). It is remarkable that this is true for two agents which in their natural host show little if any neurotropic tendencies, while many other viruses which in nature and in experimental mammalian hosts are characteristically neurotropic, are present more or less indiscriminately in various parts of the embryo or in extra-embryonic membranes and fluids. In particular, this applies to the arthropod-borne encephalitis viruses, vesicular stomatitis virus and herpes virus(7). While Gard has reported that the FA strain of Theiler's mouse encephalomyelitis virus could be isolated only from the CNS, but not from other tissues of infected eggs(8), Riordan and Sa-Fleitas found the same virus distributed throughout the egg with the highest concentration in extraneural embryonic tissue(9). The growth of rabies virus in eggs has been studied most recently by Koprowski and Cox(10). According to these authors, the concentration of the virus in the CNS of embryos is only slightly higher than in the extraneural tissues. In contrast to the findings reported above for dengue virus, significant amounts of rabies virus were present in the chorio-allantoic membrane. Yellow fever, in many respects similar to dengue virus, is also less specific than the latter in its distribution in eggs. Fox and Laemmert(2) found that the neurotropic 17D strain attained about the same titer in the CNS and muscle tissue of embryos, and only slightly lower levels in blood, skin, gut and chorio-allantoic membrane.

Despite the preferential invasion of the CNS by dengue virus, no evidence has been obtained that the infection interferes with the normal growth and development of the chicks. The available data indicate that infection does not markedly impede their hatchability, even if eggs are inoculated as early as the eighth day of incubation. In contrast, rabies virus prevents hatching of chicks inoculated before the 15th day of incubation, and the growth and development of embryos inoculated at younger stages is considerably retarded(10). Similarly, the hatchability of embryos infected with 17D yellow fever virus before the 15th day of incubation is almost completely lost, while inoculation on the 17th day fails to diminish it significantly(2). In the case of dengue-infected embryos, despite the relatively high titer of the virus in the CNS tissue, no lesions were found in it and the proliferation of cells seemed to proceed at normal rate, as judged by the large number of mitotic figures seen in the CNS.

Nevertheless, the virus persisted in nervous tissue of chicks until at least ten days after hatching, *i.e.* at least 22 days after inoculation. Comparison of the amounts of virus found in the CNS and in blood of hatched chicks suggest that the early viremia is due to "spill-over" from the CNS. While none of the infected or normal chicks survived long enough to provide material suitable for sero-

6. Koprowski, H., and Cox, H. R., *J. Immunol.*, 1947, v57, 255.

7. Beveridge, W. I. B., and Burnet, F. M., *The cultivation of viruses and Rickettsiae in the chick embryo*, London, 1946.

8. Gard, S., *Acta Med. Scand.*, 1943, Suppl. v143.

9. Riordan, J. T., and Sa-Fleitas, M. J., *Science*, 1946, v103, 499.

10. Koprowski, H., and Cox, H. R., *J. Immunol.*, 1948, v60, 533.

logical tests, it may be permissible to apply tentatively some of the findings reported for rabies and yellow fever viruses to the dengue problem. Chicks hatching from eggs inoculated with rabies virus between the 15th and 19th day of incubation either died or responded to persisting infection with production of antibody(10). Rabies virus was found to persist in the CNS of one such chick until the 27th day after hatching. Fox and Laemmert(2) did not test the CNS of yellow fever-infected chicks for virus, but found it in blood of several newly hatched chicks. Chicks which had viremia early in life later responded with antibody formation. Further work is required in order to establish whether infection with dengue virus follows a similar pattern.

The failure of hatched chicks to support multiplication of virus after intracerebral inoculation seems to fall in line with the observation that susceptibility of chick embryos decreases with progressing age. In this respect, the Hawaiian strain of dengue virus appears to simulate the 17D strain of yellow fever virus(2).

Summary. 1. The Hawaiian strain of dengue virus has been propagated in chick embryos through more than 90 passages in a series initiated with virus derived from the 101st intracerebral passage in mice. Certain characteristics of the infection of chick embryos have been described in this paper. 2. Susceptibility of chick embryos decreases with increasing age, highest titers being obtained in embryos 4 to 5 days old at the time of inoculation. 3. While sustained propagation of the virus in eggs initially required secondary incubation of more than 5 days, in later egg passages the virus attained maximum titers after 5 days. There was a slight decrease in viral concentration after the seventh day. 4. The infectivity of the virus for chick embryos is of the same order of magnitude as that for mice. 5. Infection with dengue virus does not result in discernible lesions in chick embryos, nor does it appear to affect their growth or their hatchability. 6. The concentration of virus in embryonic brain and spinal cord tissue is 100 to 1000 times higher than in other tissues, with minimal amounts found in extra-embryonic membranes and fluids. 7. Virus has been found to persist in the CNS of chicks until at least ten days after hatching, 22 days after inoculation into the egg. Minimal amounts of virus, much less than present in the CNS, have also been found in the blood of chicks two days after hatching.

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Effect of Light on Urinary Coproporphyrin Excretion in Lead-Poisoned Rabbits.* (18642)

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Certain effects of ordinary light in patients and experimental animals with lead poisoning have been recorded in the literature. Pincussen(1) observed the effect of ultra-violet light (270 mμ), infra-red light and visible light on lead poisoned rats and mice. He concluded that the exposure of these animals to the infra-red and visible light produces an increase of 10-20% in the amount of lead excreted in the urine. Pincussen also mentioned certain "chemical" effects of ultra-violet light in these animals, but did not specify their nature, and a further report has not been found. Rapoport and Rubin(2) studied lead poisoned rats and found that the

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2. Rapoport, M., and Rubin, M. I., *Am. J. Dis. Child.*, 1941, v61, 245.