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Effects of Storage on Eastern Equine Encephalitis Virus. (20987)

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Because of a lack of adequate information on the preservation of virus in field and laboratory specimens, an investigation was undertaken to explore, in part, the effects of various storage conditions upon eastern equine encephalitis virus (EEE). Some of the studies were designed to determine effects of different insect killing methods and storage temperatures upon EEE virus in infected, intact mosquitoes. Others were intended to measure the effects of carbon dioxide gas in dry ice storage upon virus in intact mosquitoes and mouse brains as well as mosquito and mouse brain suspensions. Stabilizing properties of several types of diluents on virus-containing mosquito and mouse brain suspensions were also investigated.

Materials and methods. The mosquitoes (*Aedes aegypti*) were infected with a recently isolated strain of EEE by feeding upon infected chicks and used after 12 days extrinsic incubation at 80°F. Twenty-five specimens from the infected pool inoculated into mice were all found to contain virus, which indicated uniform infection of the pool. Pools of 5 mosquitoes were used for each unit of the intact mosquito studies. After storage, each pool of 5 specimens was ground in 1.0 ml of 0.1% bovine albumin-buffered saline diluent (pH 7.2) and centrifuged 10 minutes at 2500 RPM. The supernatant was decanted into vials in an ice bath, 2.0 mg of streptomycin and 1000 units of penicillin per ml were added and serial 10-fold dilutions made in cold 0.1% bovine albumin-saline. These dilutions were inoculated immediately via the intracerebral route into 3-week-old CFW mice to determine the virus titers, with the suspension of 5 mosquitoes in 1.0 ml considered as concentrated material or 10^0 dilution. Virus titers in all tables are expressed as the inverse log of the dilution killing half the animals inoculated. When mosquito suspensions rather than intact mosquitoes were stored, they likewise were prepared in the ratio of 5 mosquitoes per ml of diluent. Infected mouse brains were stored as 10% suspensions and as intact brain tissue.

Experimental. Effects of various insect killing methods upon EEE virus in infected mosquitoes. To determine whether the most common methods of killing field-caught mosquitoes affected EEE virus they might contain, infected mosquitoes were killed in the following ways: 1) Exposed to ether fumes for 2 minutes. 2) Exposed to chloroform fumes for 2 minutes. 3) Exposed to chloroform fumes for 2 minutes, plus wetting of one specimen of each pool of 5 with chloroform. 4) Quick-frozen on dry ice in contact with

TABLE I. Effects of Various Methods of Mosquito Killing upon Virus in EEE-Infected *Aedes aegypti* Specimens, 5 Lots.

Killing method	Titers, 5 mosquitoes/ml — Sublots: a	b	c	d	e	f
Ether fumes	5.5	5.5	5.5	5.5	5.0	5.3
Chloroform fumes	5.3	5.3	4.8	5.3	5.3	5.3
Idem + wetting	4.7	4.3	5.3	3.7	4.3	broken
Quick-freezing + CO_2 gas	5.3	4.5	5.5	5.5	5.3	5.3
Quick-freezing	4.7	5.0	5.0	4.5	5.3	5.3

CO_2 gas for 10 minutes. 5) Quick-frozen at dry ice temperature but not in contact with CO_2 gas (controls).

To test each of the above killing methods for virus-inactivating effects, 6 sublots of 5 specimens each were flame-sealed in soft glass ampules and stored on dry ice 4 months before virus levels were measured. The results (Table I) reveal no evidence that virus inactivation occurred when mosquitoes were killed by methods 1), 2), 4), and 5). A suggestion of some deleterious effect was observed in method 3) where the actual wetting of one specimen of every sublot of 5 deliberately introduced free chloroform into each ampule.

Effects of storage temperature upon EEE virus in intact quick-frozen mosquitoes. Infected mosquitoes were killed by quick-freezing, then flame-sealed in ampules and stored for a 4-month period under the following different conditions: (1) –70°C without fluctuation. (2) –70°C with 13 one- to 3-minute thawings at weekly intervals. (3) –70°C with one 6-hour thawing at room temperature (25°C) after 1 month's storage. (4) –70°C with an 11-hour interval at room temperature (6 hours after 1 month's storage and 5 hours after 2 months' storage). (5) –70°C with a 17-hour interval at room temperature (6 hours after 1 month's storage, 5 hours after 2 months, and 6 hours after 3 months. (6) Fluctuation between –5°C or –15°C to –50°C weekly on the top rack of a poorly maintained dry ice chest. (7) –20°C in a mechanical freezer. (8) Fluctuation between 0 and –20°C 2-5 times weekly in the freezing compartment of a household refrigerator in use in the laboratory. Controls were not placed in storage, but were titered immediately after quick-freezing. All tests were run in quintuplicate.

After storage under these conditions, the mosquito lots were ground, diluted, and titered. The results are shown in Table II.

TABLE II. Effects of Various Temperature Conditions upon EEE Titers in Ampuled, Intact *Aedes aegypti* during 4 Months' Storage, 8 Lots.

Storage conditions	Titers, 5 mosquitoes/ml — Sublots a	b	c	d	e
Unstored controls	4.8	4.5	4.0	4.0	4.8
–70°C, constant	5.0	5.7	4.7	5.0	5.3
" , with 13 brief thaws	4.3	3.7	broken	4.2	4.3
" , " 6-hr thaw	2.7	3.2	2.0	3.5	3.2
" , " 11-hr "	2.8	2.3	1.7	2.0	2.5
" , " 17-hr "	1.3	1.3	0.6	1.5	0.7
Fluctuations, –5 to –50°C	3.2	3.4	3.3	3.7	3.3
–20°C, constant	4.8	4.2	4.3	4.0	4.3
Fluctuations, 0 to –20°C	<.0*	.0†	1.5	.7	.0

* No mice died after inoculation of the most concentrated material (5 mosquitoes ground in 1.0 ml is considered the 10^{-0} dilution).

† Half the mice died when inoculated with the most concentrated material.

At a constant temperature of –70°C, no drop in virus titer occurred. A constant temperature of –20°C appeared to give nearly comparable results, with titers only about half a log lower than in the –70° series, and on a par with the titers of unstored control specimens. Storage on the top rack in a dry ice chest, with fluctuations below the freezing point, resulted in a loss of about 1 to 1½ logs in titer. Following storage in the freezing compartment of a household refrigerator in use in the laboratory a loss of nearly all the virus in the test mosquitoes occurred.

Infected mosquitoes withstood 13 brief thawings from –70°C to near room temperature at weekly intervals with little effect on the virus they contained. However, longer

TABLE III. Effect of CO_2 Gas upon EEE Virus in Intact *Aedes aegypti* Mosquitoes Stored 4 Months on Dry Ice, 3 Experimental Lots.

Type of seal	Titer, 5 mosquitoes/ml — Sublots a	b	c	d	e
Unstored controls	5.3	4.2	4.2	4.7	5.0
Glass sealed	4.7	5.5	5.0	4.5	5.0
Rubber stoppered, taped	5.5	5.3	5.0	5.5	5.3
Open ampules	4.5	5.0	4.5	4.5	4.7

TABLE IV. Effect of CO_2 Gas and Type of Diluent upon EEE Virus in Suspensions of *Aedes aegypti* Mosquitoes Stored 4 Months on Dry Ice.

Type of seal	Titers after storage in various diluents			
	Distilled water	Saline	0.1% bovine albumin	30% horse serum
Unstored control	3.0, 3.3, <3.0	4.3, 4.3, 4.0	4.2, 5.3, 4.2	5.0, 4.7, 4.0
Flame-sealed ampules	1.5, 2.3, 1.0	2.5, 2.5, 2.7	3.0, 2.5, 3.2	5.2, 4.7, 5.0
Open ampules	1.2, 1.8, 1.7	3.3, 2.8, 3.0	3.2, 2.3, 3.3	5.3, 5.5, 5.3

periods of thawing had more drastic effects. After 6 hours at room temperature there was drop in titer of approximately 1.5-2.0 logs; after 11 hours, about 2.0-2.5; and after 17 hours, about 3.0-3.5.

Effect of CO_2 gas upon EEE virus in intact infected mosquitoes stored on dry ice. Infected mosquitoes were stored 4 months on dry ice either in flame-sealed glass ampules, open ampules, or in test tubes sealed with tightly fitting rubber stoppers bound with 3 rounds of 1-inch adhesive tape. Control mosquitoes were ground and titered immediately after quick-freezing, the others after the storage period. All tests were run in quintuplicate. The results (Table III) revealed no significant differences among the different lots of specimens, regardless of degree of exposure to carbon dioxide gas.

Effect of CO_2 gas and type of diluent upon EEE virus in suspensions of infected mosquitoes stored on dry ice. Infected mosquitoes were ground in distilled water, saline buffered at pH 7.2, 0.1% bovine albumin in buffered saline, and 30% normal horse serum in buffered saline. A portion of each suspension was titrated in triplicate immediately; the remainder of each was dispensed in triplicate into ampules which were then either flame-sealed or left unsealed, and stored 4 months on dry ice before titering.

The results (Table IV) failed to show any adverse effects of CO_2 gas upon the virus. The 30% horse serum appeared to be the superior diluent, while the 0.1% bovine albumin-buffered saline and buffered saline alone were about intermediate in stabilizing power, and distilled water alone very poor.

Effect of CO_2 gas upon EEE virus in intact mouse brain tissue stored on dry ice. Three-week-old CFW mice were inoculated intracerebrally with 1000 LD_{50} each of EEE virus. Six were sacrificed when prostrate and the brains removed and divided into right and left halves. Left halves of 3 and right halves of another 3 were flame-sealed individually in Wassermann tubes as controls. The remaining right and left halves were placed unsealed in similar tubes. All were stored on dry ice for 4 months, after which time a 10% suspension was prepared of each half brain in 0.1% bovine albumin-buffered saline and titrated intracerebrally in mice. The results, given in Table V, reveal no significant differences in titers of those portions of brain exposed to CO_2 gas during storage and those not exposed.

Effect of CO_2 gas and type of diluent upon EEE virus in 10% mouse brain suspensions stored on dry ice. Ten per cent mouse brain suspensions were prepared in distilled water, saline buffered at pH 7.2, 0.1% bovine albumin in buffered saline, and 30% normal horse serum in buffered saline, using a pool of 3 EEE-infected brains for each suspension. Part of the material from each suspension was titered in duplicate immediately as an unstored control. The remainder was dispensed in triplicate in 1.0 ml amounts in ampules and stored flame-sealed or unsealed on dry ice for 4 months before titration.

The results (Table VI) reveal little difference in titers of the different brain suspensions, regardless of type of diluent or exposure to CO_2 gas during the test period.

TABLE V. Effect of CO_2 Gas upon EEE Virus in Intact Mouse Brain Tissue (Brain Halves) Stored 4 Months on Dry Ice.

Brain half	EEE virus titer per brain half	
	CO_2 free (A)	CO_2 exposed (B)
Left (A), right (B)	8.2	7.5
Idem	7.7	7.3
"	8.0	8.0
Right (A), left (B)	7.7	7.5
Idem	7.8	7.7
"	8.0	7.8

TABLE VI. Effect of CO_2 Gas and Type of Diluent upon EEE Virus in 10% Mouse Brain Suspensions Stored 4 Months on Dry Ice.

Type of seal	Titers after storage in various diluents			
	Distilled water	Saline	0.1% bovine albumin	30% horse serum
Unstored control	7.8, 8.2	7.8, 8.8	8.7, 8.6	8.2, 8.7
Flame-sealed ampules	8.5, 8.2, 8.3	9.0, 8.7, 8.0	9.0, 8.5, 9.3	7.8, 9.3, 8.2
Open ampules	8.2, 7.7, 8.0	7.0, 8.0, 8.5	8.0, 8.5, 8.0	8.3, 8.5, 8.2

Summary. A 4-month study on the influence of certain factors upon eastern equine encephalitis virus in stored mosquitoes and brain tissue revealed the following: (1) Killing of EEE-infected mosquitoes by brief exposure to ether or chloroform fumes had no ill effects upon the virus they contained. (2) A constant temperature of −70°C or −20°C was found best for preserving EEE virus in intact, infected mosquitoes. Several hours at room temperature caused loss of viability of most of the virus present, as did fluctuations 2 to 5 times weekly from 0°C to −20°C. Fluctuations in temperature below the freezing point also caused some drop in virus titer. (3) Carbon dioxide gas in an open type dry ice chest had no apparent deleterious effect upon EEE virus in infected, intact mosquitoes, intact mouse brain tissue, or mosquito and mouse brain suspensions in distilled water, buffered saline, 0.1% bovine albumin, or 30% normal horse serum. (4) Distilled water was an unsuitable diluent for suspensions of EEE-infected mosquitoes. Buffered saline and 0.1% bovine albumin both gave better, but still rather poor results. Thirty per cent normal horse serum proved superior, since with it no drop in virus titer was observed. (5) All the above diluents gave satisfactory results in 10% EEE-infected mouse brain suspensions during the 4-month test period.

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Use of a Cation Exchange Resin for Isolation of Influenza Virus. (20988)

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The adsorption on and elution from ion exchange resins of a number of different viruses has been demonstrated by several workers. The partial purification of several neurotropic viruses was achieved by Muller (1) by the use of a cationic resin. An anionic resin was used by Lo Grippo(2) for the purification of the Lansing strain of poliomyelitis, and the extraction of poliomyelitis virus from human fecal material. That influenza virus can be adsorbed and eluted from cation resins has been shown by Muller and Rose(3), and Puck and Sagik(4). The studies presented here were undertaken to determine whether unadapted influenza virus from human sources behaves in a similar manner, and whether ion exchange resins can be used for the isolation of influenza virus.

Materials and methods. A cation exchange resin, Nalcite HCR-X12, 50-100 mesh,† was converted from the acid to the sodium form by several washings of 10% NaCl until the pH of the supernate was the same as that of the salt solution before addition to the resin. It was then washed with distilled water until washings no longer produced a precipitate when $AgNO_3$ was added. Resin which had been used was washed 3 to 5 times with distilled water then kept in a 56°C water bath for several days in 10% NaCl. This served to elute and inactivate any virus adsorbed

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