LAWRENCE, C. A., C. M. CARPENTER, AND A. W. C. NAYLOR-FOOTE. 1957. Iodophors as disinfectants. J. Am. Pharm. Assoc. **46**:500–505.

MEAD, J., AND C. L. BOMMARITO. 1948. The reliability of rectal temperatures as an index of internal body temperatures. Environmental Protection Section Rept. No. **141**. Office of The Quartermaster General, Washington.

PATTERSON, R. A. 1956. A new method for the isolation of *Mycobacterium tuberculosis*. Am. J. Public Health **46**: 1429–1430.

SOMMERMEYER, L., AND M. FROBISHER, JR. 1953. Laboratory studies on disinfection of rectal thermometers. Nursing Research **2**:85–89.

WILSON, A., G. B. GRIFFIS, AND J. C. MONTERMOSO. 1958. Effect of swelling on the properties of elastomers. Rubber World **139**:68–73.

U. S. Government Printing Office. 1955. Federal test method standard no. 601: Rubber: sampling and testing; Change in volume, Liquid immersion, method no. 6211. Washington.

Comparative Effects of β-Propiolactone on Mice, Mouse-derived Cell Cultures, and Venezuelan Equine Encephalomyelitis Virus

HENRY J. HEARN, JR., AND FREDERICK W. DAWSON[1, 2]

U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland

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ABSTRACT

HEARN, HENRY J., JR. (U. S. Army Chemical Corps, Frederick, Md.), AND FREDERICK W. DAWSON. Comparative effects of β-propiolactone on mice, mouse-derived cell cultures, and Venezuelan equine encephalomyelitis virus. Appl. Microbiol. **9**:278–282. 1961.—Studies were made comparing the toxicity of β-propiolactone (BPL) for mammalian (mouse) cells in vitro and for mice and for Venezuelan equine encephalomyelitis (VEE) virus which is highly cytopathogenic for each. The mammalian cells grown in tissue culture were found to be adversely affected by BPL in concentrations ranging from 0.001 to 0.1 mg/ml of supernatant fluid. The difference in response was influenced by the menstruum in which the BPL was suspended and the difference in cell types tested. Tenfold less BPL appeared to be required to destroy the cells when it was suspended in a balanced salt solution than when it was suspended in protein-containing solutions such as beef heart infusion broth or medium 199 plus 20% horse serum. Secondary embryonic mouse lung cells seemed slightly more adversely affected by BPL than the established embryonic lung or L cells. BPL given to mice by intranasal instillation and by intracerebral injection was lethal to half of the animals within 2 days at doses of 0.31 and 0.39 mg, respectively. Higher concentrations of BPL were required to rapidly inactivate the virus in vitro than were required to kill mice or to cause a toxic effect on cells in culture. It required 10 mg/ml of BPL to completely inactivate a high-titered VEE virus preparation in 5 min and 1 mg/ml to inactivate most, but not all, of the virus in 15 min. A concentration of 0.1 mg/ml of BPL had only a slight effect on the virus after a period as long as 60 min. Evidence is presented indicating that simultaneous inactivation of all of the properties of the VEE virus particles by BPL aerosols did not occur at the same time but that, after treatment, the virus possessed a limited ability to immunize mice despite a loss in infectivity.

Numerous studies of the effects of liquid preparations of β-propiolactone (BPL) on viruses in tissues and biological fluids have appeared in the literature. These studies and others related to killed vaccines prepared with BPL recently were reviewed by LoGrippo (1960). The number of viruses and rickettsiae reported to be inactivated by BPL vapor was extended by Dawson, Janssen, and Hoffman, (1959, 1960) to include Venezuelan equine encephalomyelitis virus and the causative agents of smallpox, yellow fever, psittacosis, and Q fever. No quantitative data seem to be available, however, comparing the virucidal levels of BPL with its toxicity levels for tissues or animals. In the present report, the toxicity levels of BPL for mice and for mouse-derived cell cultures are compared to the concentration of BPL necessary to inactivate a viral agent of known virulence for the same host.

MATERIALS AND METHODS

Preparation of cell monolayers. The preparation of monolayers of embryonic mouse lung cells was carried

[1] USN. The assertions and opinions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

[2] Present address: Wilmot Castle Company, Rochester 2, N. Y.

out according to the procedures described by Brown, Mayyasi, and Officer (1959). Briefly, excised lungs from the embryos were trypsinized to make cell suspensions of known concentrations which were then washed, resuspended in medium 199 (Morgan, Morton, and Parker, 1950) containing 20% horse serum (199/HoS), planted in T-60 Earle flasks, and incubated at 37 C. Cell sheets derived from primary cultures which were subcultured for one passage were designated secondary cultures. Those that were subcultured for ten or more consecutive passages were designated as established cell lines. Only secondary cultures and established cell lines were used in the toxicity tests. With successive subcultivation, the confluent sheets of mixed cell types that originally were present, gradually gave rise to populations resembling our present L cell cultures. The latter are shortened spindle-shaped cells almost polygonal in appearance.

Exposure and toxicity of BPL to cells in vitro. Immediately prior to use, the BPL was diluted in either cold sterile beef heart infusion broth (BHIB) or cold sterile balanced salt solution (BSS). The concentrations of BPL used ranged from 0.001 to 1.0 mg per ml of fluid. One milliliter of medium-BPL mixture was added to cell monolayers containing 10^5 to 10^6 cells in T-60 Earle flasks or 16- by 180-mm Pyrex test tubes; prior to the addition of the medium-BPL mixtures, the growth medium (199/HoS) was removed from the flasks and tubes, and the cell monolayers were washed with BSS. In one experiment, BPL diluted in cold sterile BHIB was introduced to the cells by adding the BHIB-BPL mixture directly to the growth medium. Cell cultures exposed to the medium-BPL mixtures were incubated at 37 C for 3 hr, and the pH of the mixtures was maintained at 7 to 7.4 by the addition of 2 or 3 drops of a saturated solution of sodium bicarbonate.

During the 3-hr incubation period, the cells were examined every half hour. At the end of this 3-hr period, the medium-BPL mixture, which presumably contained the products of hydrolysis (Hoffman and Warshowsky, 1958), was removed. The cell monolayers were washed with BSS; fresh growth medium (199/HoS) was added. The cells were examined at 18, 24, 48, and 72 hr thereafter for evidence of toxicity which was indicated by rounding and granularity of the cells followed by their sloughing off the glass.

Exposure of mice to BPL solutions. Test animals, 14 to 16 g, under ether anesthesia, were inoculated either intracerebrally or intranasally with 30, 3, and 0.3 mg of BPL contained in 0.03 ml of BHIB. The mice were then observed for 10 days for clinical signs of toxicity.

Preparation of tissue culture virus seeds for exposure to BPL. Venezuelan equine encephalomyelitis (VEE) virus was selected as a test agent because it provided high titered seeds, i.e., 10^8 to 10^9 mouse intracerebral (MIC) LD_{50} per ml of tissue culture fluid, and showed no loss in infectious titer during the test periods at 37 C.

The tissue culture virus seed was prepared by inoculating an L cell monolayer containing about 10^7 cells grown in medium 199/HoS with 10^5 MIC LD_{50} of a stock egg seed (PES) virus. Eighteen hours after inoculation, the medium was removed and the monolayer washed with BSS. After removing the supernatant, 15 ml of fresh BSS were placed over the cells which were reincubated at 37 C. At 36 hr the cells began to lyse. The supernatant was removed, distributed in 2.0-ml amounts in sealed ampoules and stored in a Dry Ice chest. The resulting tissue culture seed (BSV) contained $10^{9.1}$ LD_{50} per ml as assayed in mice intracerebrally and $10^{8.2}$ LD_{50} per ml as assayed intraperitoneally. The titrations were carried out in the usual manner (Hearn, 1960).

Virus inactivation with BPL solutions. Ten milliliters of a 10% tissue culture virus suspension was prepared using BSV diluted in BHIB at 37 C. Enough BPL was then added to provide concentrations of 0.1, 1.0, and 10 mg per ml of fluid. Samples were removed for immediate intracerebral titration in mice (12 to 14 g) at 5-, 15-, 30-, and 60-min intervals. In one experiment an intraperitoneal titration was performed concurrently with the intracerebral titration.

Virus inactivation with BPL vapor. Studies on the inactivation of virus by BPL vapor were carried out as follows: Gauze pads, 2 by ¼ in., were inoculated with 0.2 ml of egg embryo preparation of VEE virus containing $10^{8.6}$ mouse intracerebral LD_{50}. The pads were suspended by wire hooks attached to rubber stoppers at the top of a 4-liter cardboard drum. In one end of the drum a DeVilbiss no. 40 atomizer was inserted from which 30% BPL was sprayed. The stoppers with hooks holding the pads were inserted so that they could be removed at desired intervals during the BPL spraying. Once removed, the pads were immersed in 9 ml of BHIB and agitated to liberate the virus into the liquid. The BHIB was then assayed for virus by intraperitoneal inoculation into mice (12 to 14 g).

Results

Toxicity of BPL for cells in vitro. Results in Table 1 show that minimal toxic levels of BPL for secondary embryonic mouse lung, established mouse lung and established L cells in monolayer ranged from 0.001 to 0.1 mg BPL per ml of fluid. The differences depended upon the diluent in which BPL was suspended and the cell lines used. All three cell lines withstood ten times more BPL suspended in BHIB than in BSS. β-Propiolactone was as toxic when dissolved in tissue culture medium containing 20% horse serum as in BHIB. For one of the diluents, BHIB, common to all three cell culture types, the toxicity level for all three varied no

more than twofold. For another of the diluents, BSS, the toxicity level for all three varied no more than tenfold.

Toxicity of BPL in mice. All the test animals (20/20) given either 30 or 3 mg BPL contained in a 0.03-ml volume by intranasal instillation (IN) died within 48 hr following inoculation (Table 1). A further tenfold dilution of BPL resulted in the death of 8/20 of the injected animals. The amount of BPL necessary to kill 50% of the inoculated animals was calculated to be 0.31 mg. No deaths occurred in the control group of animals receiving an equal quantity of BHIB by the IN route. The intracerebral (IC) inoculation of either 30 or 3 mg of BPL administered in a 0.03-ml volume caused the deaths of all the test animals (20/20) within 48 hr. A tenfold dilution resulted in the deaths of 4/20 of the treated mice. Thus, 0.39 mg of BPL given IC was the calculated dose necessary to kill 50% of the infected animals.

TABLE 1. *Toxicity of β-propiolactone (BPL) for mice and mouse-derived cell cultures*

Cell type	Method of administration	Menstruum during treatment	Toxic dose* mg
Mouse cells in vitro:			
Secondary embryonic lung	Inoculated on monolayer	BHIB†	0.05
		BSS	0.001
Established embryonic lung	Inoculated on monolayer	BHIB	0.1
		BSS	0.01
L cell fibroblasts	Inoculated on monolayer	BHIB	0.1
		199/HoS	0.1
		BSS	0.01
Mice:‡			
Brain	Injected intracerebrally		0.39
Respiratory	Injected intranasally		0.31

* BPL per ml cell culture fluid (per 0.5×10^6 cells) necessary to destroy either 50% of the cells or kill 50% of the test animals within 2 days.

† BHIB = beef heart infusion broth; BSS = balanced salt solution; 199/HoS = medium 199 containing 20% horse serum.

‡ Albino mice, 14 to 16 g.

Virus inactivation with BPL solutions. BPL in concentrations of 1 and 10 mg per ml of virus suspension inactivated large amounts of virus within short periods of time. Figure 1, showing the rate of virus inactivation at various intervals posttreatment with BPL, shows that no virus could be detected after 5 min with BPL present in concentrations of 10 mg per ml of virus suspension. When 1 mg per ml was used, trace amounts of virus were found at the 30-min interval and no virus was detected after 60 min of treatment. Moreover, BPL was equally effective against virus present in a chick embryo preparation that had been diluted 1–10 in BHIB as against tissue-culture-produced virus suspended in the protein-free BSS.

When 0.1 mg BPL per ml virus suspension was used, virus inactivation was scarcely perceptible up to and including the 30-min interval. By 60 min, no more than

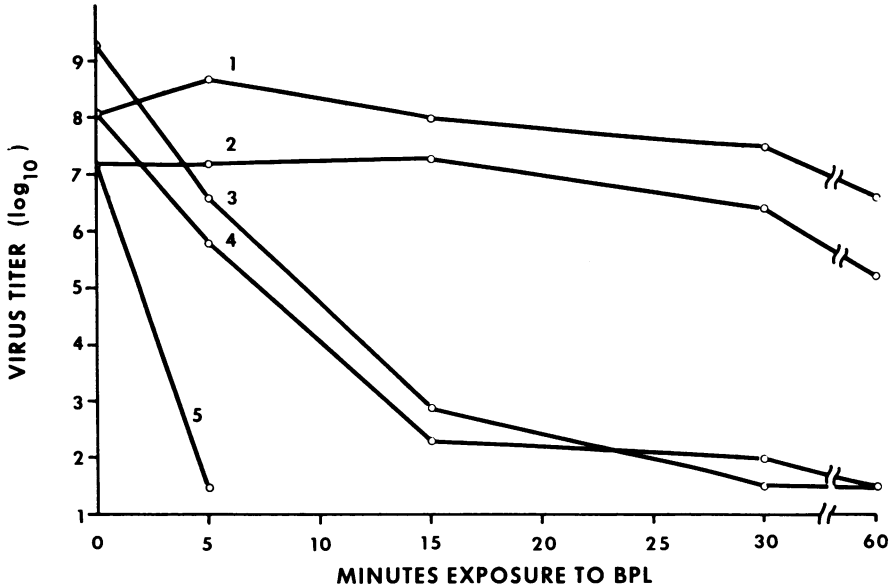

FIG. 1. *Loss of infectious titer of Venezuelan equine encephalomyelitis (VEE) virus preparation after treatment with solutions of β-propiolactone (BPL).*

1) Tissue culture virus in balanced salt solution with 0.1 mg BPL per ml in beef heart infusion broth (BHIB), titrated intracerebrally in mice (12 to 14 g).

2) Tissue culture virus in balanced salt solution with 0.1 mg BPL per ml in BHIB, titrated intraperitoneally in mice (12 to 14 g).

3) Chick embryo seed virus with 1.0 mg BPL per ml in BHIB, titrated intracerebrally in mice (12 to 14 g).

4) Tissue culture virus in balanced salt solution with 1.0 mg BPL per ml in BHIB, titrated intracerebrally in mice (12 to 14 g).

5) Tissue culture virus in balanced salt solution with 10.0 mg BPL per ml in BHIB, titrated intracerebrally in mice (12 to 14 g).

10^2 LD_{50} of virus were inactivated. Subsequent histopathological examinations of brain tissues taken from moribund mice revealed that cellular degenerative responses were more extensive in the brains of mice that received both virus and sublethal doses of BPL than in the mice that received virus alone.

Virus inactivation with BPL vapor. As reported earlier (Dawson et al., 1959), BPL vapor was found to be efficient in inactivating viruses. Complete viral inactivation, however, did not occur using the apparatus described above until the virus was exposed for 8 to 10 min to BPL. Table 2 shows that a 3-min exposure to 4.5 mg BPL per liter of air failed to inactivate the virus. All of the mice died when inoculated with BHIB into which the gauze pad had been placed and agitated to release the virus. After 5-min exposure to BPL, however, only 4/20 of the inoculated mice died leaving survivors which resisted a challenge of 100 LD_{50} of VEE virus 3 weeks later. This suggested that a 5-min exposure destroyed almost all the infectious activity of the virus without destroying the immunogenic activity. Increased exposure periods of 7 and 9 min resulted only rarely in mouse death. Survivors from BPL exposures for this length of time, however, failed to resist challenge with untreated virus.

Discussion

Tests in vitro showed that a liquid preparation of BPL was a highly efficient disinfectant for VEE virus. Large amounts of virus were destroyed after short periods of contact with small concentrations of BPL in either the liquid or vapor state. BPL was equally effective against virus prepared in an egg embryo seed suspension as against tissue-culture-produced virus suspended in a balanced salt solution.

As first reported by Hoffman and Warshowsky (1958), the bactericidal activity of BPL plus its rapid inactivation and ease of application suggested that this chemical was of potential value as a gaseous decontaminant for large areas (rooms, laboratories, etc.). Prospects of its efficacy gained in importance upon the demonstration that BPL efficiently inactivated many different viral agents (Dawson et al., 1959, 1960; LoGrippo, 1960). In view of the present work, however, it is suggested that this chemical be used with precautions as described recently by Feazel and Lang (1959) and Spiner and Hoffman (1960). Among the reasons for this is the fact that mammalian cells (mouse) showed a low level of tolerance to the toxic effects of BPL as tested in tissue culture and by injection into crucial anatomical areas such as the brain or respiratory tract of mice. In fact, it was found that 10 to 1,000 times more BPL was needed for rapid virus inactivation than could be withstood by the tissue cultures and 2.5 to 30 times more than could be withstood by mice.

TABLE 2. *Inactivation of VEE virus on gauze pads after various exposure periods to β-propiolactone (BPL) vapor*

Minutes exposure of virus* to BPL vapor†	No. dead/no. inoculated‡	Per cent survivors	Immune to reinfection with 100 mouse intraperitoneal LD_{50} of virus§
3	20/20	0	
5	4/20	80	+
7	1/20	95	−
9	0/20	100	−

* Virus seed ($10^{8.6}$ MIC LD_{50}) inoculated onto gauze pad 2 by ¼ in.

† BPL vapor concentration = 4.5 mg/liter of air.

‡ Determined by inoculating mice (12 to 14 g) with BHIB into which the gauze pad was placed and agitated to release the virus.

§ Untreated egg embryo seed virus.

Histopathologic examination of mouse brain sections disclosed that the degenerative response to active virus and subtoxic BPL was more intense than when either BPL or virus was given alone. This suggested that the biological and chemical agents may have acted synergistically during the early intervals of virus inactivation.

While using one method of inactivating VEE virus with BPL, it appeared that total inactivation of all properties of the virus did not occur at the same time. Exposure of the virus to constant amounts of aerosolized BPL for various periods of time resulted in virus preparations of low lethality for mice and with a limited capacity to immunize. Mice injected with BPL-treated virus were able to withstand a challenge of 100 LD_{50} of virulent virus.

Somewhat analogous results were obtained in studies of the aerosol inactivation of *Coxiella burnetii* in which serum from guinea pigs inoculated with inactivated rickettsial preparations showed a low-grade capacity to fix complement (Dawson et al., 1960). It is suggested that the test animals in these instances were inoculated with BPL-treated preparations in which the end point of infectivity had been reached and the mice that survived had received only inactive organisms possessing sufficient antigenicity to produce low-grade immune responses. In contrast to this, in other experiments in which a live, attenuated strain of VEE virus was used, the immunogenic response of the host was much greater (Hearn, 1960).

LITERATURE CITED

Brown, A., S. A. Mayyasi, and J. E. Officer. 1959. The toxic activity of vaccinia virus in tissue culture. J. Infectious Diseases, **104**:193–202.

Dawson, F. W., H. J. Hearn, and R. K. Hoffman. 1959. Virucidal activity of β-propiolactone vapor. I. Effect of β-propiolactone vapor on Venezuelan equine encephalomyelitis virus. Appl. Microbiol. **7**:199–201.

Dawson, F. W., R. J. Janssen, and R. K. Hoffman. 1960. Virucidal activity of β-propiolactone vapor. II. Effect on

the etiological agents of smallpox, yellow fever, psittacosis, and Q fever. Appl. Microbiol. **8**:39–41.

FEAZEL, C. E., AND E. W. LANG. 1959. New vapor phase disinfectant. Soap Chem. Specialties **35**:113–121.

HEARN, H. J. 1960. A variant of Venezuelan equine encephalomyelitis virus attenuated for mice and monkeys. J. Immunol. **84**:626–629.

HOFFMAN, R. K., AND B. WARSHOWSKY. 1958. β-Propiolactone vapor as a disinfectant. Appl. Microbiol. **6**:358–362.

LOGRIPPO, G. A. 1960. Investigations of the use of β-propiolactone in virus inactivation. Ann. N. Y. Acad. Sci. **83**:578–594.

MORGAN, J. F., H. J. MORTON, AND R. C. PARKER. 1960. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. **73**:1–8.

SPINER, D. R., AND R. K. HOFFMAN. 1960. Method for disinfecting large enclosures with β-propiolactone vapor. Appl. Microbiol. **8**:152–155.

Testing Thermal Death Data for Significant Nonlogarithmic Behavior[1]

ARTHUR E. HUMPHREY[2] AND JOHN T. R. NICKERSON

Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts

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ABSTRACT

HUMPHREY, ARTHUR E. (Massachusetts Institute of Technology, Cambridge), AND JOHN T. R. NICKERSON. Testing thermal death data for significant nonlogarithmic behavior. Appl. Microbiol. **9**:282–286. 1961.—The thermal death behavior of *Bacillus stearothermophilus* spores in distilled water suspensions is nonlogarithmic in dynamic as well as static tests. The explanation of heat lags as the cause of nonlogarithmic behavior is incorrect.

The controlled death of bacterial spores and knowledge pertaining thereto are important factors in the manufacture of canned foods and in the sterilization of broth for fermentations processes. Indeed, without such knowledge, many sterilization operations cannot be accomplished successfully. For this reason, considerable research effort has been devoted to the study of death of bacterial spores. After almost 60 years of intensive research, however, little understanding has been gained of the exact nature of bacterial death.

In the design of all sterilization operations it has been assumed that the death of microorganisms can be adequately described by a logarithmic-type survivor curve. The amount of data which have appeared in the literature to substantiate logarithmic behavior has been so overwhelming that notable workers such as Rahn (1945) have stated "The order of death (of microorganisms) is logarithmic." Even Ball and Olson (1957) in their book *Sterilization in Food Technology* have written ". . .it is not unreasonable to believe that perfect experimentation will eventually show this (survival) curve to be truly exponential. . . ."

In spite of these resolute statements, nonlogarithmic survivor curves are continually being reported in the literature; *see* Anderson, Esselen, and Fellows (1949), Collins et al. (1956), Davis and Williams (1948), El-Bisi and Ordal (1956*a*,*b*), Frank and Campbell (1957), Kaplan, Lichtenstein, and Reynolds (1953), Kaufman and Andrews (1954), LaBaw and Disrosier (1954), Malin (1952), Reed, Bohrer, and Cameron (1951), Reynolds and Lichtenstein (1952), Sugiyama (1951), White (1953). Typical of such data are those obtained by the method of Stern and Proctor (1954) for the thermal death of spores of *Bacillus stearothermophilus* strain FS 7954 suspended in distilled water (*see* Fig. 1.). Halvorson (1958) has explained behavior of this type as due to the use of insufficiently heat-shocked suspensions. However, for the low temperature portion of these data the lag time is much longer than the time necessary for adequate heat activation. Ball and Olson (1957) have attributed nonlogarithmic behavior to heat lags; but, in these experiments the heat lags were carefully measured and accounted for in the results.

Because of this confusing and contradictory nature of findings relative to the death of bacterial spores by heating, it was felt worthwhile to investigate further the character of thermal destruction of bacterial spores. In particular, it was felt that "dynamic characterization" of such data was needed.

To date all thermal destruction studies on spores have been carried out in a static or isothermal manner,

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[2] Present address: School of Chemical Engineering, University of Pennsylvania, Philadelphia 4, Pa.