Overwintering of Western Equine Encephalomyelitis Virus in Experimentally Infected Garter Snakes and Transmission to Mosquitoes. (26006)

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Since viremia of long duration and high titer results from the experimental inoculation of garter snakes with Western equine encephalomyelitis (WEE) virus(1), the possibility that garter snakes serve as an overwinter reservoir of WEE virus was investigated. A hibernation cage was constructed for the purpose of holding snakes and mosquitoes under simulated natural conditions.

Materials and methods. The hibernation cage, as illustrated in Fig. 1, was composed of 3 compartments and dug into the bank of a small stream. Each compartment was filled with a mound of decayed logs and debris covered by a thick layer of rocks and soil. A layer of straw was provided as insulation. Throughout the lower part of the compartment, and leading from exposed surface to area of decayed logs, wooden ducts of various lengths provided passageways for snakes and mosquitoes. A pan of water and cotton pads wet with a 10% solution of honey were kept in each compartment as long as there was evidence of mosquito activity. Temperatures during the winter were much more severe than long time averages for the area:

Month	Minimum	Maximum	Median
Nov.	−24°F	68°F	29°F
Dec.	7	56	28
Jan.	−21	49	19
Feb.	− 9	50	28
Mar.	− 9	72	25
Apr.	25	78	44
May	25	84	51

The strain of virus used was originally isolated from *Culex tarsalis* from the state of Washington in 1957 and had undergone one chick passage and 3 suckling mouse passages.

Two species of wild garter snakes (*Thamnophis sirtalis parietalis* Say and *Thamnophis ordinoides vagrans* Baird and Girard) were inoculated intraperitoneally with 1 million suckling mouse LD_{50} in 0.05 ml virus-infected mouse brain suspension and placed in the hibernation cage. As snakes began to appear following hibernation, they were captured and held in small cages in the laboratory until May 1 when they were placed within the screened entrance of the hibernation cage. Blood was obtained from snakes by snipping their tails and tested for virus content in suckling mice. Numbers of snakes used in the study, as well as the date on which each group was placed in hibernation cage, are shown in Table I.

C. tarsalis for hibernation study were collected as larvae near Provo, Utah. They were fed as adults, either on a non-infected chick or on one that had been inoculated with virus, and were placed immediately in the hibernation cage. Numbers of mosquitoes of each group, and date they were placed in the hibernation cage, are shown in Table I. Uninfected *C. tarsalis* used to demonstrate transmission of virus from overwintering snakes to

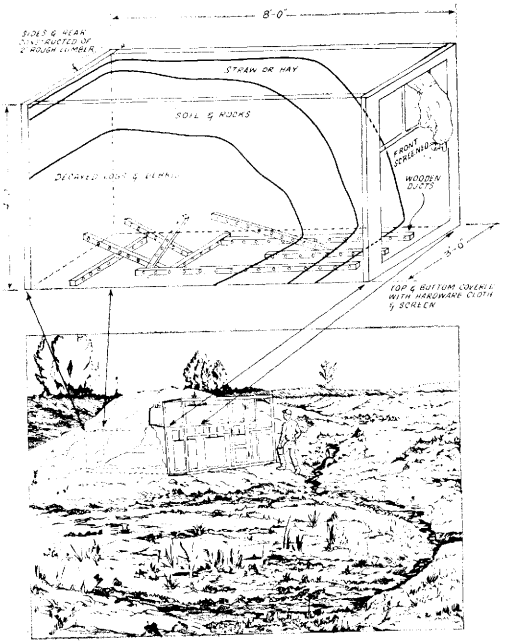

FIG. 1.

TABLE I. Mosquito and Snake Combinations in Hibernation Cage.

Compartment 1			Compartment 2			Compartment 3		
	No.	Date		No.	Date		No.	Date
Infected mosq.	30	9/11/59	Normal mosq.	?		Infected mosq.	155	8/28/59
(total 80)	50	10/20/59				(total 270)	40	8/31/59
			Infected snakes	5	9/14/59		25	9/ 1/59
Normal snakes	20	9/11/59	(total 50)	10	9/18/59		50	10/20/59
(total 30)	10	11/ 6/59		10	9/21/59			
				6	9/28/59	Snakes	0	
				19	11/ 6/59			

1-day-old chicks were obtained from the Communicable Disease Center, Greeley, Colo., through the courtesy of Mr. John S. Blackmore. These mosquitoes were fed on post-hibernating snakes which had viremia and held from 9 to 23 days before allowing them to feed on a non-infected 1-day-old chick.

Positive identifications of WEE virus isolated from snake bloods, mosquitoes, and chicks used to demonstrate transmission of virus were made using the mouse neutralization test(2). All virus titrations and identifications were made in suckling mice (0.05 ml, I.P.). The Reed-Muench method was used to calculate LD_{50}(3).

Results. No mosquitoes were found in any compartment following hibernation.

Twenty-five of 30 snakes were recovered from Compartment 1 between 3-23-60 and 5-9-60. No virus was detected in these snakes in a series of 3 bleedings at weekly intervals.

As shown in Table II, 26 of 50 inoculated snakes were recovered from Compartment 2. Virus was isolated from 23 of the 26 snakes with positive identification of WEE virus from 16 of the 23 snakes. Questionable virus isolations were made from 2 snakes—Nos. 6074 and 6358. No virus was detected from snake No. 5433. Virus was detected in snakes at least 70 days following recovery of snakes

TABLE II. WEE Virus Isolations from Snakes (Hibernation Study 1959-60).

Snake No.	Snake recovered from cage	Days after snake recovered on which blood tested for presence of virus	Snake died
5328	3/23/60	(0), *4*, 9	4/ 3
5326	3/24/60	*0*, 3, 8, 13	4/12
5327	3/24/60	(0), *3*, 8, 13, 19, 26, 34	5/ 6
5324	3/25/60	(0) (2), *4*, 7, 12, 18, 25, (33) (42) (53) (61) (68) (74) (81) (88) (95) (102) (109) (115)	8/ 3
5325	3/25/60	(0), *2*, 7, 12, 18, 20, 21, 25, 33, 42	5/17
5331	3/27/60	*0*, 5	4/ 3
5432	4/ 3/60	1, *3*, 9, 16, 24, 33, 44, 52, 59, 65	6/14
5433	4/ 4/60	(0) (2) (7) (15) (23) (32) (43) (51) (58) (64) (71) (78) (85) (92) (99) (105)	
5445	4/ 4/60	1, 2, *8*, 15, 23, 32, 43, 51, 58	6/ 7
5446	4/ 4/60	(1), 2, 7, *15*, 23, 32, (43) (51) (58) (64) (71) (78) (85) (92) (99) (105)	7/18
5448	4/ 5/60	(0), 1, *6*, 14, 22, 31, 42, 50, 57, 63	6/14
5449	4/ 5/60	(0) (2), 6, 14, 22, (31), 42, 50, 57, 63, 70, (77) (84) (91) (98) (104) (111)	8/ 1
5451	4/ 5/60	(0), 2, *6*, 7, 14, 22, 31, 42, 50, *57*, 63, 70	6/21
5504	4/ 8/60	(0), *4*, 11, 19, 28, 39, 47, 54, 60, 67, (74) (81) (88) (95)	7/18
6018	5/ 6/60	(0) (5) (11) (19), 26, 32, 39, 46, *53*, (60)	7/12
6019	5/ 6/60	(0) (5), 11, 19, *26*, 32, 39, 46, 53, (60), 67, (73) (80)	8/ 1
6020	5/ 6/60	(0) (5) (11) (19) (26), 32, 39, 46	6/28
6021	5/ 6/60	(0) (5), 11,* 19,* 26, 32, 39, 46, *53*, 60	7/12
6022	5/ 6/60	(0), 5,* 11,* 19, 26, 32, 39, 46, 53, 60, 67, (73) (80)	8/ 1
6051	5/ 9/60	(0), 2,* 8,* (16), 23,* 29,* *36*, 43, 50, 57, (64)	7/14
6055	5/10/60	0, 7, 15	6/ 1
6074	5/12/60	(0) (7) (15) (22) (28), 35*	6/21
6075	5/12/60	(1) (8) (16) (23) (29) (36) (43), 50, 57, 64, 70	7/19
6357	6/ 1/60	0, 2, 6,* (13) (20) (27) (34) (41) (47)	
6358	6/ 1/60	(0) (2), 6,* 13,* (20) (27)	7/ 5
6416	6/ 3/60	(0), 4, 11, (18) (25) (32) (39) (45)	

* Questionable isolation. ( ) = no virus detected. Italicized figures indicate day virus identified.

TABLE III. WEE Virus Titrations of Snake Bloods (Hibernation Study 1959-60).

Snake No.	Snake recovered from cage	Virus titrations: Day blood collected	Virus titrations: LD_{50}
5328	3/23/60	4, 9	5.8, 5.0
5326	3/24/60	3, 8, 13	2.8, 3.2, 6.3>
5327	3/24/60	8, 13, 19, 26, 34	3.9, 6.0, 4.5>, 4.3, 4.3
5324	3/25/60	7, 12, 18, 25	3.4, 2.3, <1.0, <1.0
5325	3/25/60	2, 7, 12, 18, 20, 21, 25, 42	2.4, 4.9, 4.8, 3.3, 3.4, 3.9, 3.3, 2.8
5331	2/27/60	0, 5	1.8, 2.3
5432	4/ 3/60	3, 33, 44, 52, 59, 65	5.5, 3.0, 3.3, 4.4, 2.8, 3.4
5433	4/ 4/60		
5445	4/ 4/60	1, 2, 15, 32, 43, 51, 58	2.6, 2.7, 5.3>, 5.0>, 3.5>, 2.7, 2.5
5446	4/ 4/60	15, 32	1.5, 1.5
5448	4/ 5/60	14, 31, 42, 50, 57, 63	4.8, 4.0, 2.5>, 4.3, 3.4, 2.9
5449	4/ 5/60	14, 42, 57, 63, 70	1.2, 3.5, 1.0, 2.5>, 1.0
5451	4/ 5/60	7, 14, 31, 42, 50, 57, 63, 70	4.0, 3.8, 3.2, 4.0, 3.4, 3.5, 4.3, 3.0
5504	4/ 8/60	11, 28, 39, 47, 54, 60, 67	2.2, 1.5, 2.5, 3.0, 4.0, 2.0, 1.7
6018	5/ 6/60	39, 46, 53	3.3, 2.6, 2.0
6019	5/ 6/60	11, 19, 26, 32, 39, 46, 53, 67	3.3, 2.4, 2.6, 4.5>, 1.9, 2.4, 1.3, 1.3
6020	5/ 6/60	32, 39, 46	3.3, 4.5>, 5.0>
6021	5/ 6/60	26, 32, 39, 46, 53, 60	1.5>, 3.5>, 4.1, 3.4, 3.3, 2.5
6022	5/ 6/60	26, 32, 39, 46, 53, 67	1.5>, 3.4>, 4.1, 2.6, 2.5>, 1.3
6051	5/ 9/60	36, 43, 50, 57	4.4>, 5.0, 2.8, 2.1
6055	5/10/60	7, 15	2.9, 4.4
6074	5/12/60		
6075	5/12/60	50, 64, 70	4.5, 3.4, 2.4
6357	6/ 1/60	2	2.3
6358	6/ 1/60		
6416	6/ 3/60	4, 11	4.0, 0.9

> = or greater; < = or less.

from hibernation. In many instances no virus was detected in the first blood specimen collected from the post-hibernating snakes; however blood collected after these snakes had been exposed to room temperature a few days yielded virus in most instances. In cases where the snake was bled and returned to outside conditions, there was a longer period of time during which no virus was detected.

As shown in Table III, a virus titer as high as $10^{6.3}$ LD_{50} was observed.

Virus transmissions were obtained from 4 hibernating snakes. In the case of snake No. 5324, WEE virus was isolated from snake blood, 6 of 30 mosquitoes following an extrinsic incubation period of approximately 3 weeks, and chicks that these mosquitoes fed on. WEE virus was isolated from blood of snake No. 5331, 1 mosquito that fed upon it, and the chick that the mosquito fed upon. In the case of snakes Nos. 5432 and/or 5445, the 2 snakes were placed together in the cage for mosquito feeding. Nine mosquitoes were fed on them. WEE virus was isolated from blood of both snakes, the 1 mosquito tested, and from the chick that it fed on. Virus was detected in snake bloods before and after mosquitoes fed on them in dilutions of $10^{2.3}$ to $10^{3.4}$ for snake No. 5325, $10^{1.8}$ to $10^{2.3}$ for snake No. 5331, and $10^{2.7}$ to $10^{5.5}$ for snakes Nos. 5432 and 5445.

Discussion. The failure to recover post-hibernating mosquitoes might be attributed to a number of causes, *e.g.*, the small number of mosquitoes under study and an inadequate hibernation-conditioning period following the blood meal, since the mosquitoes were placed in the cage immediately after they became engorged with blood. The unusually rapid drop in temperature from 68°F on 11-2-59 to -24°F on 11-16-59 may have caused high mosquito mortality.

Infected mosquitoes were confined with normal snakes in Compartment 1 to determine if after hibernation virus-infected mosquitoes transmitted virus to normal snakes. Since no mosquitoes survived overwinter, it was to be expected that no virus would be detected from these snakes.

Experimentally infected garter snakes were

shown to hibernate overwinter and to circulate virus in high titer and for long periods the following spring. Normal mosquitoes became infected by feeding on the emerged snakes and transmitted virus to chicks after an extrinsic incubation period of approximately 3 weeks. These data demonstrate a possible overwintering mechanism of WEE virus, but this virus has not, as yet, been isolated from garter snakes collected in the field.

Summary. Garter snakes were inoculated in September and November with Western equine encephalomyelitis (WEE) virus and were caused to hibernate under simulated natural conditions. They emerged during March, April, May and June. After varying periods when no virus was detectable in their blood, virus was detected in concentrations as high as $10^{6.3}>$ and for a period up to 70 days following emergence of snakes. Normal mosquitoes became infected by feeding on these snakes and after an extrinsic incubation period of approximately 3 weeks transmitted WEE virus to 1-day-old chicks. These data demonstrate that snakes may serve as a natural overwintering mechanism for WEE virus.

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ECHO-4 Viruses: Improved Methods and Strain Selection for Identification and Serodiagnosis.*† (26007)

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The majority of the Enteroviruses propagated in cell culture are readily identified by standard roller tube neutralization technics (1,2). A notable exception is ECHO-4(2,3). Plaque-forming strains of ECHO-4 virus may be identified by the plaque reduction technic (2,3). The applicability of the complement fixation (CF) technic for identification of the first 13 ECHO viruses except ECHO-4 was first demonstrated by Archetti *et al.*(4) using virus antigens prepared in HeLa cells. Halonen *et al.*(5,6) later found that the fluorocarbon procedure of Gessler *et al.*(7) as applied by Manson *et al.*(8) to viral antigens grown in cell culture removed anticomplementary activity of rhesus monkey kidney cell culture (RhMKCC) fluids and greatly reduced reactions between cell culture antigens and antibodies thereto, factors otherwise posing problems in interpretation of tests used for viral identification. This report, although limited to studies on ECHO-4 virus, describes a further modification of the CF test which virtually eliminates all antibody reactions with cell-culture antigens.

Another problem in diagnostic work involving ECHO-4 infections is the general inability to demonstrate neutralizing antibodies in the convalescent serum of the patient. A strain of virus is described which has been found to be readily neutralizable in tube culture tests.

Materials and methods. 1. Cell culture. Trypsinized RhMKCC were grown in 0.5% lactalbumin hydrolysate in Hanks' (LAH) balanced salt solution (BSS) containing 2% calf serum and maintained in lactalbumin

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