Infectivity of Nucleic Acid and Adsorptive Properties of Venezuelan Encephalitis Virions Inactivated by Lipid Solvents[1] (34839)

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A property of most arthropod-borne animal viruses (arboviruses) is inactivation (*i.e.*, destruction of infectivity) by lipid solvents, in particular, sodium deoxycholate (SDC), diethyl ether, and chloroform (1–3). Yet, despite the common use of these chemicals to characterize newly isolated arboviruses, the mechanism of viral inactivation, especially with diethyl ether and chloroform, has not been studied with a variety of arboviruses. Inactivation by lipid solvents has been attributed to removal of "peripheral structural lipids" with subsequent "degradation" of virions (4), a conclusion supported by the observation that SDC in high enough concentrations (0.2–1%) released infectious ribonucleic acid (IRNA) from purified suspensions of arboviruses (5). However, SDC is structurally unlike diethyl ether or chloroform, and their actions on arboviruses might, therefore, be dissimilar.

In this study SDC, diethyl ether, and chloroform were examined at commonly employed, inactivating concentrations to learn whether inactivated virions still yielded IRNA upon phenol treatment, and adsorbed to susceptible animal cells. A group A arbovirus, Venezuelan encephalitis, was selected because similar studies had been done with a group B arbovirus, Murray Valley encephalitis (6). That group A arboviruses might behave differently from group B was suggested by the resistance of group A but sensitivity of group B viruses to SDC in serum and to proteases (7, 8).

Methods. VE virus. Strain 63U2, isolated from a sentinel hamster in Mexico during 1963 (9), was utilized after six or seven suckling mouse brain passages or after six mouse brain and one primary chicken embryonic cell culture (CEC) passage. Brains were removed from Swiss albino mice about 24 hr after intracranial inoculation, and virus suspensions were supernatant fluids (10^4g, 1 hr, 0°) from 10% tissue in 1% bovine albumin in Hanks' solution at pH 8.0, containing 100 U of penicillin and 100 μg of streptomycin per ml (BA). CEC were prepared and used as described elsewhere (10); fluids were harvested 20 hr after adsorption of virus at multiplicities of 100–1000 and were centrifuged at 10^4g for 1 hr at 0° before supernatant fluids were mixed with an equal volume of BA and stored in an electric —60° box in screw-capped vials. For experiments with crude virus suspension, virus from CEC was thawed, centrifuged at 10^5g for 2 hr at 5°, and the pellets were resuspended in volumes of BA equivalent to the uncentrifuged virus. *Virus was partially purified by treating* CEC harvests with final concentrations of 2 μg/ml of pancreatic ribonuclease for 20 min at 37°, centrifuging for 2 hr at 80,000*g* at 5° and discarding supernatant fluids to remove enzyme. Sediments were resuspended in phosphate-buffered saline at pH 7.2, equivalent to the original volume of virus suspension, and centrifuged again (2 hr, 80,000*g*, 5°). Virus sediments were suspended in one-fifth the original volume of saline, and sodium dextran sulfate was added to make 0.2% to preserve viral infectivity; they were then shaken for 10 min at 5° with 0.5 vol of trichlorotrifluoroethane (Freon; Matheson, Coleman and

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Bell Co., East Rutherford, New Jersey). Fluorocarbon-water emulsions were centrifuged at 400*g* for 5 min at 5° to separate the water and fluorocarbon phases. The water phase was diluted to the original volume with saline and centrifuged at 80,000*g* for 2 hr at 5°. Virus pellets were resuspended in BA and stored at −60°. In tests for residual RNase adhering to virions after partial purification, virus was suspended in Hanks' solution and used after storage for 9 months at −60°.

VE viral IRNA was prepared by standard methods of hot and cold phenol extractions (11, 12). Versene (ethylene diamine tetracetate) at approximately 0.001 *M* final concentration was added to CEC virus preparations prior to attempts to extract IRNA with phenol or lipid solvents.

Treatment of VE virus or IRNA with SDC, diethyl ether, or chloroform. IRNA or virus suspensions were mixed with equal volumes of solvents (see subsequent tables for concentrations), and the mixtures shaken continuously at approximately 280 oscillations per minute, employing an electric shaker (Khan, Adams, Clay-Adams Co., New York, New York) with a half-inch stroke at 3°. After 1 hr, ether and chloroform mixtures were centrifuged at 400*g* for 5 min at 0° to separate aqueous and solvent phases. The aqueous phase was then decanted and nitrogen bubbled through it to remove as much volatile solvent as possible. SDC was special enzyme grade (Mann Research Laboratories, New York, New York), diethyl ether was anhydrous analytical grade (Mallinckrodt Chemical Works, St. Louis, Missouri) washed five times with 4 vol of glass-distilled water just before use, and chloroform was reagent grade (Merck & Co., Rahway, New Jersey). For control purposes, IRNA was inactivated by incubation for 20 min at 22–25° with RNase (bovine pancrease, crystalline code K, Worthington Biochemical Corp., Freehold, New Jersey) 0.1 μg/ml final concentration.

Assay of VE virus and IRNA. VE virus and IRNA were assayed by counting plaques on 18-cm^2 sheets of primary chicken embryonic cells in cultures, prepared as described elsewhere (10) except that 0.15% instead of 0.25% lactalbumin hydrolyzate was in the overlay medium. Virus was diluted in isotonic Hanks' solution, and IRNA in hypertonic saline (1.0 *M* NaCL in 0.1 *M* Tris–HCl, pH 8.2). After adsorption of virus to cells for 1 hr at 37°, the inoculum was not removed nor were cell sheets rinsed. For IRNA assays, cell sheets were rinsed with 2–3 ml of Hanks' solution without calcium and magnesium ions and then with 0.5 *M* NaCl, IRNA was adsorbed in 0.2 ml for 15 min at 22–25°, and the inoculum was removed and cell sheets washed once with 3.0 ml of Hanks' solution. Agar medium (10) was then placed over cells, and plaques counted after 2–4 days at 37°.

Adsorption to CEC of hemagglutinin in lipid solvent-inactivated virus suspensions. Inactivated virus with hemagglutinating activity was prepared as described above, from infectious virus harvested from CEC except that ether was used for 4 hr, chloroform for 2.5 hr and SDC (final concentration 0.1%) for 6 hr followed by dialysis against Hanks' solution for 24 hr at 3° to reduce the SDC concentration below that detrimental to CEC and goose erythrocytes used in hemagglutination tests. Inactivated or untreated virus suspensions were added in 0.1 ml to either 18-cm^2 sheets of CEC (washed beforehand with 2–4 ml of Hanks' solution two to four times to remove hemagglutinin-inhibitors in serum-containing medium) or to empty bottles. At selected times, fluids were tested for hemagglutinating activity as described elsewhere (13), except that approximately 24,000 goose erythrocytes per cubic millimeter were used; tests were conducted at pH 6.2 and incubated at 37° for 1 hr.

Interference tests. A control preparation of inactivated VE virus which would interfere with infectious VE virus was made by placing 3 ml of virus suspension from CEC in a 100-mm petri dish and exposing it for 3 min to ultraviolet light 5 cm below a Westinghouse sterilamp 782L-30; it was then diluted 1:2 with maintenance solution (MS) (10) and incubated for 20 min at 50°. Virus from CEC was inactivated with ether, or virus from mouse brain diluted 10^{-2}, with chloro-

form, by shaking equal volumes of solvent and virus suspension for 6 hr. This time period was necessary to inactivate virus to a residual infectious virus level less than the 10 PFU used for challenge. CEC monolayers containing approximately 10^5 cells in 16 × 125 screw-capped test tubes were incubated for 24 hr at 37° with 0.3 ml of (a) inactivated virus suspension (undiluted for uv–heat and chloroform and 10^{-1} dilution for ether), or (b) MS for controls. This small number of cells per culture was used to gain a multiplicity of inactivated virus of approximately 100 based on preinactivation infectious virus titers. Two tube cultures were used for uv–heat-treated virus, three or four for ether- or chloroform-treated virus and three for controls. Cell sheets were washed twice with 1 ml of Hanks' solution, and 0.1 ml of infectious VE virus containing approximately 10 PFU (*i.e.*, the maximal countable plaques on the small cell sheets used) was adsorbed to each cell monolayer for 1 hr at 37°, and cells were covered with agar medium. Plaques were counted after incubation at 37° for 4 days.

TABLE I. Appearance of IRNA in Crude Suspensions of VE Virus After Treatment with Inactivating Concentrations of SDC, Diethyl Ether, and Chloroform.

Chemicals and source of virus	Exp.	Infectivity titer as negative $\log_{10}$ PFU/ml assayed in isotonic or hypertonic solution[a]				Release of IRNA
		Before treatment	After treatment			
		Isotonic	Isotonic	Hypertonic	Hypertonic + RNase	
Controls						
Hot phenol (40°)						
Mouse brain	1	9.4	<1.9	6.3	<1.9	Yes
CEC	2	8.5	<1.9	4.8	<1.9	Yes
Cold phenol (3°)						
Mouse brain	3	9.7	<1.9	6.0	2.3	Yes
	4	9.5	<1.9	6.2	<1.9	Yes
CEC	5	8.6	<1.9	5.3	<1.9	Yes
	6	8.6	<1.9	5.0	<1.9	Yes
Tests						
SDC (0.1%)						
Mouse brain	7	9.2	4.2	6.1	4.2	Yes
	8	9.3	4.4	6.3	4.1	Yes
CEC	9	8.6	4.0	5.0	4.2	Yes
	10	8.6	3.8	5.1	3.8	Yes
Diethyl ether (50%)						
Mouse brain	11	9.2	4.0	6.3	<4.0	Yes
	12	9.3	3.8	6.2	3.2	Yes
CEC	13	8.6	3.2	4.3	3.0	Yes
	14	8.6	3.2	4.7	3.0	Yes
Chloroform (50%)						
Mouse brain	15	9.4	4.3	<3.0	<3.0	No
	16	9.2	3.7	3.2	3.2	No
CEC	17	8.6	3.3	4.7	3.0	Yes
	18	8.3	3.6	4.0	<3.0	Yes

[a] For assays in isotonic and hypertonic solution, see *Methods*.

TABLE II. Attempts to Release IRNA from Preparations of Partially Purified VE Virus by Treatment with Inactivating Concentrations of SDC, Diethyl Ether, and Chloroform.

		Infectivity titer as negative $\log_{10}$ PFU/ml assayed in isotonic or hypertonic solution				
		Before treatment	After treatment			
Chemicals	Exp.	Isotonic	Isotonic	Hypertonic	Hypertonic + RNase	Release of IRNA
Controls						
Hot phenol (40°)	19	9.0[a]	<1.9	5.0	<1.9	Yes
	20	8.9	<1.9	4.0	<1.9	Yes
Cold phenol (3°)	21	9.1	<1.9	2.7	<1.9	Perhaps
	22	9.0	<1.9	<1.9	ND[b]	No
SDC (1.0%)	23	9.0	<2.9	4.3	<2.9	Yes
	24	9.1	3.8	4.7	3.5	Yes
Tests						
SDC (0.1%)	25	9.0	4.0	3.7	3.7	No
	26	9.0	4.6	4.6	4.5	No
Diethyl ether (50%)	27	9.1	3.4	3.0	3.2	No
	28	8.8	3.2	3.4	ND	No
Chloroform (50%)	29	9.0	3.6	3.7	3.6	No
		9.2	<3.8	<3.8	ND	No

[a] Titer in hypertonic solution 8.5 and in hypertonic solution plus RNase, 8.5. For assays in isotonic and hypertonic solution, see *Methods*.

[b] ND = not done.

Results. Release of IRNA from SDC- , diethyl ether- , or chloroform-inactivated, partially purified VE virions by phenol extraction. It was necessary to use partially purified virions for these experiments because crude virus suspensions from mouse brain or CEC inactivated with SDC or diethyl ether, or from CEC inactivated with chloroform yielded IRNA apparently from incomplete virion, *i.e.*, those with unstable, or totally without, envelopes and capsids (Table I)[3]. In contrast, partially purified infectious virus preparations yielded no IRNA after treatment with commonly employed, inactivating concentrations of SDC (0.1%), diethyl ether (50%), or chloroform (50%) (Table II). Only after exposure to hot phenol, 1.0% final concentration of SDC, or sometimes cold phenol, was IRNA released from partially purified infectious virions (Table II).

Detection of IRNA after treatment of virus with SDC, diethyl ether, or chloroform was not related to the capacity of these chemicals to inactivate RNase in viral preparations because only chloroform and phenol inhibited RNase.[4] The possibility that IRNA was released from purified virus, but was

[3] IRNA was not found in chloroform-treated mouse brain viral suspensions, but since the infectivity titer of phenol-prepared IRNA was reduced 200-fold when it was mixed with normal mouse brain and chloroform it is possible that chloroform yielded small amounts of IRNA from brain suspensions which were then destroyed by a product of a brain–chloroform interaction.

[4] RNase, 0.01 or 0.1 μg/ml, was mixed in equal volume with 0.1 or 1% SDC, 50% ether, 50% chloroform, or 50% hot or cold phenol and shaken for 1 hr at 3°. The aqueous phase was separated and residual RNase activity was assayed by adding an equal volume of VE IRNA prepared by hot phenol extraction of virus from CEC and incubating at 22–25° for 20 min. IRNA titers were reduced >1.9 $\log_{10}$ PFU/ml after exposure to SDC- or ether-treated RNase, but were insignificantly reduced (<0.6 $\log_{10}$ PFU/ml) with chloroform- or phenol-treated RNase.

TABLE III. Release of IRNA from Lipid Solvent-Inactivated, Partially Purified VE Virions by Treatment with Hot and Cold Phenol.

Virus[a] inactivated by:	Exp.	Infectivity as negative $\log_{10}$ PFU/ml in hypertonic solution without and *with RNase* after treatment with: No phenol	Hot phenol	Cold phenol
SDC (0.1%)	31	3.0, *3.0*	5.1, *<1.9*	
	32	3.5, *3.5*		4.2, *<1.9*
Diethyl ether (50%)	33	3.0, *3.0*	5.3, *<1.9*	
	34	<3.3, *<3.3*		4.3, *<1.9*
Chloroform (50%)	35	3.1, *2.9*	5.2, *<1.9*	
	36	<3.3, *<3.3*		4.2, *<1.9*

[a] Original titer before any treatment was 8.5 $-\log_{10}$ PFU/ml in hypertonic solution assay.

destroyed by RNase adherent to virions also seemed unlikely because RNase activity could not be demonstrated adhering to ether-inactivated or stored virions in sufficient concentrations measurably to affect IRNA.[5]

IRNA was also released by hot phenol extractions of SDC-, diethyl ether-, and chloroform-inactivated, partially purified virions (Table III), and the titer of this IRNA was similar to that obtained with hot phenol extraction of untreated virions (Table II, experiments 19 and 20 vs Table III, experiments 31, 33, and 35). Cold phenol yielded substantially more IRNA from lipid solvent-inactivated virions than from untreated partially purified virions (Table II, experiments 21 and 22 vs Table III, experiments 32, 34, and 36). These findings suggested that SDC, diethyl ether, and chloroform changed VE virions in a way which enhanced the ability of cold phenol to release IRNA. Noteworthy also was the release of VE IRNA from lipid solvent-inactivated virus suspensions which had significantly lower infectious virus titers (about 10^3 PFU/ml) than those usually considered necessary to yield IRNA with phenol (10^{6-8} or more PFU/ml).

Thus, it seemed that VE virions which were inactivated by lipid solvents remained intact sufficiently to retain their IRNA, and that inactivation was not due to a complete rupture of the virion, but probably rather to more subtle changes in its surface. Inactivation was clearly not due to a direct effect of SDC, diethyl ether, or chloroform on VE IRNA because when phenol-extracted IRNA from CEC viral suspensions was exposed to inactivating concentrations of SDC, ether, and chloroform, its infectivity titer, measured in hypertonic solution, remained unchanged (Table IV) like IRNA from other arboviruses (5, 6, 14, 15). Therefore, the possibility that surface changes might affect the virion's ability to adsorb to cells was studied next.

Attempts to detect adsorption of VE virions inactivated by SDC, diethyl ether, and chloroform to tissue cells. Since Semliki Forest arbovirions inactivated by ether or caseinase C in Tris-buffered saline still hemagglutinated (16, 17), hemagglutination was tried as a method to assay inactivated VE virus during adsorption experiments in CEC. It was found that hemagglutinin in lipid solvent-inactivated suspensions of VE virus did not adsorb to CEC monolayers within 60 min, while hemagglutinin in untreated suspensions adsorbed in 30 min (Fig. 1). These observations suggested that lipid

[5] Phenol-extracted IRNA was incubated for 20 min at 25° with suspensions of partially purified virions inactivated (a) by ether to a residual infectious virus titer of 3.7 $-\log_{10}$ PFU/ml (equal parts of virus and ether shaken 1.5 hr at 3°), or (b) by storage for 9 months at −60° in Hanks' solution which yielded a residual virus titer of 3.5 $-\log_{10}$ PFU/ml. Negative $\log_{10}$ PFU/ml of VE IRNA were 4.3 before and after incubation with equal parts of either inactivated virus preparation, but decreased from 4.3 to <1.9 after similar incubation with RNase (0.1 μg/ml).

TABLE IV. Failure of SDC, Diethyl Ether, and Chloroform to Inactivate IRNA Extracted from VE Virus with Phenol.

Phenol-extracted IRNA treated with:	Infectivity titer in hypertonic solution as negative $\log_{10}$ PFU/ml	
	Before treatment	After treatment
Control		
RNase (0.1 μg/ml)	5.1, 4.3	<1.9, <1.9
Test		
SDC (0.1%)	5.1	5.2
Diethyl ether (50%)	5.1	5.3
Chloroform (50%)	5.1, 4.3	5.2, 4.3

solvent-inactivated VE virions did not adsorb to susceptible animal cells. However, they were also compatible with detachment of active hemagglutinin from virions during chemical treatment, in which case, inactivated virions, intact except for their hemagglutinins, could still have adsorbed.

Therefore, the possibility was examined that inactivated virions might adsorb to cells and interfere with active virions. As described in *Methods,* small cell sheets had to be used to obtain large multiplicities (100) of inactivated virus, but as a consequence, only small numbers of PFU of infectious VE virus could be counted on these sheets and thus used for challenge. Ultraviolet–heat-inactivated VE virions were employed as controls to demonstrate that interference would occur in the system utilized. The numbers of PFU were equal in cultures unexposed and exposed to lipid solvent-inactivated virus (Table V), suggesting that under the conditions of these experiments, ether- and chloroform-inactivated VE virions did not interfere with small amounts of challenge infectious VE virus. Since interference probably requires adsorption of interfering virus even when mediated through interferon, these results suggested that ether- or chloroform-inactivated virus did not adsorb to chicken embryonic cells in culture.

Discussion. These results showed that when a group A arbovirus, Venezuelan encephalitis (VE), was treated with common-

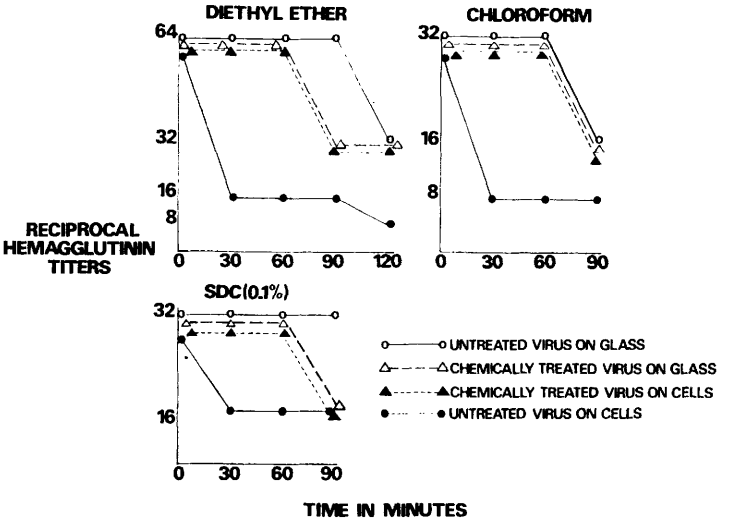

FIG. 1 Attempts to adsorb VE virus inactivated by diethyl ether, chloroform, or SDC onto primary chicken embryonic cells cultured *in vitro*.

TABLE V. Attempts to Demonstrate Interference Between VE Virus Inactivated by Diethyl Ether and Chloroform and Infectious VE Virus.

	Average numbers of PFU of infectious VE virus in 2–4 CEC cultures treated with:			
		Virus inactivated by:		
Exp.	No virus	Uv, heat	Ether	Chloroform
1	10	0	7	ND[a]
2	11	ND	10	ND
3	6	0	ND	8

[a] ND = not done.

ly employed, inactivating concentrations of sodium deoxycholate (SDC), diethyl ether, or chloroform, the virion was not totally disrupted because infectious ribonucleic acid (IRNA) was not released until inactivated virions were treated with phenol. Phenol-extracted IRNA of VE virus was not adversely affected by inactivating concentrations of SDC, diethyl ether, or chloroform indicating that VE virus was not inactivated by these lipid solvents through a direct action on IRNA. These observations with a group A arbovirus were like those obtained with Murray Valley encephalitis virus from group B, because MVE virus inactivated by ether, chloroform, or phospholipase A also did not release IRNA until it was treated further with phenol (6). Arboviruses inactivated by other agents which presumably do not involve lipids, *viz.*, heat- or nitrous acid- (but not ultraviolet light-) inactivated VE and eastern encephalitis viruses, also have yielded IRNA upon phenol treatment (18). Thus, studies to date indicate that surface components of arbovirions containing lipids, possibly phospholipids and probably proteins, can be modified by selective treatments in such a way as to destroy infectivity of the whole virion but retain structural integrity and infectivity of the nucleic acid within the virion.

The function of infectivity sites presumably on the arboviral envelope remains to be determined. These experiments attempted to relate them to viral adsorption to susceptible cells by measuring (a) hemagglutinating activity, and (b) interference induced by inactivated virus upon contact with cells. The failure of the hemagglutinating activity of SDC- , diethyl ether- , or chloroform-inactivated VE virus to disappear from fluid overlying chicken embryonic cells, and of inactivated virus to interfere with infectious VE virus were compatible with a relationship between infectivity sites of the viral envelope and adsorption to cells. Recently reported studies with Sindbis virus support this concept since SDC-treated, noninfectious virions with labeled nucleic acid did not attach to tissue cells (19). Further studies including direct microscopic examination of virus–cell interactions and physical characterization of the virus particles after treatment with these lipid solvents should also be done to establish this concept unequivocally.

Summary. Venezuelan encephalitis (VE) virions, inactivated by 0.1% sodium deoxycholate (SDC), 50% diethyl ether, or 50% chloroform yielded infectious ribonucleic acid (IRNA) upon hot or cold phenol treatment, but they could not be shown to adsorb to cultured chicken embryonic cells as measured by hemagglutinin in cultural fluid or by interference with homologous virus. These lipid solvents in the above concentrations did not inactivate phenol-extracted IRNA nor did they release IRNA from purified VE virions, although it was found after treatment of crude suspensions of virus which apparently contained incomplete, unstable virions. Therefore, it is postulated that SDC, diethyl ether, or chloroform inactivate VE virus by altering viral surface properties related to adsorption to susceptible cells rather than by disruption of the capsid with release of IRNA or by destruction of IRNA within the virus.

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