THE SUSCEPTIBILITY OF FATHEAD MINNOW CELLS TO COXSACKIEVIRUSES B-1 AND B-2

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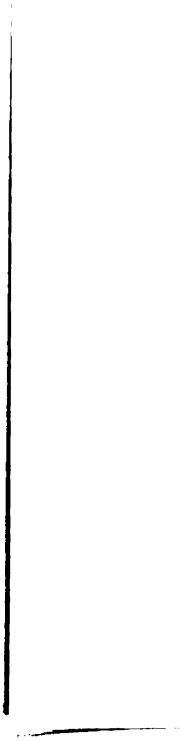

ABSTRACT

THE SUSCEPTIBILITY OF FATHEAD MINNOW CELLS
TO COXSACKIEVIRUSES B-1 AND B-2

By

Carolyn Jacobs Merritt

Two methods were used to study the susceptibility of fathead minnow (FHM) cells, *Pimephales promelas*, to Coxsackievirus types B-1 and B-2. One method used acridine orange-labeled viruses, and the other used FHM cells treated after viral inoculation with dimethyl sulfoxide.

Acridine orange-labeled viruses were prepared by growing stock viruses in susceptible cells containing acridine orange. Studies showed that the labeled viruses were inactivated by light. When shielded from light and grown in susceptible cells without acridine orange, the acridine orange-labeled viruses produced unlabeled, light resistant progeny at 36°C and 30°C. Inoculation of FHM cells with acridine orange-labeled Coxsackieviruses did not result in the isolation of light resistant viruses.

Virus inoculated FHM cells were treated with 4% concentrations of dimethyl sulfoxide in an attempt to by-pass the necessity of virus-host receptor site specificity. Results indicated that, at the concentration of dimethyl sulfoxide tolerated by FHM cells, there was no enhancement of FHM cell susceptibility to Coxsackievirus types B-1 and B-2.

The poikilothermic cells of fathead minnows, using the techniques of these studies, were not susceptible to infection by Coxsackievirus types B-1 and B-2.

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Carolyn Jacobs Merritt

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INTRODUCTION

The enteroviruses are known to be inhabitants of the gastrointestinal tract of man and some lower warm blooded animals. The mode of transmission of these viruses is by the oral route. With an ever increasing population and a lag in sewage treatment modification, our fresh and marine waters are becoming grossly contaminated with potentially dangerous chemical, bacterial and viral agents.

The impact of chemical pollution on aquatic life has been studied extensively; however, the effects of mammalian viruses on the aquatic environment lack adequate study. We have only limited knowledge of the susceptibility of fish to mammalian viruses and even less knowledge concerning the ability of fish to harbor viruses in their digestive systems and their tissues. Solis and Mora (66) have studied the viral susceptibility of fathead minnow (FHM) cells to 19 mammalian viruses. In their study these cells were susceptible to 13 of the 19 viruses. Only two enteroviruses were used, poliovirus types 1 and 3, and neither of these viruses replicated in FHM cells. Gravell and Malsberger (22) determined that ECHO-11, an enterovirus, did replicate in FHM cells yielding high titers with little apparent cytopathic effect (CPE).

Mammalian Group B Coxsackieviruses are known to be the most pathogenic Coxsackieviruses to man and only infect species of homeothermic cells. Whether FHM cells are susceptible to infection by members of this group of viruses has not been determined. In this

study FHM cells were inoculated with Coxsackievirus types B-1 and B-2 and examined microscopically for the development of discernible CPE. Under normal conditions no visible CPE was evident. The absence of CPE was not taken as proof that these poikilothermic cells were insusceptible to the two viruses. Two methods were used to determine further the susceptibility of FHM cells to the Coxsackieviruses. The first method used acridine orange-labeled, photosensitive viral particles. Hiatt and Moore (29) and Schaffer (64) showed that polioviruses grown in susceptible cells and in a proflavin medium produced proflavine-labeled progeny that were sensitive to light. The objective of this portion of the study was to demonstrate viral replication, if any, in FHM cells containing no vital dye as indicated by the appearance of non-labeled, photoresistant viral progeny. No photoresistant viruses were recovered using this method. To circumvent the possibility of host-virus receptor site differences, a mediator of membrane penetration, dimethyl sulfoxide (DMSO), was used. A concentration of 4% DMSO did not result in any apparent enhancement of viral penetration or infection.

Although attempts to infect FHM cells with Coxsackievirus types B-1 and B-2 were unsuccessful, the method of employing vital dye labeled viruses was successful in differentiating new progeny from input virus in cell systems whose viral susceptibilities have not been determined.

REVIEW OF THE LITERATURE

Fish Tissue Culture

Historical. In 1914 Osowski (49) made the first report of fish tissue culture. Using Ringer's solution and frog lymph, Osowski was able to maintain fry and embryonal trout explants for 24 hours. In 1921 and 1924 Dederer and Goodrich (16,21), respectively, grew Fundulus in Locke's physiological saline solution with glucose and fish bouillon.

The breakthrough in fish tissue culture came in 1949 with the experimental work of Schlumberger. Using mammalian-type medium, he (Schlumberger) was able to culture neoplastic cells from the adult goldfish, *Carassius auralis* (65). The etiology of the tumor was never established; however, this was probably the first application of fish tissue culture in virology.

The first detailed studies of fish tissue culture and its application in fish virology were reported in 1956 by Grutzner (23,24). In 1958 Grutzner introduced the first trypsinization technique for fish tissue that would yield cells in monolayers (25). This method utilized low temperature (20°C.) during trypsin digestion of fish tissues. With the successful subculturing of these cells, fish tissue culture techniques were begun.

In 1962 Wolf and Quimby (74) prepared monolayer cell cultures from the enzymatically digested gonadal tissue of rainbow trout, *Salmo gairdneri*. These cell cultures were the first permanent poikilothermic cells ever established. Three years later Gravell and Malsberger (22)

using tissue posterior to the anus of normal adult male and female fathead minnows, established growth of the epithelial-like cells of the fathead minnow (FHM), *Pimephales promelas*.

Other researchers have been successful in establishing poikilothermic cell lines. Fryer *et al.* (20) in 1965 reported the establishment of five different cell lines from embryonic Pacific salmon and rainbow trout hepatoma. In the area of marine teleosts it is important to note the work of Clem (10), who in 1961 initiated the first cell line of *Haemulon flavolineatum*, a species of marine origin.

Culture of Poikilothermic Cells

The techniques for *in vitro* cultivation of poikilothermic cells are similar to those used for *in vitro* cultivation of homeothermic cells. The major differences are encountered in temperature requirements and tolerances and osmolarity of saline solutions and media.

Balanced Salt Solutions. Based upon the work of Black (6) and Lockwood (39) the freezing points of freshwater teleosts' bloods have been determined. Black found the freezing temperature to be -0.57°C. and Lockwood determined a narrower but comparable mean of -0.54°C. The mean freezing points of two mammalian and one teleost balanced salt solutions (Earle's, Hanks' and Cortland) are -0.58, -0.59 and -0.58°C., respectively (75).

The freezing point determinations and constituents of these three salt solutions are very similar. Based upon these similarities, it has been found that balanced salt solutions used in homeothermic tissue culture are quite suitable for freshwater teleost tissue culture (6).

Media. The nutritional requirements for fish cells grown *in vitro* have not been determined. Nutritional supplementation for *in vitro* cultivation of poikilothermic cells has been based upon the nutritional requirements of homeothermic cells grown *in vitro*. Poikilothermic cells will grow in most media if the media contain adequate inorganic salts, vitamins, amino acids and generous supplements of serum (6).

Serum. Calf serum, either fetal or full-term, has been used and reported almost continuously as the serum of choice for fish tissue culture (75). Fetal bovine serum has been noted for its lack of toxicity, growth stimulating properties and lack of virus neutralizing activity (6). When used to supplement media in which fish cells are grown, the usual level of serum is 10-15%, but fish cells can be maintained on as little as 2% (6).

pH. The pH of the medium is not critical for good growth of fish cells. Most cells seem to fare well in the range of 7.3-7.4. Wolf and Quimby (74) found that rainbow trout gonadal (RTG-2) cells grew best when the initial pH was about 7.3. A neutral pH was tolerated and in old cultures cells remained viable at a pH of 6.8. Gravell and Malsberger (22) recommended a pH range of 7.2-7.4 for effective growth of fathead minnow (FHM) cells.

Temperature. Poikilothermic cultures grow at rates which are temperature dependent. Gravell and Malsberger (22) determined the growth rates of

FHM cells and found that FHM cells will multiply over a wide temperature range. The maximum rate of cell growth occurred at about 34°C. Little multiplication occurred at 4°C. At incubation temperatures of 38°C. and above cell death ensued. Over the temperature range of 14° to 34°C. an approximate twofold increase in growth occurred for each 10° increase in temperature.

Plumb and Wolf (52), using RTG-2 cells, determined that the growth rates of these poikilothermic cells were also temperature dependent. The RTG-2 cells grew well over a range of 5-25°C. but did not survive at 30°C. Optimum growth was observed at 20°C.

Susceptibility of Poikilothermic Cells to Mammalian Viruses

Research in the area of poikilothermic susceptibility to mammalian viruses has been limited. A majority of the studies have used FHM and RTG-2 cells as the host systems. Some studies have included cell lines derived from Salmonid fish and intact Northern quahogs (*Mercenaria mercenaria*).

Officer (47), using RTG-2 cells, demonstrated that these poikilothermic cells, when grown at 22°C., could support the proliferation of two arborviruses, Venezuelan equine encephalitis (VEE) virus and Eastern equine encephalitis (EEE) virus. The use of RTG-2 cells aided in the differentiation of the two equine encephalitic viruses. Microscopic examinations of the infected cells indicated that the EEE virus was more cytopathogenic for RTG-2 cells than the VEE virus. In EEE virus infected cells there was an intense cytopathic effect (CPE) with no persistent infection. In contrast, VEE virus infected cells revealed only slight CPE with a persistent infection lasting the duration of the study.

The homeothermic virus, ECHO-11, has also been propagated in high titers in FHM with no extensive cell destruction or other CPE (22).

It has been reported in the literature that FHM cells are sensitive to the Mahoney strain of type 1 poliovirus and to type 3 adenovirus (22).

Solis and Mora (66) determined the viral susceptibility range of FHM cells to 19 mammalian viruses. These viruses were representative members of the entero-, myxo-, arbo-, herpes-, pox-, and rhabdovirus groups and one member of the psittacosis-lymphogranuloma trachoma virus group. Thirteen of these viruses replicated in the FHM cells. Poliovirus 1 and 3, infectious bursal agent and infectious bronchitis viruses failed to replicate in FHM cell cultures using their infecting techniques.

Oie and Loh (48) inoculated FHM cells with reovirus and determined that there was no propagation of the virus; neither was there formation of inclusion bodies. FHM cells were found to be susceptible to frog virus 3, FV_3. However, inoculation of this virus to FHM cells previously challenged with reovirus resulted in virtually no visible effect and a slight increase in virus titer. An antiviral substance having physical and chemical properties similar to interferon was detected.

Nims *et al.* (46) used two cell lines derived from Salmonid fish to study the replication of two fish viruses, sockeye salmon virus and Sacramento River chinook virus, and two viruses of warmblooded vertebrates, western equine encephalitis (WEE) and Newcastle disease virus (NDV). Both Salmonid cell lines supported the growth of WEE virus, but neither supported the growth of NDV.

Shellfish have also been used to detect their ability to support the growth of or serve as reservoirs for human enteroviruses. Chang *et al.* (9), using Northern quahogs and proflavine bound enteroviruses (polio-, Coxsackie-, and ECHO viruses), found that at varying time

intervals after inoculation there was no increase in virus titers, but that each virus survived for long periods of time in the intact quahogs.

Picornavirus Group. The name Picornavirus designates a group of agents having a particle diameter of 17 to 30 mμ, cubic symmetry, single stranded ribonucleic acid (RNA) core, absence of a lipid envelope and cationic stability to thermal inactivation (34). This group of viruses includes two subgroups, those of human origin and those of lower animal origin. Human picornaviruses include the enteroviruses and the rhinoviruses. Those picornaviruses of lower animals are exemplified by the virus of foot and mouth disease and Theiler's virus of mice.

Enteroviruses. This subgroup was named in 1957 by the Committee of the Enteroviruses (11) to group all viral agents which were normal inhabitants of the human gastrointestinal tract. The enterovirus subgroup has been divided into the polioviruses (antigenic types 1-3), Group A Coxsackieviruses (antigenic types 1-23), Group B Coxsackieviruses (antigenic types 1-6) and the enteric cytopathogenic human orphan viruses, ECHO (antigenic types 1-33) (11). The incidence of infections caused by these viruses is seasonal, being highest in the summer months, the period when enteroviruses are most readily isolated from raw sewage and polluted waters (34). These viral agents are transmitted generally by the oral-fecal route; hence, presumably all viruses of this group have the potential for transmission in polluted waters (4).

Infections caused by the enteroviruses are many and varied. The most well documented clinical diseases are those associated with

infection and destruction of the central nervous system, i.e., paralytic poliomyelitis, acute aseptic meningitis or meningoencephalitis. Herpangina, myocarditis, pleurodynia and, in some isolated cases, encephalitis are all clinical manifestations associated with or caused by one or more of the enteroviruses.

Coxsackieviruses

Historical. The original strains of Coxsackieviruses were isolated in 1948 by Dalldorf and Sickles from the fecal specimens taken from two boys suffering from what appeared to be paralytic poliomyelitis (13). The isolations of these two Group A Coxsackieviruses were the first indication of the existence of numerous unsuspected enteric agents. Originally, the Coxsackieviruses were studied to determine their relationship and similarities to polioviruses. With the isolation and confirmation of the Group B Coxsackieviruses as causative agents of Bornholm disease (epidemic pleurodynia) and epidemic myocarditis (43,45,73), the Coxsackieviruses were no longer studied as synergistic agents in poliomyelitis infections, but realized as a distinct group of enteric viruses.

Physical and Chemical Properties. Coxsackieviruses have the general properties of size, shape, stability and molecular weight shared by other enteroviruses. Members of both groups (A and B) are spherical particles and have estimated diameters of about 25 to 30 mμ (44) and molecular weights in the range of 7×10^6 daltons (41). The virus particle contains a single stranded RNA core which differs markedly in base composition from that of poliovirus in the proportion of quanine, which is greater in Coxsackievirus RNA, and adenine, which is greater in

poliovirus RNA (41). These viruses, like polioviruses, are relatively stable to physical and chemical agents. They can withstand exposure to a wide range of pH, i.e., pH 2.3 to 9.4 for 1 day and 4.0 to 8.0 for 1 week (59). In the presence of high concentrations of divalent cations, Mg^{++}, the Coxsackieviruses are stable at 50°C. for 1 to 3 hours (72). Coxsackieviruses are resistant to inactivation by dimethyl ether, phenol, deoxycholate and antibiotics but susceptible to formalin and chlorine (51).

Coxsackieviruses are distinguished from other enteroviruses by two unique characteristics: their much greater pathogenicity for suckling rather than adult mice, and their inducement of lesions of the striated muscles (51). The Group A Coxsackieviruses produce generalized myositis accompanied by inflammation and necrosis of fibers of the voluntary muscles in laboratory animals (57). Group B Coxsackieviruses produce inflammatory changes in the brain, dorsal fat pads, pancreas, myocardium and focal changes in skeletal muscle tissue in laboratory aniamls (57). The type and location of lesions in susceptible laboratory animals are the criteria used in identifying and classifying Coxsackieviruses into their specific groups.

Replication. The infectious cycle of Coxsackievirus is similar to that of poliovirus (14). The virus is adsorbed on lipoprotein receptor sites on the plasma membrane of the susceptible cells (31). The attachment of the virus depends upon the concentration of electrolytes and is relatively independent of temperature (32). It has been suggested that not only do the lipoproteins adsorb the virus, but they also mediate, perhaps by some enzymatic action, in the alteration of the virus capsid to aid in the release of the viral RNA (32,33). After this alteration

has occurred, the virus is pinocytosed and its viral RNA is released by proteolysis within a pinocytosis vacuole (33). The replication of infectious virus particles then follows the general pattern of single-stranded RNA viruses with replication taking place in the cytoplasm of the cell. The fully assembled Coxsackieviruses, not like the polioviruses which are released almost immediately by a bursting of the infected cell, tend to remain intracellularly and are not released immediately into the culture medium (14).

Growth of Coxsackieviruses in susceptible cultured cells, like other enteroviruses, is characterized by rounding-up, shrinkage and refractileness of the cells, marked nuclear pyknosis and eventual sloughing-off of the cells from the growing surface (34).

Pathogenicity. The manifestations of infection in man by individual Coxsackieviruses are not uniform; however, certain of these viruses have been recognized as the etiological agents of many infections.

Most Coxsackievirus related diseases, like poliomyelitis, have a tendency to occur periodically and seasonally. Infections caused by these viruses are universal and appear during the summer and fall months (34). Group A Coxsackieviruses, frequently found in the feces of normal persons, have definitely been identified as the causative agents of many "minor" illness. Herpangina, a warm weather disease of children, has been found to be caused by Group A Coxsackieviruses (35). The disease is characterized by shallow ulcers and vesicles of the orapharynx, fever and sore throat. Coxsackievirus A10 has been implicated in epidemic acute lymphonodular pharyngitis among children (67). This disease is similar to herpangina in symptoms of sore throat,

fever and lesions, but differs from herpangina in the non-vesicularity of the lesions (34). Human-hand foot and mouth disease, maculopapular rash,has been attributed to an A16 Coxsackievirus (58).

Group B Coxsackieviruses are considerably more pathogenic for man than are most Group A Coxsackieviruses. Infections caused by these viruses are varied in symptomatology. Such infections include epidemic pleurodynia (Bornholm disease), myocarditis of newborn infants, meningo-encephalitis and hepatitis of infants, acute, benign pericarditis of adults, vesicular pharyngitis and aseptic meningitis (57).

Immunity to the Coxsackieviruses has been tested using pooled human sera. Most of the Group A Coxsackieviruses were neutralized by these sera. Group B Coxsackieviruses were neutralized to a lesser degree. It appears that antibodies against the Group B viruses are less stable than those against the Group A viruses (51).

Acridine Dyes

Historical. In 1870 Graebe and Caro reported the isolation of a new basic material, acridine, from the anthracene fraction of coal tar. Physical and chemical analyses of this substance revealed that it was a heterocylic compound having the skeletal structure of $C_{13}H_9N$, a moderately weak base, sparingly soluble in water, faintly yellow in color, and when rubbed the powder emitted light (1). Future analyses determined that acridine absorbed light in the ultraviolet spectrum (1).

Aminoacridines. The acridines of biological importance belong to the aminoacridine group with no C-substituents (no substitutions at the carbons except for amino groups). Members of this group include aminacrine, proflavin and acridine orange (1).

The aminoacridines have the unique ability of staining nuclei *in vivo* (15). Because of this ability and the ease with which their presence can be demonstrated by fluorescence microscopy, the aminoacridines have emerged as a useful tool in the investigation of cellular metabolism *in vivo* and *in vitro*.

The first experiments demonstrating the photosensitizing effect of a dye upon microorganisms were reported by Raab (54) in 1900. He found that paramecia grown in the presence of acridine and shielded from light flourished, while exposure of cultures to sunlight caused death. The phenomenon was studied extensively by Tappeiner and Jodlbauer (70) and was named "photodynamic action." In 1941 Blum (7) defined photodynamic action as the photosensitization of a biological system by a "substance which serves as a light absorber for photochemical reactions in which molecular oxygen takes part."

Tappeiner and Jodlbauer (70) also noted that photodynamic action could occur with other dyes and in other biologic systems. A wide variety of biological substances can be sensitized for photodynamic action. Fowlks (18), in a review, described the destructive effects of photodynamic action upon amino acids, proteins, nucleic acids, plant and animal viruses, bacteria, red blood cells and multicellular organisms.

Photosensitization of Animal and Bacterial Viruses. Pedrau and Todd (50) found that the viruses of vaccinia, herpes, fowl plague, louping-ill, Borna disease, Fujinami's bird tumor and canine distemper were readily inactivated by visible light in the presence of methylene blue, a basic dye. In 1937 Rosenblum *et al.* (60) found poliomyelitis virus to be susceptible to the photodynamic action of methylene blue.

As a result of these early studies, a number of experimenters became interested in the effects of vital dye incorporation into developing and mature plant, animal and bacterial viruses.

Yamamoto (76,77) studied the photodynamic action of acridine orange, neutral red and toluidine blue upon coliphages. He found that photodynamic activity against these phages was confined mainly to dyes of the acridine and thiazine series and concluded that these compounds combined at some critical site on the viral nucleic acid. Further research in this area (37,38) determined that in high concentrations, acridines are bound to the phosphate groups of the DNA backbone on the outside of the double helix, and in lower concentrations the acridine molecules became inserted, or intercalated, between adjacent base-pairs in the DNA.

The experimentation of LoGrippo and Basinski (40) revealed that acridine orange had no effect on mature poliovirus and Coxsackievirus. Hiatt *et al.* (28), in two separate studies, determined that mature enteroviruses cannot be inactivated by toluidine blue and light and that the increased susceptibility of phages to the action of a vital dye and light was due to the permeability of the phage's protein coat (27).

Schaffer (63) concluded that mature enteroviruses were resistant to photoinactivation because their protein coats were impermeable to the vital dyes, thus preventing the interaction of the dyes with sensitive sites on the nucleic acid. Schaffer (64) and Hiatt *et al.* (30) found that when grown in the presence of proflavin, poliovirus firmly bound the dye and became photosensitive. In 1961 Crowther and Melnick (12) showed that both neutral red and

acridine orange sensitized poliovirus particles when the virus was grown in cells containing the vital dye. Using a concentration of 5.0 µg/ml of acridine orange, Mayor and Diwan (42) were able to demonstrate by direct examination in the fluorescence microscope a definite difference between preparations of normal poliovirus particles and those grown in the presence of the dye and unfixed. The former particles did not fluoresce and the latter fluoresced a brilliant green. The deviation from the normal fluorescence of fixed RNA viruses was attributed to the limited amount of dye incorporated into the developing virus.

Photosensitization of Living Cells in Culture. Hill *et al.* (30), using L-strain fibroblasts, found that acridine orange in concentrations of 150 µg/100 ml had no measurable effect on cell multiplication, protein synthesis or DNA replication if cultures were shielded from light. However, exposure of cultures to light caused prompt cessation of growth, net protein synthesis and DNA replication. They could not demonstrate any metabolic utilization of acridine orange, but did observe some metabolic control over the dye.

Mode of Action of Acridine Orange. When acridine orange is bound to polynucleotides, at least three types of complexes, Complex I, II and III, are formed, depending upon the concentration ratio of polynucleotide to dye. Complex I is formed at low ratios of polynucleotide to dye and Complex II at high ratios (71). Complex III has been described by Sastry and Gordon (61) and is composed of ribonucleic acid-dye and paramagnetic metal ions. Complex I is inactive; there is no sensitization of the nucleic acid and no fluorescence (61). In contrast, the nucleic acid-dye association in Complex II is sensitive

to light and fluoresces (62). The paramagnetic metal ions of Complex III protect the nucleic acid against photodynamic inactivation (61).

Hiatt (27) has proposed a reaction scheme for the photodynamic inactivation of viruses. First the dye is combined at critical sites in the virus particle to yield a dye-virus complex that is excited by absorbing one photon of light energy during irradiation. The excited complex then is combined with oxygen resulting in the loss of viral infectivity.

Attempts were made by Sastry and Gordon (62) to elucidate the mechanism of photodynamic inactivation by acridine orange. Their results suggested that inactivation occurred without a cleavage of the ribose-phosphate backbone of the nucleic acid. The possibility of base destruction was considered. All four nucleosides were irradiated in the presence of acridine orange. Only guanosine was destroyed. Further tests showed that there was a total breakdown of the guanosine molecule, and that this attack could occur on at least five points of the guanosine molecule (62).

Dimethyl Sulfoxide

Chemical and Physical Properties. Dimethyl sulfoxide (DMSO), a by-product of paper manufacturing, is an extraordinary chemical. The compound was first synthesized by Alexander Saylzeff in 1866. DMSO is a highly stable, polar, aprotic solvent having a pyrimidal structure composed of a highly polar sulfur-oxygen bond at the apex and two methyl groups at the corners. The broad solvent characteristics of DMSO can be attributed to its ability to form stable solvates by dipole-dipole interactions or solvent-solute associations (55). DMSO is highly hygroscopic and miscible with water, lipoids and organic solvents (36).

Because of its highly associated nature, DMSO is thermolabile between 48°C. and 60°C. (55).

DMSO has been shown to penetrate cell membranes with apparently little or no permanent damage (36). Because of its extraordinary penetrant and solvent properties, DMSO has been used as a mediator in topically applied drugs (8), an anti-inflammatory adjunct (68), a cell and tissue preservative during freezing (17), a cell protector against ionizing radiation (3), and a bacteriostatic agent (53). The penetrating capacity of DMSO has also been shown to enhance the uptake of viral nucleic acids. Amstey and Parkman (2) determined that within a few minutes after treatment with varying concentrations of DMSO (5-80%) a significant amount of poliovirus RNA was absorbed into African green monkey kidney cells. Recently, Stewart *et al.* (69) found that treatment of human tumor cells, rhabdomyosarcoma cell line, first with 5-iododeoxyuridine (IdU) and then with DMSO, increased the production of C-type virus from these cells approximately tenfold.

Mode of Action. Dimethyl sulfoxide's mode of action is still unclear. However, it is known that DMSO, unlike other hypertonic agents, increases the rate of movement of molecules across cell membranes in the inward direction only (19). The rate of movement of the transported molecule across the cell membrane increases with size (19).

MATERIALS AND METHODS

Cell Cultures

Continuous cell cultures of human carcinoma of the larynx epithelium cells (HEp-2) were obtained from the School of Public Health, University of Michigan, Ann Arbor, Michigan, and Grand Island Biological Company (GIBCO), Grand Island, New York. These cultures were routinely grown and maintained at 36°C. in GIBCO medium 199 (M-199) containing 10 percent and 2 percent heat inactivated calf serum, respectively. All medium contained 100 units of penicillin and 100 µg of streptomycin per milliliter. Monolayer cultures were enzymatically digested with 5 ml of .25 percent trypsin for 45 seconds, rinsed in Hanks' balanced salt solution (HBSS), fed 18 ml of M-199, shaken vigorously to disperse the cells and supplemented with 2 ml of inactivated calf serum. Ten milliliter aliquots were seeded to 8 oz. milk dilution bottles for stock cultures and 1 ml aliquots were seeded to tissue culture tubes for virus titrations. Cells were maintained in M-199 containing 2 percent inactivated serum (2% M-199).

Fathead minnow (FHM) epithelial cell cultures, *Pimephales promelas*, were obtained from the Grayling Fish Hatchery, Grayling, Michigan, and GIBCO. The cells were grown in 8 oz. milk dilution bottles in GIBCO, Hanks' base minimal essential medium (MEM) supplemented with heat inactivated fetal bovine serum in final concentrations of 15 percent for growth and 2 percent for maintenance, 100 units of penicillin, 100 µg of streptomycin and 2 mM of L-glutamine per milliliter of

medium. Subcultures were prepared by gently agitating medium across the surface of the monolayers to dislodge the cells. Volumes of medium and serum and seeding procedures were the same as those used for HEp-2 cells.

Stock Virus Preparation

Coxsackieviruses B-1 and B-2 were obtained from the Michigan Department of Public Health, Lansing, Michigan. Six tubes of HEp-2 cells, washed and not containing medium, were inoculated with .1 ml of the original virus suspensions. Three tubes were inoculated with Coxsackievirus B-1 and three tubes were inoculated with Coxsackievirus B-2. Controls, not infected with the two viruses, were run in conjunction with the infected cells. The infected cells and controls were incubated for 1 hr. at 36°C. After incubation, .9 ml of 2% M-199 was added to each cell culture, and the cells were incubated at 36°C. When upon microscopic examination 50-75% of the infected cells showed cytopathic effect (CPE), the infected cells were shell frozen and thawed 3 times *in situ*, clarified and the appropriate viral suspensions were pooled. Monolayer cultures of HEp-2 cells grown in 8 oz. milk dilution bottles were inoculated with 1.0 ml of each pooled virus suspension. The second passage of the Coxsackieviruses was harvested as stated previously and stored at freezer temperatures (-22°C.) until titered.

Titration of Stock Viruses

To 4.5 ml of 1X Hanks' BSS .5 ml of each stock virus, Coxsackievirus B-1 and B-2, was added and serial tenfold dilutions were made. Titrations of each virus were done in duplicate using 3 tubes of HEp-2 cells per dilution. All cells were washed 3 times with 1X Hanks' BSS, inoculated with .1 ml of each virus dilution and incubated for 1 hr. at

36°C. After incubation, the cells were fed .9 ml of 2% M-199 and incubated at 36°C. and examined microscopically for CPE. The control cells were inoculated with .1 ml 1X Hanks' BSS and treated in the same manner as the infected cells. Virus titers were calculated by the method of Reed and Muench (56).

Shell-Freezing and Clarification of Virus Infected and Virus Inoculated Cells and Fluids

All infected and inoculated cells (HEp-2 and FHM) and fluids were harvested after freezing in a solution of 95% ethanol and solid CO_2 and thawing in a 36°C. waterbath for 3 times *in situ*. Fluids collected in this manner will be designated lysates or lysate fluids. All lysate fluids were clarified by centrifugation in a refrigerator centrifuge at 2000 rpm for 15 min. The lysate fluids were stored at freezer temperatures until used in future tests.

Labeling of Viruses with Acridine Orange

A stock solution of acridine orange (AO) containing .01 g/ml was autoclaved at 15 lbs. pressure 121°F. for 15 min. A 1:10 dilution of the stock was prepared and .5 ml of this dilution was added to 99.5 ml of 2% M-199. Thus, the final concentration of acridine orange was 5.0 µg/ml of medium.

Two sets of tests were performed; one for each of the test viruses. Both sets included a series of four controls and the AO-labeled virus stock. The controls were: 1) AO-labeled virus, 2) unlabeled virus, 3) uninfected cells maintained in AO-medium and 4) uninfected cells maintained in medium without AO.

A group of 8 oz. bottles containing monolayer HEp-2 cell cultures were individually infected with .5 ml of each test virus,

Coxsackievirus B-1 ($TCD_{50}/ml = 10^{-7.25}$) and Coxsackievirus B-2 ($TCD_{50}/ml = 10^{-6.0}$). After 1 hr. adsorption at 36°C., 10 ml of 2% M-199 with AO were added to two bottles of each set. These bottles were then wrapped in aluminum foil to exclude light and incubated at 36°C. To two bottles of each set 10 ml of maintenance medium without AO were added. These virus controls were incubated at 36°C.

Virus infected cells maintained in medium without AO were used to microscopically monitor CPE. When these controls showed 50-75% cellular destruction, one bottle of infected cells maintained in medium with AO was simultaneously checked and compared. Those labeled viruses exposed to light during examination were discarded. Additional tests were performed to determine the cytotoxic effect of the AO-concentration used in these experiments on HEp-2 cells. Uninfected cells were maintained on medium with and without AO. The AO treated cells were shielded against light with aluminum foil. The treated and untreated cells were examined at the time of virus harvesting.

Titration of AO-Labeled and Unlabeled Viruses Replicated at 36°C.

Acridine orange-labeled and unlabeled viruses were harvested when advanced CPE was evident by freezing and thawing *in situ* and clarification. Virus titers were determined for both the AO-labeled and unlabeled viruses. All labeled stock viruses were handled in darkness or subdued light during replication, harvesting and subsequent testing.

Exposure of Viruses to Incandescent Light

Tubes containing 2.5 ml of fluid with AO-labeled viruses were exposed to 100 W of incandescent light at a distance of 2.5 cm for 2 hrs. All tubes were immersed in ice water throughout the exposure period.

Multiplication of AO-Labeled Viruses in HEp-2 Cells at 30°C. and 36°C.

A number of tubes with HEp-2 cells were infected with .1 ml of the AO-labeled Coxsackieviruses, shielded and incubated at 30°C. for 1, 3, and 5 days and at 36°C. for 1, 12, and 24 hrs. Five tubes were collected at each time interval, frozen and thawed, and clarified prior to pooling the fluids. Half of the pooled fluids, approximately 2.5 ml, were exposed to incandescent light and half were left unexposed. Virus titers were determined for both the exposed and unexposed fluids.

Inoculation of FHM Cells with AO-Labeled Viruses

Two sets of FHM cells were inoculated with .1 ml of the AO-labeled virus stock shielded and incubated at 30°C. for 4 days. Five tubes from each set were frozen and thawed 3X, pooled and clarified. Half of the pooled fluids were exposed to light and half were unexposed. Titrations of the exposed and unexposed fluids were made on HEp-2 cells.

Cytotoxic Effect of DMSO on FHM Cells

Growth medium (10% MEM) was decanted from 1 week old cultures of FHM cells. The cells were washed 3 times with 1X Hanks' BSS, fed maintenance medium (2% MEM) and incubated at 30°C. for 24 hrs. After 24 hrs., each tubed culture was checked microscopically for any changes in cellular morphology and confluency of monolayers. Only tubed cultures having a confluent monolayer were used for the test. Prior to DMSO treatment, maintenance medium was decanted and the cells were washed in the routine manner.

Varying concentrations of DMSO (50, 40, 30, 20, 15, 10, 5, 4, 3, 2, and 1%) were made in 2% MEM.

All tests were performed in duplicate. One milliliter of each DMSO concentration was added to each of two tubes of FHM cells and left at room temperature for the appropriate time interval (1, 15, 30 and 60 mins.). After the addition of the varying concentrations of DMSO, the tubed cultures were rotated in their racks to a position that would eliminate prolonged and untimed exposure to DMSO. When the time of exposure could be monitored, the cultures were rotated to a position that would allow complete contact of monolayers with the DMSO solutions.

At the end of each timed interval the DMSO solutions were decanted, and the FHM cells were washed and fed 2% MEM.

Controls consisted of untreated FHM cells which were washed and fed in the same manner as the treated cells.

Prior to incubation at 30°C. for 1 week, all FHM cultures, DMSO treated and untreated, were examined microscopically for evidence of immediate cytotoxic effects of DMSO concentrations. Those cultures showing signs of complete monolayer destruction, in comparison with the controls, were recorded and discarded. Cultures showing monolayer fixation, partial monolayer destruction, granularity or no destruction were incubated at 30°C. and monitored daily microscopically for 1 week.

Toxicity of DMSO to Coxsackieviruses B-1 and B-2

Serial tenfold dilutions of the test viruses were made using .5 ml of the stock virus suspensions diluted with 4.5 ml of 4% DMSO-Hanks' BSS mixture. The viruses were treated at room temperature for 30, 60, and 90 mins. At the end of each time interval, three tubes of HEp-2 cells were inoculated with .1 ml of each virus dilution. Adsorption, feeding and microscopic examinations were performed in the

same manner as previously stated. Virus titers were calculated in the routine manner.

Inoculation of FHM Cells with Coxsackieviruses

Minimal essential growth medium was decanted from 1 week old FHM cells, and the cells were washed 3 times. Each of five tubes of FHM cells was inoculated with .1 ml of stock Coxsackievirus B-1 (TCD_{50}/ml = $10^{-7.25}$), and each of five tubes was inoculated with .1 of stock Coxsackievirus B-2 (TCD_{50}/ml = $10^{-6.0}$). Controls consisted of FHM cells not inoculated with either virus. All cells were incubated for 1 hr. at 36°C. At the end of the first incubation period .9 ml of 2% MEM was added to each cell culture and all cultures were incubated for an additional hour at 36°C. After the second incubation period, the supernatant fluids were removed. The cells were washed 4 times with 1 ml of 1X Hanks' BSS to remove unadsorbed viruses. The supernatant fluids and the first and fourth washes were collected and stored. All inoculated cells and controls were fed 2% MEM and incubated for 1 week at 30°C. Microscopic examinations of the inoculated cultures were made daily. At the end of 1 week the virus inoculated cells were shell-frozen and thawed 3 times *in situ*. The lysate fluids were clarified and collected.

Inoculation of 4% DMSO Treated FHM Cells with Coxsackieviruses B-1 and B-2

Ten tubes of FHM cells were inoculated and incubated in the same manner as above. After the first incubation period, .9 ml of a 4% DMSO mixture (4 ml DMSO to 96 ml 2% MEM) was added to each virus inoculated cell culture and five control cultures. All cell cultures were incubated a second time for 1 hr. at 36°C. The same procedures were

used previously for collection of supernatant and wash fluids, incubation, microscopic examinations, shell-freezing, thawing and clarification were repeated.

Passage of Fluids from 4% DMSO Treated and Untreated FHM Cells Inoculated with Coxsackieviruses B-1 and B-2 on HEp-2 Cells

All passages were done in duplicate on HEp-2 cells. Three day old HEp-2 cells were inoculated with .1 ml of the supernatant, wash and lysate fluids. Inoculated cells were incubated for 1 hr. at 36°C. After incubation, .9 ml of 2% M-199 was added to each tubed culture and all tubes, including uninoculated controls, were incubated at 36°C. and checked daily microscopically for 1 week. Maintenance medium was changed every second day. Those cultures showing CPE were recorded as positive (+) and those showing no CPE were recorded as negative (-).

RESULTS

Multiplication of AO-Labeled and Unlabeled Coxsackieviruses at 36°C.

The susceptibilities of two cell lines, HEp-2 cells and FHM cells, to infection by acridine orange-labeled, photosensitive, Coxsackieviruses B-1 and B-2 were tested. Recovery of unlabeled, photoresistant viruses from lysate fluids of cell cultures inoculated with the two AO-labeled viruses constituted the basis upon which susceptibilities were determined.

The results of this experiment are shown in Table 1. Unlabeled Coxsackievirus B-1 had a TCD_{50} of $10^{-7.12}$ while the labeled virus titer decreased only slightly to $10^{-6.5}$. This approximated a half log drop in titer. The Coxsackievirus B-2 was found to have a TCD_{50} of

Table 1. Multiplication of acridine orange-labeled and unlabeled Coxsackieviruses in HEp-2 cells at 36°C.

Virus	Titer (TCD_{50}/ml) AO-labeled	Unlabeled
Coxsackievirus B-1	$10^{-6.50}$	$10^{-7.12}$*
Coxsackievirus B-2	$10^{-5.50}$	$10^{-6.75}$

*Average of two titrations.

$10^{-6.75}$ normally and labeling produced an approximate one and a quarter log drop in the TCD_{50}, $10^{-5.5}$. The results show that, if proper

precautions of shielding from light are taken, vital dye labeling of these two virus strains leads to very little reduction of virus infectivity.

Multiplication of AO-Coxsackieviruses at 36°C. in HEp-2 Cells

The results from titration of lysate fluids from HEp-2 cells infected with the two vital dye labeled viruses and incubated at 36°C. for 1, 12, and 24 hours are summarized in Tables 2 and 3. One hour

Table 2. Multiplication of acridine orange-labeled Coxsackievirus B-1 in HEp-2 cells at 36°C.[a]

Time after infection (hr.)	Virus yield (TCD_{50}/ml)	
	Unexposed	Exposed[b]
1	$10^{-4.75}$	negative[c]
12	$10^{-7.50}$	$10^{-6.75}$
24	$10^{-7.75}$	$10^{-7.25}$

[a]HEp-2 cells maintained in medium without acridine orange.

[b]Virus exposed to 100 W incandescent light at 2.5 cm for 2 hrs.

[c]Negative at a 10^{-1} dilution.

after infection viruses were recovered in lysate fluids of infected cultures unexposed to light. Neither Coxsackieviruses B-1 nor B-2 were recovered 1 hour after infection from lysate fluids of cultures exposed to incandescent light, indicating that exposure to light had destroyed viral infectivity. Twelve to twenty-four hours after infection viruses were recovered from lysate fluids of cultures infe-ted with both viruses and exposed to light. This indicates that light-resistant

Table 3. Multiplication of acridine orange-labeled Coxsackievirus B-2 in HEp-2 cells at 36°C.[a]

Time after infection (hr.)	Virus yield (TCD_{50}/ml)	
	Unexposed	Exposed[b]
1	$10^{-2.50}$	negative[c]
12	$10^{-5.00}$	$10^{-2.75}$
24	$10^{-4.75}$	$10^{-3.75}$

[a]HEp-2 cells maintained in medium without acridine orange.

[b]Virus exposed to 100 W incandescent light at 2.5 cm for 2 hrs.

[c]Negative at a 10^{-1} dilution.

progeny were being produced, at least, 12 hours after infection. Lysate fluids from cultures infected with AO-Coxsackievirus B-1, harvested 12 and 24 hours after infection and unexposed to light, had TCD_{50}'s of $10^{-7.5}$ and $10^{-7.75}$, respectively, while virus titers of exposed lysate fluids from the same cultures decreased slightly to $10^{-6.75}$ and $10^{-7.25}$, respectively, over the same period. These decreases resulted in a three quarter and a one quarter log drop, respectively, in titer of viruses in lysate fluids exposed to light. A more drastic reduction in titer was observed for lysate fluids from Coxsackievirus B-2 infected cultures harvested 12 and 24 hours after infection and exposure to light (table 3). Unexposed lysates gave virus yields of $10^{-5.0}$ and $10^{-4.75}$ 12 and 24 hours after infection, respectively. In contrast, exposed lysate fluids gave virus yields of $10^{-2.75}$ 12 hours after infection and $10^{-3.75}$ 24 hours after infection. This signifies a two and one quarter and a one log drop in titer, respectively, for lysate fluids exposed to light. These variances in titer could possibly have

been due to errors in pipetting, errors inherent in the titration method employed or accidental exposure of vital dye labeled viruses to light during the initial infection period.

Multiplication of AO-Coxsackievirus at 30°C. in HEp-2 Cells

The results of this experiment are shown in Tables 4 and 5. Coxsackieviruses B-1 and B-2 were recovered 1 day after infection from lysate fluids of AO-labeled virus infected cultures exposed to light, indicating that both viruses were capable of replicating light-resistant progeny at 30°C., at least, 1 day after infection of susceptible cells. Increases in virus titer of unexposed lysates containing both viruses were observed over the time periods examined. Titers of exposed lysate fluids containing Coxsackievirus B-1 collected at the same intervals remained almost constant (Table 4). Virus yields

Table 4. Multiplication of acridine orange-labeled Coxsackievirus B-1 in HEp-2 cells at 30°C.[a]

Time after infection (days)	Virus yield (TCD_{50}/ml)	
	Unexposed	Exposed[b]
1	$10^{-5.75}$	$10^{-6.50}$
3	$10^{-7.50}$	$10^{-6.00}$
5	$10^{-7.25}$	$10^{-6.25}$

[a]HEp-2 cells maintained in medium without acridine orange.

[b]Virus exposed to 100 W incandescent light at 2.5 cm for 2 hrs.

of exposed lysate fluids containing Coxsackievirus B-2 increased from $10^{-2.5}$ after the first day to $10^{-5.5}$ after the fifth day (Table 5).

Table 5. Multiplication of acridine orange-labeled Coxsackievirus B-2 in HEp-2 cells at 30°C.[a]

Time after infection (days)	Virus yield TCD_{50}/ml)	
	Unexposed	Exposed[b]
1	$10^{-4.50}$	$10^{-2.50}$
3	$10^{-5.25}$	$10^{-4.75}$
5	$10^{-6.50}$	$10^{-5.50}$

[a]HEp-2 cells maintained in medium without acridine orange.

[b]Virus exposed to 100 W incandescent light at 2.5 cm for 2 hrs.

This represents an increase of three logs in titer over a five day period. Variances in virus yields of unexposed and exposed lysate fluids containing both viruses were observed and attributed to experimental errors and/or accidental exposure of AO-labeled viruses to light.

Multiplication of AO-Labeled Coxsackieviruses in FHM Cells Incubated at 30°C.

Results of titration of lysate fluids from FHM cells inoculated with AO-labeled test viruses and incubated at 30°C. for 4 days are shown in Table 6. Light-resistant viruses were not detected in exposed lysate fluids of FHM cells inoculated with either vital dye-labeled virus. Lysate fluids from Coxsackievirus B-1 and B-2 inoculated FHM cells and unexposed to light had TCD_{50}'s of $10^{-3.25}$ and $10^{-2.5}$, respectively. These results indicate that there was sufficient virus

present to cause infection if FHM cells were susceptible to the two virus strains used.

Table 6. Titrations on HEp-2 cells at 36°C. of exposed and unexposed lysate fluids from FHM cells inoculated with AO-labeled Coxsackieviruses B-1 and B-2 and incubated at 30°C. for 4 days

Virus Strains	Titer (TCD_{50}/ml)	
	Unexposed	Exposed
AO-Coxsackievirus B-1	$10^{-3.25}$	negative
AO-Coxsackievirus B-2	$10^{-2.50}$	negative

Cytotoxic Effect of DMSO on FHM Cells

Previous studies showed that fathead minnow cells were unable to support the growth of AO-labeled Coxsackieviruses B-1 and B-2. In an attempt to determine whether a chemical agent such as dimethyl sulfoxide (DMSO) could enhance the susceptibility of FHM cells to the two test viruses, FHM cells were inoculated with the viruses and treated with DMSO.

Table 7 shows the cytotoxic effect of DMSO in concentrations from 50 to 1% on FHM cells. Concentrations above 5% were consistently toxic to FHM cells causing either fixation of cells to the surfaces of the culture containers, partial or complete destruction of monolayers or death of the cells. Concentrations of 50% DMSO caused fixation of cells at each time interval of treatment. Fixation of cells resulted after treatment periods of 30 and 60 min. with 40% DMSO while monolayer destruction was observed 1 and 15 min. after treatment with the same concentration of

Table 7. Cytotoxic effect of various concentrations of DMSO on FHM cells

DMSO concentration	Time (min.) 1	15	30	60
50%	F	F	F	F
40%	D	D	F	F
30%	D	D	D	D
20%	D	D	D	D
15%	D	D	D	D
10%	D	D	D	D
5%	N	D	N	D
4%	N	N	N	N
3%	N	N	N	N
2%	N	N	N	N
1%	N	N	N	N

F = cells became fixed and no evidence of cellular metabolism after 48 hrs.

D = destruction of monolayer and cells unrecoverable.

N = neither cellular morphology nor metabolism altered.

DMSO. Cultures appearing fixed were supplemented with medium without DMSO, incubated at 30°C. for 1 week and checked macroscopically and microscopically for signs of cellular metabolism. No changes in pH of the maintenance medium were observed in fixed cultures during the incubation period, indicating that the cells were utilizing little, if any, of the nutrients. Some fixed cells became dislodged from the surface of the culture containers, and others remained attached but showed no signs of cellular division. DMSO mixtures ranging in concentrations from

30 to 10% caused either complete or partial destruction of cell monolayers. Attempts were made to recover cells dislodged post-DMSO treatment; however, subculturing resulted in few cells attaching to the surfaces of culture bottles. Cells remaining attached after treatment were supplemented with medium lacking DMSO, incubated and examined daily. Growth of these cultures was slow with no confluent monolayers forming after 1 week, and some cells showed granularity of the cytoplasm. At concentrations of 5% DMSO half of the cells tested showed some cytotoxic effects. A 4% concentration of DMSO was the highest concentration used that caused no visible changes in cellular morphology, growth or the formation of a monolayer. As a result of these findings, concentrations of 4% DMSO were used in all experiments using DMSO.

Toxicity of DMSO to Coxsackieviruses B-1 and B-2

The results of these experiments are listed in Tables 8 and 9. As shown, 4% DMSO has no viricidal effect on Coxsackievirus B-1.

Table 8. Test for toxicity of 4% DMSO on Coxsackievirus B-1[a]

Exposure time (min.)	Virus titer (TCD_{50}/ml)[b]
30	$10^{-7.25}$
60	$10^{-7.50}$
90	$10^{-7.25}$

[a]Stock Coxsackievirus B-1 (TCD_{50}/ml = $10^{-7.25}$) was used to make virus dilutions.

[b]Titrations were made on HEp-2 cells.

Table 9. Test for toxicity of 4% DMSO on Coxsackievirus B-2[a]

Exposure time (min.)	Virus titer (TCD_{50}/ml)[b]
30	$10^{-5.25}$
60	$10^{-5.50}$
90	$10^{-5.50}$

[a]Stock Coxsackievirus B-2 (TCD_{50}/ml = $10^{-6.0}$) was used to make virus dilutions.

[b]Titrations were made on HEp-2 cells.

Variances in titer of the stock Coxsackievirus B-1 suspension and titers after exposure to 4% DMSO for 30 and 90 min. were not observed. A quarter log increase in titer of virus exposed to 4% DMSO for 60 min. was observed; however, this rise was not considered significant. Slight decreases in titer were evident in tests using Coxsackievirus B-2. Stock suspensions of Coxsackievirus B-2 had a TCD_{50} of $10^{-6.5}$ prior to treatment with 4% DMSO. After 30 min. of treatment with 4% DMSO, a one and a quarter log drop in titer was observed. Drops of one log in titer were evidenced after 60 and 90 min. exposure to 4% DMSO. These drops in titer could possibly have been due to experimental errors in titration or some viricidal effects of 4% DMSO. Results from these studies indicate that exposure of Coxsackievirus B-1 to 4% DMSO causes no significant changes in titer after 90 min. Titers of Coxsackievirus B-2 were slightly altered during the periods of treatment.

Passage of Fluids from Virus Inoculated FHM Cells

Tables 10 and 11 summarize the results of passage on HEp-2 cells of supernatant, wash and lysate fluids from FHM cells inoculated with Coxsackieviruses B-1 and B-2. All supernatant and 1st wash fluids from

Table 10. Passages of undiluted supernatant, wash and lysate fluids from FHM cells inoculated with Coxsackievirus B-1 on HEp-2 cells

Test	Supernatant*	1st wash	4th wash*	lysate*
1	+	+	-	-
	+	+	-	+
2	+	+	-	-
	+	+	-	-
3	+	+	-	-
	+	+	-	-
4	+	+	+	-
	+	+	+	-
5	+	+	-	-
	+	+	-	-

+ = evidence of CPE on HEp-2 cells.
- = no evidence of CPE on HEp-2 cells.
* = positive tests on passage were treated on HEp-2 cells.

virus inoculated FHM cells, when inoculated to HEp-2 cells, caused typical enteric virus CPE indicating that unadsorbed viruses were being removed by mechanical means up to the 1st wash. Fourth wash fluids from FHM cells inoculated with both viruses gave fewer positive responses (CPE). Only one 4th wash fluid from these same cells inoculated with Coxsackievirus B-1 gave positive responses on HEp-2 cells. Three samples of 4th wash fluids from FHM cells inoculated with Coxsackievirus B-2, when inoculated on HEp-2 cells, gave positive responses. Fewer

Table 11. Passages of undiluted supernatant, wash and lysate fluids from FHM cells inoculated with Coxsackievirus B-2 on HEp-2 cells

Test	Supernatant*	1st wash	4th wash*	lysate*
1	+	+	+	+
	+	+	+	-
2	+	+	-	+
	+	+	-	-
3	+	+	+	-
	+	+	-	-
4	+	+	-	-
	+	+	-	-
5	+	+	-	-
	+	+	+	-

+ = evidence of CPE on HEp-2 cells.
- = no evidence of CPE on HEp-2 cells.
* = positive tests on passage were treated on HEp-2 cells.

positive responses of 4th wash fluids showed that at least a majority of the unadsorbed viruses were removed prior to incubation and subsequent lysis of the inoculated FHM cells. Microscopic examinations over the 7 day incubation period revealed no CPE in FHM cells inoculated with the two enteric viruses. Lysate fluids from FHM cells inoculated with Coxsackievirus B-1 gave one positive response when inoculated to HEp-2 cells while lysates from FHM cells inoculated with Coxsackievirus B-2 gave two positive responses on HEp-2 cells. Results from passage of these lysate fluids indicate that some viruses were present after four washings and incubation at 30°C. for 1 week. Whether viruses in the lysate fluids were the result of residual inocula or progeny of replication in FHM cells could not be determined by these methods. Results of titration of fluids positive on passage are explained elsewhere.

Passage of Supernatant, Wash and Lysate Fluids from Virus Inoculated and 4% DMSO Treated FHM Cells

Results from these studies are listed in Tables 12 and 13. Passage of supernatant and 1st wash fluids from Coxsackievirus inoculated FHM cells treated with 4% DMSO gave positive responses on HEp-2 cells. Each 4th wash sample from DMSO treated and Coxsackievirus B-1 inoculated FHM cells gave at least one positive response when passed in duplicate on HEp-2 cells. Only one positive response (Table 10) was detected for FHM cells inoculated with the same virus but not treated with 4% DMSO. Whether this increase of positive responses of 4th wash fluids from DMSO treated FHM cells was significant could not be determined using the methods employed in this study. Fourth wash samples from DMSO treated FHM cells inoculated with Coxsackievirus B-2

Table 12. Passage of undiluted supernatant, wash and lysate fluids from FHM cells treated with 4% DMSO and incubated with Coxsackievirus B-1 on HEp-2 cells

Test	Supernatant*	1st wash	4th wash*	lysate*
1	+	+	+	-
	+	+	+	-
2	+	+	+	-
	+	+	-	-
3	+	+	+	-
	+	+	+	-
4	+	+	+	-
	+	+	-	-
5	+	+	+	-
	+	+	-	-

+ = evidence of CPE on HEp-2 cells.
- = no evidence of CPE on HEp-2 cells.
* = positive tests on passage were treated on HEp-2 cells.

Table 13. Passage of undiluted supernatant, wash and lysate fluids from FHM cells treated with 4% DMSO and inoculated with Coxsackievirus B-2 on HEp-2 cells

Test	Supernatant*	1st wash	4th wash*	lysate*
1	+	+	-	-
	+	+	-	-
2	+	+	-	-
	+	+	-	-
3	+	+	-	-
	+	+	-	-
4	+	+	-	-
	+	+	-	-
5	+	+	-	-
	+	+	-	-

+ = evidence of CPE on HEp-2 cells.
- = no evidence of CPE on HEp-2 cells.
* = positive tests on passage were treated on HEp-2 cells.

gave no positive responses on HEp-2 cells. Microscopic examinations of virus inoculated-DMSO treated cells showed no apparent CPE typical of enteric viruses. Samples of lysate fluids from both virus inoculated-DMSO treated fish cells elicited no positive responses when passed on HEp-2 cells.

Titration of Supernatant, Wash and Lysate Fluids from Virus Inoculated 4% DMSO Treated and Untreated FHM Cells Positive when Passed on HEp-2 Cells

Results of titrations of supernatant fluids from DMSO treated and untreated virus inoculated FHM cells are listed in Tables 14, 15, 16, and 17. Supernatant fluids from DMSO treated Coxsackievirus B-1 inoculated FHM cells had an average TCD_{50} of $10^{-3.85}$ (Table 16), while supernatant fluids from fish cells inoculated with the same virus but untreated with

Table 14. Titrations of positive passage supernatant fluids from FHM cells inoculated with Coxsackievirus B-1

Test	Titer (TCD_{50}/ml)*
1	$10^{-3.75}$
2	$10^{-3.50}$
3	$10^{-4.25}$
4	$10^{-3.75}$
5	$10^{-5.50}$
	average TCD_{50}/ml = $10^{-4.15}$

*Titrations were done on HEp-2 cells.

Table 15. Titrations of positive passage supernatant fluids from FHM cells inoculated with Coxsackievirus B-2

Test	Titer (TCD_{50}/ml)*
1	$10^{-1.75}$[a]
2	$10^{-3.25}$
3	$10^{-2.25}$[a]
4	$10^{-1.50}$[a]
5	$10^{-2.50}$
	average TCD_{50}/ml) = $10^{-2.25}$

*Titrations were done on HEp-2 cells.

[a]Virus titers are listed using inocula of undiluted supernatant fluids.

Table 16. Titrations of positive passage supernatant fluids from FHM cells treated with 4% DMSO and inoculated with Coxsackievirus B-1

Test	Titer (TCD_{50}/ml)*
1	$10^{-4.00}$
2	$10^{-4.25}$
3	$10^{-3.50}$
4	$10^{-4.25}$
5	$10^{-3.25}$
	average TCD_{50}/ml = $10^{-3.85}$

*Titrations were done on HEp-2 cells.

Table 17. Titrations of positive passage supernatant fluids from FHM cells treated with 4% DMSO and inoculated with Coxsackievirus B-2

Test	Titer (TCD_{50}/ml)*
1	$10^{-2.75}$
2	$10^{-2.50}$
3	$10^{-3.25}$
4	$10^{-2.50}$
5	negative[a]
	average TCD_{50}/ml = $10^{-2.2}$

*Titrations were done on HEp-2 cells.

[a]One tenth milliliter inocula of undiluted supernatant fluid resulted in no visible CPE after one week.

DMSO had average TCD_{50} of $10^{-4.15}$ (Table 14). This represents a quarter log difference in titer between the two supernatant fluids. Titers for some supernatant fluids (test 1, 3 and 4, Table 15) from DMSO treated-Coxsackievirus B-2 inoculated FHM cells could not be calculated when the fluids were diluted ten times. All HEP-2 cells inoculated with .1 ml of undiluted supernatant fluids from these cells showed CPE. Thus, the undiluted inocula were considered a 10^{-0} dilution, and calculations were based on this dilution. Titers of supernatant fluids from Coxsackievirus B-2 inoculated fish cells when averaged gave a TCD_{50} of $10^{-2.2}$ (Table 17) for DMSO treated cells and an average titer of $10^{-2.25}$ (Table 15) for untreated cells. The variance in titer was five hundredths of a log and was not considered significant. These results revealed that titers of viruses present in supernatant fluids were not significantly altered by the addition of 4% DMSO to FHM cells inoculated with either Coxsackievirus B-1 or B-2.

Fourth wash fluids from virus inoculated -4% DMSO treated and untreated FHM cells, positive on passage (Tables 10, 11, and 12), when diluted 10^{-1}, resulted in no CPE on HEp-2 cells. Lack of CPE production upon dilution showed that, at least, four washings of the virus inoculated fish cells were adequate in removing most of the unadsorbed viral particles. Lysate fluids positive on passage from virus (Tables 10 and 11) inoculated untreated fish cells caused no CPE when diluted 10^{-1} and inoculated to HEp-2 cells. Since there were no evidences of increases in virus titer in lysate fluids, the positive responses observed on passage could not be attributed to replication of the viruses in FHM cells, but could possibly be attributed to residual viral particles left after washing.

DISCUSSION

Two methods were used to determine the susceptibility of FHM cells to two enteric viruses, Coxsackievirus types B-1 and B-2. The first method used acridine orange (AO)-labeled viruses to inoculate FHM cells. Hiatt (29) suggested that vital dye-labeled viruses could be useful in studying the dynamics of virus replication in various cell systems. The second method employed virus inoculated FHM cells treated and untreated with DMSO. Enteric virus tropism for primate cells has been attributed to the presence of lipoprotein receptor sites (31). A search of the literature failed to reveal any studies related to the receptor site composition of FHM cells. To possibly bypass the necessity of specific receptor sites, DMSO was used as an enhancer of virus penetration across FHM cell membranes. DMSO has been helpful in enhancing polio-RNA infectivity in primate cells (2).

Initial inoculation of FHM cells with the test viruses resulted in no apparent CPE after incubation at 30°C. for 1 week. The absence of cell destruction was not taken as absolute evidence that the cells were unable to support the growth of the viruses, since Gravell and Malsberger (22) found that another enteric virus, ECHO-11, replicated in FHM cells in high titers with little cell destruction.

Coxsackievirus types B-1 and B-2 were grown on HEp-2 cells in medium containing 5 µg/ml acridine orange. The concentration of acridine orange used in these experiments had no apparent effect on shielded HEp-2 cells after 24 and 48 hr. incubation periods. Viruses

labeled with the vital dye were sensitive to light; however, if proper precautions of shielding from light were taken during replication, harvesting, storage, titration and inoculation, very little of the viral infectivity was lost due to exposure to light. Both AO-labeled viruses replicated in HEp-2 cells at the temperatures investigated. The rate of replication of Coxsackievirus B-1 was significantly influenced by temperature. After 3 days of growth at 30°C., the titer of Coxsackievirus B-1 was equivalent to 12 hours of growth at 36°C. At the end of 24 hrs. (1 day) the titers of Coxsackievirus B-2 were almost equivalent at both temperatures, indicating that temperature had a lesser influence on this virus' replication. The photosensitivity technique was capable of distinguishing new progeny from input virus. At 36°C. both viruses were light-sensitive 1 hr. after infection of HEp-2 cells and light-resistant thereafter, indicating that a new unlabeled generation of viruses had been replicated in cells susceptible to the viruses. Infected HEp-2 cells incubated at 30°C. and titered 1 day after infection showed the presence of light-resistant viruses. Lysate fluids from FHM cells inoculated with the AO-labeled viruses and incubated at 30°C. resulted in no new viral progeny resistant to light after a 4 day incubation period. Titration of unexposed fluids from these same cells on HEp-2 cells indicated that input viruses were present in quantities sufficient to cause infection if the cells were susceptible to the two test viruses. Using proflavine-labeled enteric viruses (polio-, Coxsackie- and ECHO viruses) and Northern quahogs, Chang *et al.* (9) obtained similar results. Digestive diverticulum and mantle cavity fluids from quahogs inoculated with proflavine-labeled enteroviruses contained no light-resistant viral progeny after incubation at 25°C. for 3 days.

Preliminary DMSO toxicity experiments determined the concentrations of DMSO tolerated by FHM cells and the test viruses. At a concentration of 4%, DMSO had no cytotoxic effect on the cells and caused no decrease in titer of stock Coxsackievirus B-1 suspensions. Decreases in titers of Coxsackievirus B-2 preparations were observed. The approximate 1 log drop in titers was not considered drastic and could have been due to experimental errors in titration or loss of viral activity as a result of DMSO treatment. Passage of undiluted fluids from DMSO treated and untreated FHM cells on HEp-2 cells showed that virus particles were mechanically removed, in most cases, up to the 4th washing. After four consecutive washings, the virus inoculated-DMSO treated and untreated cells were incubated at 30°C. for 1 week. Daily microscopic examinations revealed no visible CPE typical of enteric viruses and no changes in growth patterns of FHM cells over this period. Lysate fluids from these cells, when passed undiluted on HEp-2 cells, gave few positive responses. Titrations of these fluids revealed that at a 10^{-1} dilution the lysate fluids were negative. These results indicate that after a period of one week at 30°C. there was no increase in titer of the test viruses in DMSO treated and untreated FHM cells. The positive responses on passage were possibly due to the presence of residual viruses.

Averages of the titers from virus inoculated -4% DMSO treated and untreated FHM cells showed that there was no significant decrease in titers of Coxsackieviruses in the supernatant fluids. It was not possible to conclude from these data that enhancement of virus penetration did not occur with DMSO treatment, because no electron microscopy work was done. However, Amstey and Parkman (2) found that there was a linear increase in polio-RNA infection in HeLa cells when DMSO was

increased from a concentration of 5% to 40%. The 4% concentration of DMSO tolerated by the FHM cells could possibly have been too dilute to facilitate the passage of the intact Coxsackieviruses across the membrane in appreciable amounts to warrant a decrease in the initial titer of the supernatant fluids. Undiluted lysate fluids from these same treated cells did not give typical enteroviral CPE when passed on HEp-2 cells, indicating that even if the 4% DMSO facilitated membrane passage of the virus, the titration methods employed in this study did not detect, after cell rupture, any increase in virus titer.

It can be concluded from results obtained by methods used in this study that a 4% concentration of DMSO has no influence in enhancing viral susceptibility of FHM cells to two enteric viruses, Coxsackievirus types B-1 and B-2. The lack of enhancement could possibly be due to the dilute concentration of DMSO tolerated by FHM cells. As a result of using AO-labeled viruses, one may also conclude that under the stated experimental conditions, at least Coxsackievirus types B-1 and B-2 did not replicate in FHM cells. The inability of FHM cells to support the growth of these two viruses was probably due to the absence of specific enteric virus receptor sites.

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