Different Effects of Deoxycholate, Ether, Chloroform, Hydrocarbons, and Alcohols on Venezuelan Encephalitis Viral Infection, Hemagglutination, and Complement-Fixation[1] (34550)

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Arboviruses are thought to contain lipids essential for infectivity because certain lipid solvents reduce infectivity titers (*i.e.*, inactivate) at least 133 of the more than 200 currently listed arboviruses (1). Presumably the commonly employed chemicals (*i.e.*, a surfactant, sodium deoxycholate (SDC), an ether, diethyl ether, and a polyhalogen, chloroform) dissolve peripheral structural lipids and degrade the virion (2). However, there are other lipid solvents, such as straight chain and cyclic hydrocarbons and alcohols, which have not been extensively studied for their effects on arboviruses. Straight chain and cyclic, saturated hydrocarbons, like SDC, diethyl ether, and chloroform, are poor solvents for lipoproteins, but are good solvents for unbound lipids, phospholipids, neutral fats, and steroids (3–6). Alcohols, on the other hand, dissolve lipoproteins as well as unbound lipids and affect proteins.

A study was therefore made of nine lipid solvents to learn if they had similar or different effects on the abilities of a model arbovirus, Venezuelan encephalitis (VE) virus, to infect, hemagglutinate, and fix complement in the presence of antibody. The study was based on the premise that if the chemicals primarily dissolved certain lipids essential for these functions of the virion, they should have similar effects, whereas if different lipids or nonlipid compounds were involved, effects of the chemicals would differ.

Materials and Methods. VE virus. Strain 63U2 (7) was utilized after six or seven suckling mouse brain passages or after six mouse brain and one chicken embryonic cell culture (CEC) passage. Virus suspensions from mouse brain were supernatant fluids (10^4g, 1 hr, 0°) from 10% tissue, suspensions in 1% bovine albumin in Hanks' solution at pH 8.0, containing 100 units of penicillin and 100 μg of streptomycin per ml (BA). Virus suspensions from CEC, prepared and utilized as described elsewhere (8), were cultural fluids harvested 20 hr after adsorption of viral multiplicities of 100–1000 pfu/cell; they were centrifuged at 10^4g, for 1 hr, at 0°, and mixed with an equal volume of BA. Virus suspensions were stored at −60°, or were lyophilized by exposure to pressures of 15–20 μ for 6 hr; lyophilized virus was sealed under vacuum in glass ampoules and kept at −60° until reconstituted with sterile glass-distilled water.

For certain hydrocarbon experiments fluid was harvested 3 days after inoculating CEC with about 0.001 pfu/cell and incubating at 37°. Virus was purified by centrifuging at 1000*g* for 15 min at 5°. The supernatant fluid was then centrifuged in 30- to 35-ml aliquots layered on 9 ml of 15% sucrose, at 73000*g* for 3 hr at −5°. The sediment was resuspended in 1% bovine albumin in 0.05 *M* Tris at pH 8, stored at 5° overnight, treated with RNase (0.2 mg/ml final concentration) at 25–28° for 30 min, and the mixture centrifuged on 15–30% sucrose gradients (80,000*g*, 2.5 hr, −5°.). The distinct white bands

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present about 15–20% of the gradient height from the bottom were removed with capillary pipettes, pooled, and used promptly for hydrocarbon experiments.

Treatment of VE virus with lipid solvents. Aliquots of virus suspensions were mixed with equal volumes of solvents or Hanks' solution for controls, and the mixtures were 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°. With lyophilized virus, the volume of lipid solvent equalled the volume of reconstituted virus suspension. At selected time intervals, the mixtures were centrifuged at 400*g* for 5 min at 0° to separate the aqueous and solvent phases in those cases where two phases were separable. The aqueous phase was then decanted and nitrogen bubbled through it to remove as much volatile solvent as possible. Assays were done promptly considering the aqueous phase as 10^0. SDC was special enzyme grade (Mann Research Laboratories, New York, New York), diethyl ether was anhydrous and analytical grade (Mallenckrodt Chemical Works, St. Louis, Missouri) washed five times with 4 vol of glass-distilled water just before use, and chloroform was reagent grade (Merck & Company, Rahway, New Jersey). Hexane and benzene were from Matheson Coleman and Bell, (East Rutherford, New Jersey), pentane from that source or Fisher Scientific, Fair Lawn, New Jersey, pentanol from Eastman Organic Chemicals, Rochester, New York, butanol from J. T. Baker Chemical Company, Phillipsburg, New Jersey, and absolute ethanol from Commercial Solvents Corporation, Newark, New Jersey.

Assays for infectious VE virus. CEC were prepared and virus assayed as described for Japanese encephalitis virus (8) except that 1:400 (w/v) cell suspensions were used to inoculate bottles, and 0.15% lactalbumin hydrolyzate was incorporated into the solid medium. VE virus plaques were counted after incubation at 37° for 2–4 days.

Hemagglutination tests were done according to methods already described (9) except that approximately 24,000 goose erythrocytes per cubic millimeter, as determined by count in a hemocytometer, were used, and tests were conducted at pH 6.2 and incubated at 37° for 1 hr.

Complement-fixation tests were done in disposable plastic trays covered with Saranwrap, employing 0.025 ml of antigen, 0.025 ml of antibody, 0.05 ml (2 full units) of complement, and 0.05 ml of sensitized sheep erythrocytes (a mixture of equal parts of sheep erythrocytes, 500,000/mm^3, and ambocepter, 3 units/0.25 ml). Incubation of antigen, antibody, and complement was at 5° for 16 hr after which sensitized cells were added, and tests were read after 30 min at 37°. Antigen titers were expressed as the highest dilution yielding 0–25% cell hemolysis with 4 units of VE virus mouse antiserum.

Results. Effects of various lipid solvents on infectivity of VE virus. Virus suspensions from two sources, mouse brain and chicken embryonic cell cultures (CEC), were examined to detect possible differences in virions from two types of host cells. As expected, the conventional organic solvents (SDC, diethyl ether, and chloroform) reduced titers of infectious virus by a factor of 10^7 or more, but, surprisingly, hydrocarbons, although similar in lipid solvent efficiency to the conventional solvents (3–6), inactivated little or no virus from either source (Fig. 1). Benzene did not inactivate CEC virus, but slight inactivation ($10^{1.4}$ pfu) occurred with mouse brain virus after 24 hr. However pentane and hexane failed to inactivate either virus even after 24 hr of treatment. Interestingly in another experiment, CEC virus was inactivated ($>10^1$ pfu) when shaken with a mixture of benzene and normal mouse brain (equal parts of each), while benzene or normal mouse brain alone did not affect CEC virus. Thus, the effect of benzene on mouse brain virus might have been indirect and related to an initial interaction of benzene with mouse brain tissue which then resulted in viral inactivation.

The alcohols, ethanol, pentanol, and butanol promptly and essentially completely inactivated VE virus (Fig. 1). Likewise in

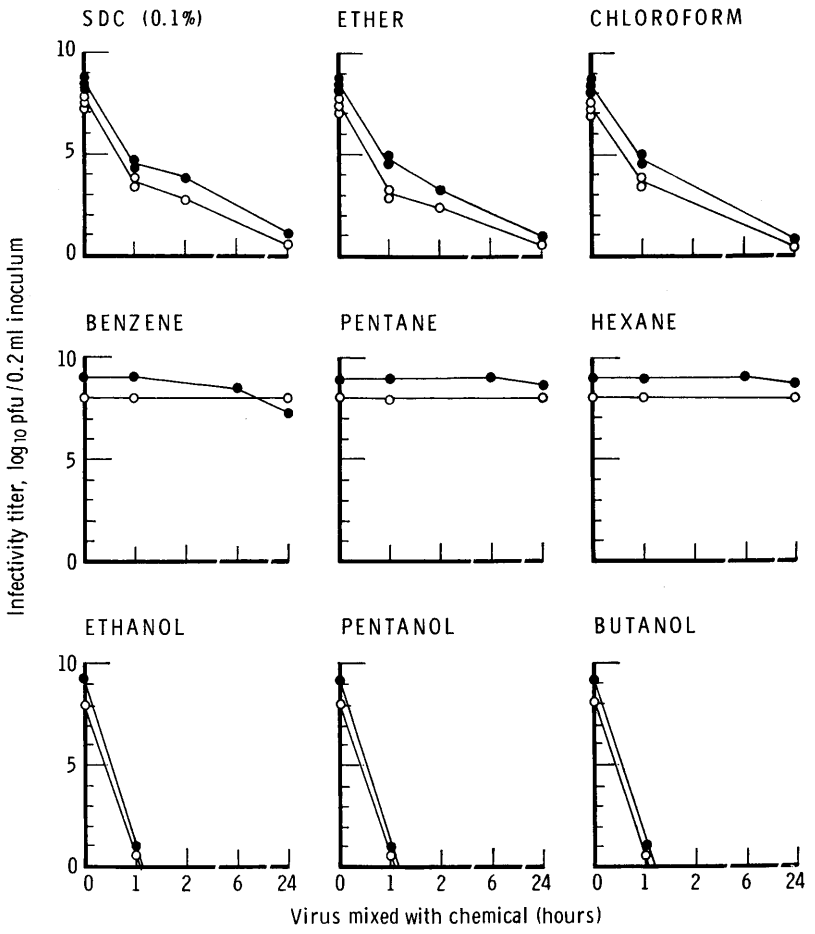

FIG. 1. Different effects of lipid solvents at 3° on infectivity of crude suspensions of VE virus.

other experiments, phenol, in contrast to benzene, inactivated virus ($>10^7$ pfu) within 1 hr. The effects of water molecules on these results was determined by parallel experiments with lyophilized virus. Results were similar to those with aqueous virus suspensions except that absolute ethanol did not inactivate lyophilized virus (Table I).

Because the failure of hydrocarbons to destroy infectivity of crude suspensions of VE virus could have been due to binding of hydrocarbon by extraneous materials, these experiments were repeated with centrifuged, RNase-treated, density-gradient purified virus from CEC. Again benzene did not inactivate virus and only minimal, slow inactivation occurred with pentane or hexane when compared with ether or chloroform or with salt solution controls (Fig. 2). Thus, even with purified virus, cyclic and straight-chain hydrocarbons had relatively little or no effect on viral infectivity, especially when compared with ether or chloroform.

Effects of lipid solvents on hemagglutinin (HA) and complement-fixing (CF) properties of VE virus. Lipid solvents affected HA and CF properties of VE virus differently from infectivity. Treatment for 24 hr with SDC (final concentration 0.1%) or with hydrocarbons did not reduce HA titers of mouse brain or CEC virus (Fig. 3). Diethyl ether and chloroform, on the other hand, decreased

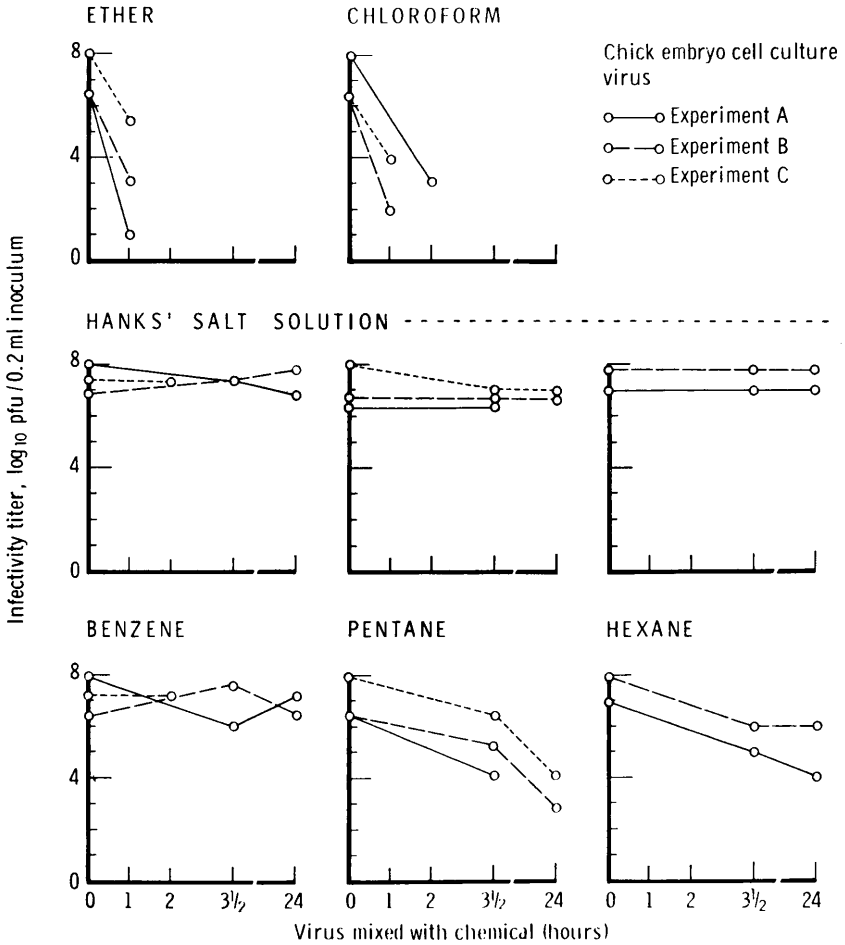

FIG. 2. Different effects of lipid solvents at 3° on infectivity of purified suspensions of VE virus.

HA titers of virus from both sources within 2–6 hr. Actually there were initial 2- to 4-fold increases in HA titers of mouse brain virus treated with these lipid solvents; these change, occurring within a minute of exposure to 0.1% SDC and within an hour after ether, chloroform, and hydrocarbons, presumably resulted from removal or inactivation of nonspecific lipid inhibitors (10). Alcohols destroyed HA from both mouse brain and CEC within 1 hr (Fig. 3).

SDC, diethyl ether, chloroform, and hydrocarbons did not affect CF antigen titers of VE virus suspensions after continuous treatment for up to 24 hr at 3° (Fig. 4). However, alcohols rapidly reduced CF antigen titers to undetectable levels.

Discussion. The different effects of SDC,

TABLE I. Effects of Lipid Solvents on Infectivity of Lyophilized VE Virus.

Lipid solvents	Log_{10} reduction of plaque-forming units in CEC	
	Lyophilized virus[a]	Reconstituted virus
Sodium deoxycholate (0.1%)	3.6	4.3
Diethyl ether	4.6	4.2
Chloroform	2.6	4.3
Pentane	0.0 0.3	0.1 0.2
Hexane	0.3	0.4
Benzene	0.0	0.1
Ethanol (95%)	≧7.3	≧7.3
Ethanol (100%)	0.0 0.0 0.0	≧7.3

[a] Virus was from CEC.

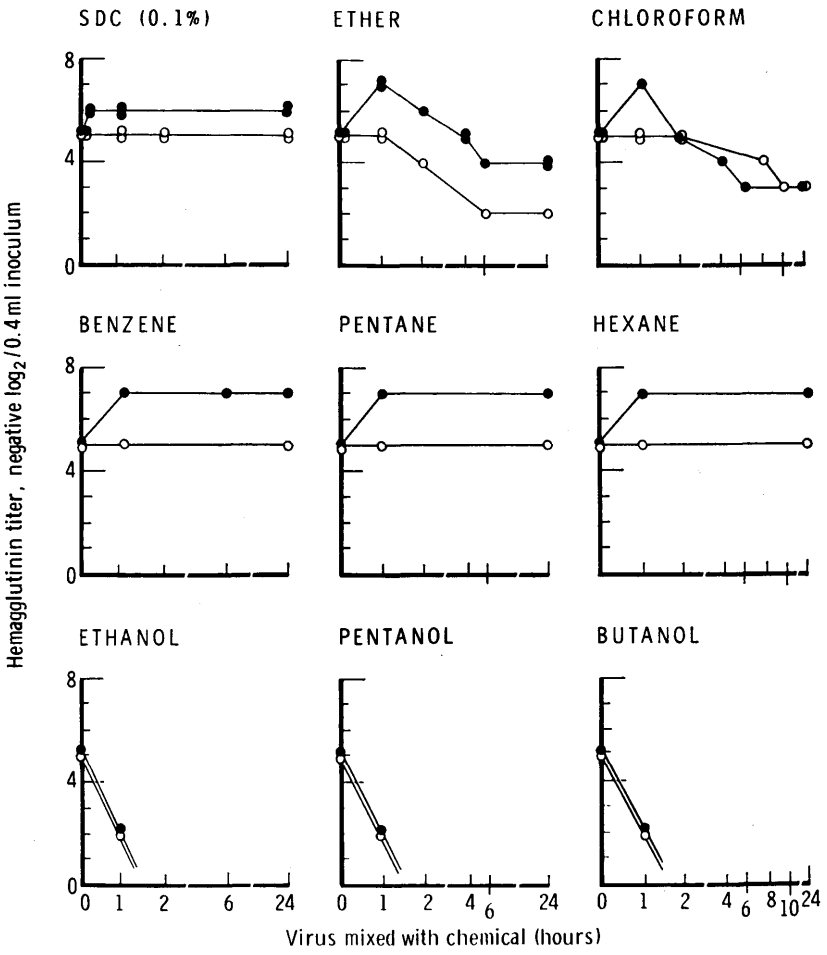

FIG. 3. Different effects of lipid solvents at 3° on hemagglutinating activity of crude suspensions of VE virus.

diethyl ether, and chloroform on infectivity, HA and CF properties of VE virus suggested involvement of different chemical entities of the virion in these processes. Perhaps infectivity and HA properties of VE virus have lipids in common since they were destroyed by diethyl ether and chloroform. However, HA and infectivity did not seem to involve identical viral entities, since infectivity was more rapidly destroyed by lipid solvents than HA. Similar conclusions were made from a study with caseinase C and the properties of infectivity and hemagglutination of Semliki Forest virus (11). Upon treatment with caseinase C in Tris-buffered saline, Semliki Forest virions retained infectivity but lost hemagglutinating activity and surface projections which were visible in untreated negatively stained virus. With caseinase C in phosphate buffer, both hemagglutinating activity and infectivity were lost, the envelope was degraded and sometimes destroyed, but the number of remaining virions was not reduced, and they still released IRNA upon treatment with high concentrations of SDC. The identity of surface projections with hemagglutinating activity was also supported by their presence on small (15–35 mμ) he-

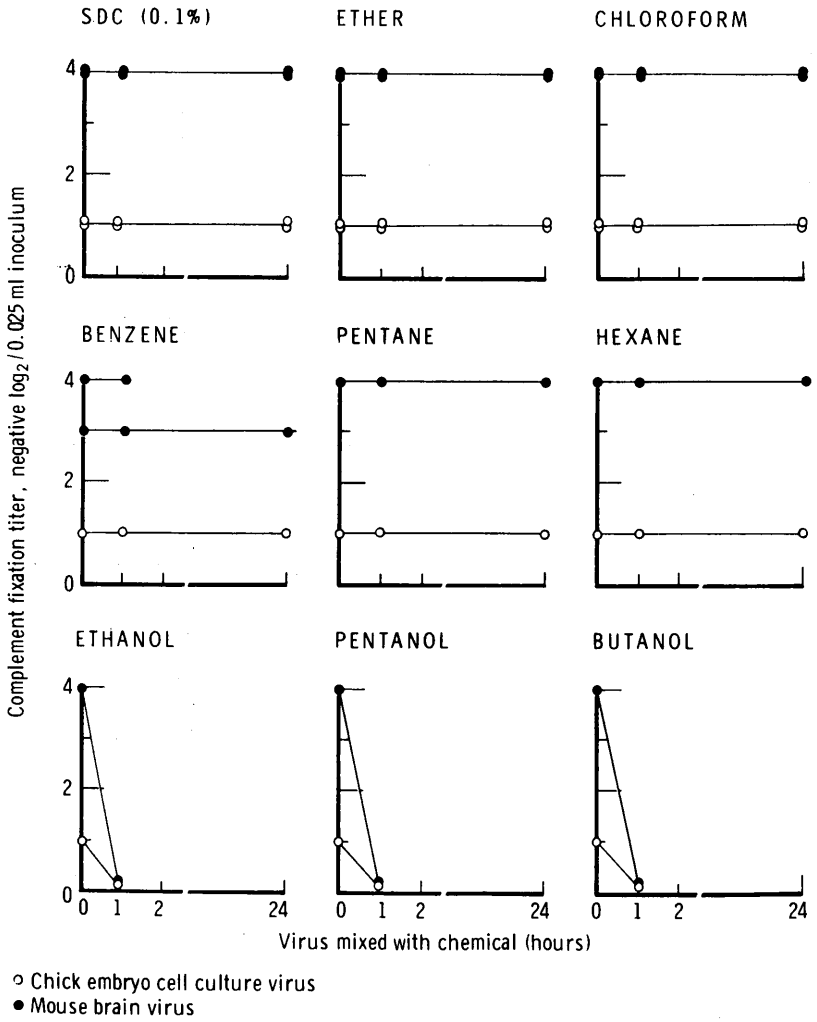

FIG. 4. Different effects of lipid solvents at 3° on complement-fixing activity of crude suspensions of VE virus.

magglutinating, noninfectious particles derived from Sindbis virus by Tween 80–ether treatment (12).

The chemical entities concerned with fixing complement in the presence of VE virus antibody may not involve lipids since this virus property was not affected by lipid solvents. However, it is possible that subviral particles either initially separate from virions or separated from them by lipid solvents, functioned as soluble CF antigens to fix complement with antibody, and thus maintain the CF capabilities of viral suspensions after treatment with lipid solvents.

It was surprising to find that the straight-chain hydrocarbons, pentane and hexane, did not inactivate VE virus so rapidly as SDC, diethyl ether, or chloroform. Even more unexpected was the lack of inactivation with a cyclic hydrocarbon, benzene. These results occurred despite use of purified virus suspensions to minimize binding of solvents by extraneous materials. Thus, the differences in rates of inactivation of VE virus by various lipid solvents remain unexplained.

The drastic action of alcohols on the three properties of VE virus contrasted with the differential actions of the other lipid solvents

and may have been due to effects on both lipids and proteins. Whatever the mechanism is, it seemed to require water molecules since absolute ethanol, in contrast to 95% ethanol, did not affect lyophilized VE virus. Additional evidence that hydrophilic hydroxyl groups are important in inactivation of VE virus was provided by the observation that pentanol and phenol, as opposed to pentane and benzene, destroyed VE viral properties.

Further studies are required fully to learn the role of lipids in the functional integrity of VE virions. If one assumes that all lipid solvents employed in these experiments removed lipids from virions, then lipids are not essential to VE virus since benzene did not inactivate virus. Perhaps SDC, ether, and chloroform destroy VE virus infectivity by reaction with other compounds in addition to lipids or by removing lipids in a critically different manner than hydrocarbons. Unfortunately, the inactivation of arboviruses by lipoidal enzymes (13, 14) does not clarify the situation since their specificities and mechanisms of action are subject to unknown considerations similar to lipid solvents.

Summary. Venezuelan encephalitis virus suspensions were affected differently by various lipid solvents. Infectivity titers of both crude virus suspensions from mouse brain or primary chicken embryonic cell cultures and density gradient-purified virus from cultures were reduced more and faster by sodium deoxycholate (SDC), diethyl ether, or chloroform than by pentane or hexane. Benzene failed to affect infectivity titers of even density gradient-purified virus. Hemagglutinin (HA) titers decreased after 2–6 hr of treatment of virus with diethyl ether or chloroform, but remained unchanged for 24 hr with SDC, pentane, hexane, or benzene. Complement-fixing antigen titers of crude virus suspensions were unchanged by these lipid solvents. Alcohols rapidly destroyed all three viral properties, although water was essential for alcohol inactivation since lyophilized virus was unaffected by absolute ethanol. Evidently hydrophilic hydroxyl groups are important in viral inactivation by these chemicals since pentanol and phenol were much more rapidly virucidal than pentane and benzene.

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