

1034

THE EFFECTS OF β -PROPIOLACTONE ON BACTERIAL SPORES

HAROLD R. CURRAN AND FRED R. EVANS

EASTERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE, WASHINGTON 25, D.C.

THE EFFECTS OF β -PROPIOLACTONE ON BACTERIAL SPORES

HAROLD R. CURRAN AND FRED R. EVANS

Eastern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Washington 25, D.C.

The widespread use of pooled blood and blood derivatives as therapeutic agents has focused attention upon a health hazard arising from the transmission of infectious hepatitis (homologous serum jaundice)—a serious disease of viral origin. In an attempt to resolve this problem, Hartman, Kelly, and their associates¹⁻⁷ conducted a systematic search for compounds that would inactivate the virus in blood or plasma, without residual activity and without material change in the constituent proteins. The screening of 400 compounds yielded 7 that were effective and provisionally acceptable. Of these, β -propiolactone (hereafter abbreviated (BPL)) was the most promising and it is

now undergoing extensive clinical tests. The unusual properties of this compound, its potential usefulness as a preserving or sterilizing agent, and the almost complete absence of information concerning its activity toward spores, prompted the present study.

MATERIALS AND METHODS

The following species were tested: *Bacillus subtilis* (15u), American Can Company and strains 6051, 6634, American Type Culture Collection; *Bacillus cereus* (401), N. R. Smith; *Bacillus stearothermophilus* (1518); *Clostridium botulinum* (62A); *Clostridium* spp. (3679), National Canners Association; *Escherichia coli* (228), R. P. Tittler.

The spores were produced, collected, and washed as previously described.⁸ Clumps were dispersed by shaking with small glass beads.

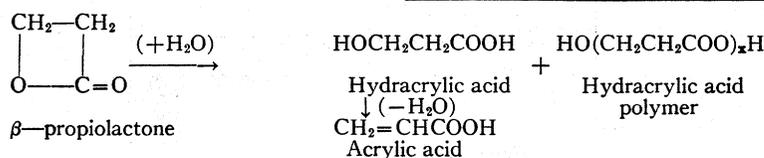
Washed spores were seeded into different substrates, treated with purified BPL* with or without previous activation of the spores by heating at 85 or 100 C for 10 or 15 minutes. After the desired period of contact, the action of BPL was stopped by the addition of sodium thiosulphate, which in the quantities used had no sporistatic or sporicidal activity. When sterile milk was the substrate, the pH was adjusted to 6.8 before treatment to preclude precipitation of the casein. Spore counts in plates or tubes were made before and after treatment. The counting medium for *B. subtilis*, *B. cereus*, *B. stearothermophilus*, and *Esch. coli* was glucose nutrient agar containing 0.1% soluble starch. The clostridia were counted in Prickett tubes containing Yesair pork infusion agar with 0.1% starch plus 0.1% filter-sterilized NaHCO₃ and sealed with 1.5% agar containing BBL thioglycollate supplement.

- Received for publication May 21, 1956.
1. Mangum, G. H., Kelly, A. R., Saunders, B. E., Piepes, S. L., Wallbank, A. M. and Hartman, F. W. 1951, Some virucidal agents and their chemical and pharmacological properties. *Federation Proc.* **10**: 220.
 2. Hartman, F. W., Piepes, S. L. and Wallbank, A. M. 1951, Virucidal and bactericidal properties of beta-propiolactone. *Federation Proc.* **10**: 358.
 3. Kelly, A. R. and Hartman, F. W. 1951, Beta-propiolactone; its toxicity, degradation products and comparison with nitrogen mustard. *Federation Proc.* **10**: 361.
 4. Kelly, A. R. and Hartman, F. W. 1952, Biological effects of beta-propiolactone. *Federation Proc.* **11**: 419.
 5. Kelly, A. R. 1952, Effects of virucide beta-propiolactone and its degradation products on blood platelets. *Federation Proc.* **11**: 419.
 6. Hartman, F. W. and Kelly, A. R. 1953, Tissue toxicity of beta-propiolactone and its degradation products. *Federation Proc.* **12**: 390.
 7. Kelly, A. R., Rupe, C. E., Tazuma, J. J. and Hartman, F. W. 1954, Toxicity of beta-propiolactone degradation products in the dog and man. *Federation Proc.* **13**: 434.
 8. Evans, F. R. and Curran, H. R. 1943, The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. *J. Bact.* **46**: 513-523.

* Obtained through the courtesy of Dr. T. L. Gresham, B. F. Goodrich Research Center, Brecksville, Ohio.

RESULTS

BPL, in aqueous substrates, at room temperatures and above, undergoes rapid hydrolysis with decrease in pH. The reaction proceeds as follows:



The principal product of hydrolysis is hydracrylic acid with traces of acrylic acid and hydracrylic acid polymer. The rate of hydrolysis or disappearance of BPL is very dependent upon temperature and is greatly increased in the presence of salts, amines, and mercaptans.

Time survivor curves are shown (fig. 1) for the spores of 3 species, at 37 C in skim milk containing 0.3% BPL. Under these conditions, the drug was rapidly sporicidal for each organism. The spores did not die at a uniform rate, although the death rate was essentially linear for 99+% destruction. A few spores

survived the treatment, even after 5 hours, which suggests that they differ in some way from the majority. The period of contact beyond 2 hours had little influence upon the final result,

since by this time hydrolysis of the BPL was practically complete and the products of the reaction—unlike the undegraded compound—have little or no sporicidal activity. The majority of spores of *B. subtilis* were more resistant to BPL than those of *Cl. botulinum* or the ultra-heat-resistant *B. stearothermophilus* (1518). Figure 2 shows survivor curves of the resistant *B. subtilis* (15u) strain treated with BPL in distilled water, in nutrient broth and in skim milk, following preliminary sublethal heating of the spores in the test mediums; the lower spore survival in the nutrient broth as compared with dis-

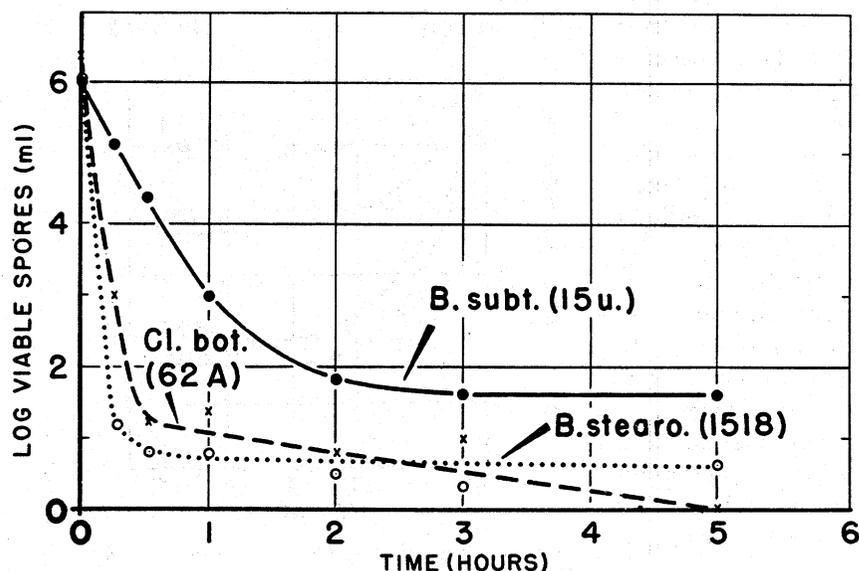


FIG. 1.—The effects of β -propiolactone (0.3%) on bacterial spores in skim milk at 37 C.

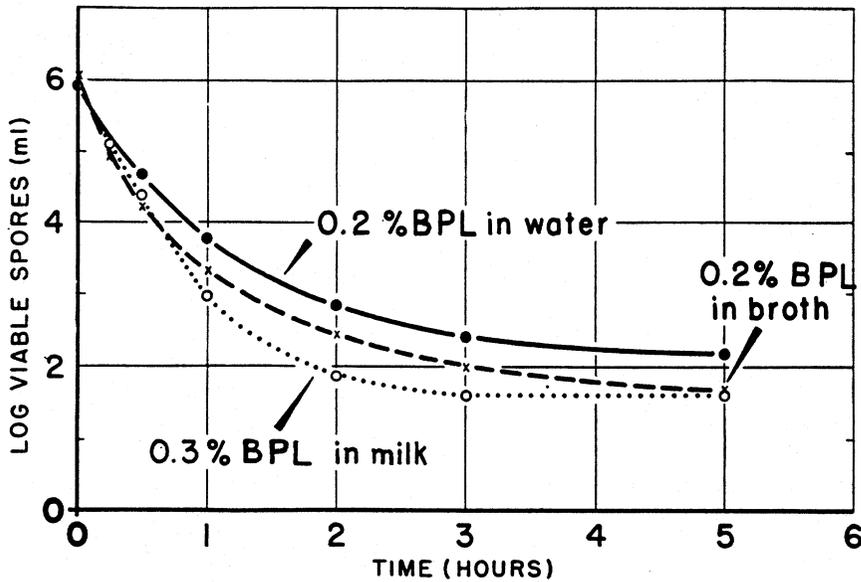


FIG. 2.—Sporicidal activity of β -propiolactone for *B. subtilis* (15u) in distilled water, nutrient broth, and skim milk at 37 C.

tilled water may be attributed, entirely or in part, to the greater heat activation of the spores in the nutrient medium. Skim milk treated with 0.2% BPL, with the pH adjusted to that of untreated milk, supported active growth of *Esch. coli* (223), although multiplication was much less rapid than in the control; the spores of *B. subtilis* (15u) became heat-labile as rapidly in the treated milk as in the control.

In figure 3 is shown the sporicidal activity of BPL in relation to its concentration. Rapid and marked destruction of the spores occurred only at the higher concentration levels. The pronounced effect of temperature upon the activity of BPL is shown (fig. 4). At 3 C, relatively few spores became non-viable over the 5-hour period, whereas, at 25 C, and higher, sporicidal activity was greatly increased, indicating a near-to-normal temperature coefficient. The changes in pH reflect progressive stages in the hydrolysis of BPL (fig. 5); at 3 C, the pH changed but slightly, indicating

that most of the drug remained in the undegraded form, although as already noted, very little sporicidal activity occurred at this temperature. At 25 and 37 C, the pH dropped rapidly, yielding curves not unlike the activity curves for these temperatures. Hydrolysis of BPL was only slightly more rapid at 45

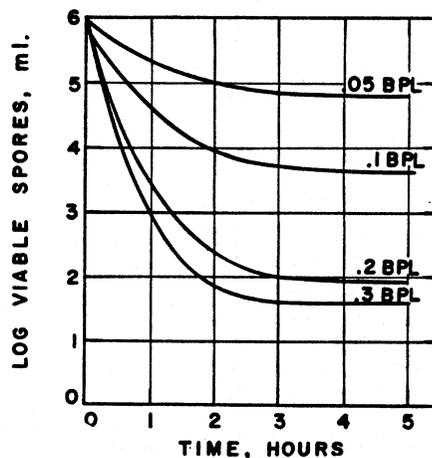


FIG. 3.—The effect of varying concentrations of β -propiolactone on its sporicidal activity for *B. subtilis* (15u) in skim milk at 37 C.

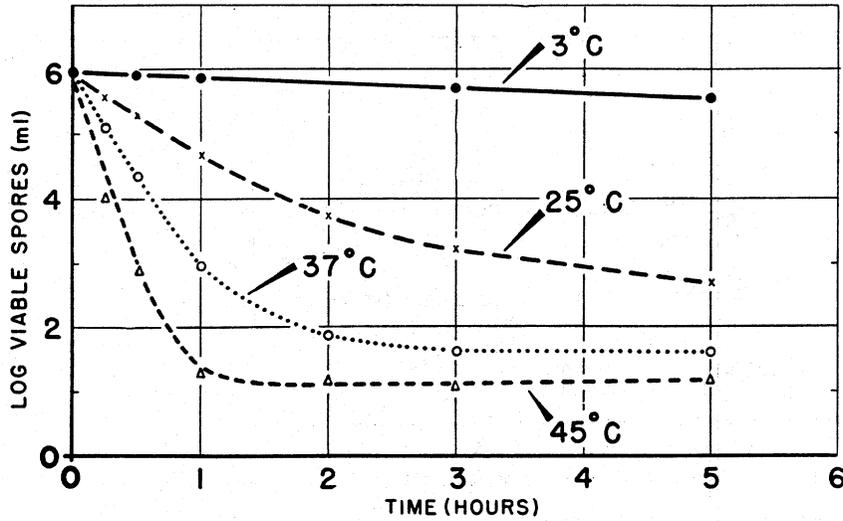


FIG. 4.—The effect of different temperatures upon the sporicidal activity of β -propiolactone (0.3%) for *B. subtilis* (15u) in skim milk.

than 37 C, although the spores died much more rapidly at the higher temperature.

It has been seen that BPL is rapidly sporicidal at 0.2 to 0.4% levels in protein mediums at 25, 37 and 45 C; also that such treatment was not sufficient to achieve sterility in the presence of a

rather heavy seeding of spores (1×10^6 /ml). Higher concentrations of BPL are apparently not practicable for the treatment of milk, since permissible adjustment of the pH is not sufficient to stabilize the milk against the acid of hydrolysis. Nutrient broth is less subject to this limitation and may be steri-

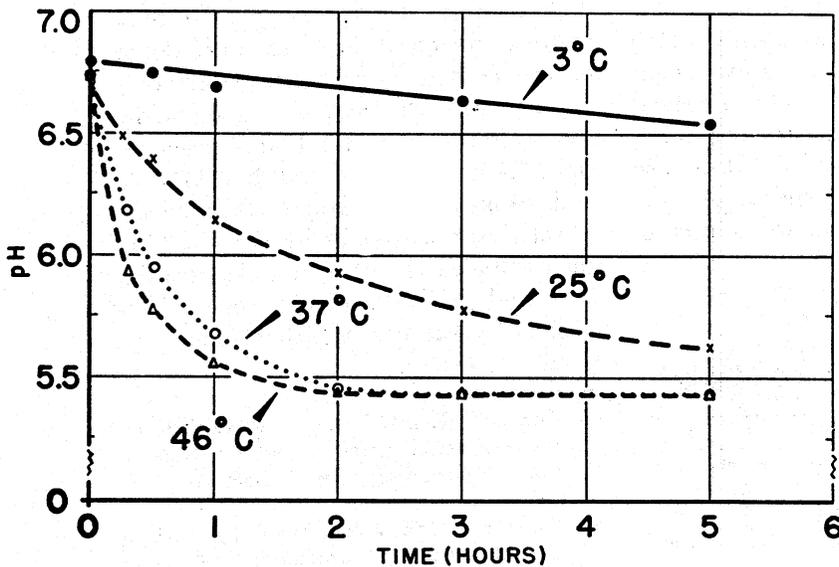


FIG. 5.—The effects of β -propiolactone (0.3%) on the pH of skim milk at different temperatures.

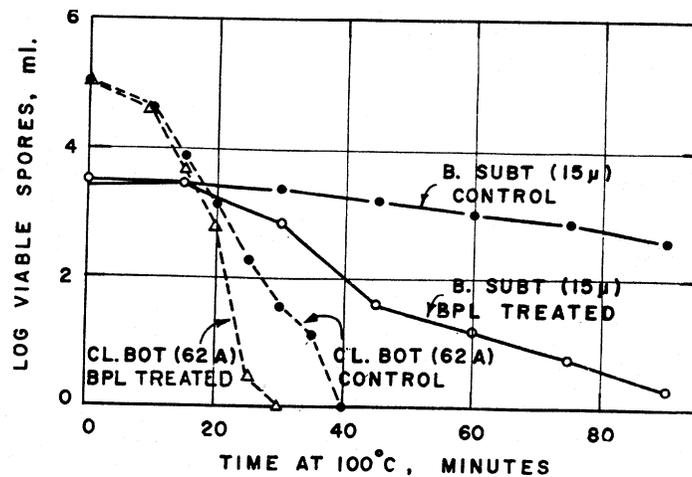


FIG. 6.—The effects of β -propiolactone on the subsequent heat resistance of bacterial spores. Heavy suspensions (76×10^6 /ml) were treated with β -propiolactone, sufficient to reduce the count to 1.5×10^6 /ml. After neutralization, the treated spores, together with untreated control spores were suspended in phosphate buffer at pH 7.0 and heated.

lized by BPL, although relatively high concentrations are required (table 1); a lighter spore load and/or a longer period of contact might be expected to reduce the quantity of BPL required. In this experiment, spore clumps in the inoculum were not broken up before use, and the test suspensions were not activated by heat before chemical treatment.

It is well known that spores which survive a particular destructive agent may be more easily killed by a second quite different lethal influence than are untreated or control spores. It is also recognized that when spores are exposed to the combined action of certain

lethal agents, the sequence of treatment materially affects the number of spores which will survive the treatment.⁹ When spores were treated with BPL and then heated at 100 C (fig. 6), they become nonviable much more rapidly than spores similarly heated without previous exposure to BPL. This effect was evident after a short coming-up-to-temperature period; differences in the slopes of the control curves reflect inherent differences in the heat resistance of the two cultures. Somewhat analogous results have been reported by Smolens and Stokes,¹⁰ who found that virus-infected serums could be sterilized by the combined use of ultraviolet irradiation and BPL—a result not accomplished by the use of either agent, applied separately.

TABLE 1.—Sporicidal activity of β -propiolactone in nutrient broth.

Organism	Viable spores after treatment with β -Propiolactone		
	0.5%	1.0%	1.5%
	per ml	per ml	per ml
<i>B. cereus</i> (401)	0	0	0
<i>B. subtilis</i> (6634)	0	0	0
<i>B. subtilis</i> (6051)	3100	6	0
<i>B. subtilis</i> (15u)	40	0	0
<i>B. stearothermophilus</i> (1518)	1230	1	0
<i>Cl. botulinum</i> (62A)	240	0.3	0
<i>Cl. spp.</i> (3679)	6	0	0

Viable spores before treatment 1×10^8 /ml.
Exposure time 30 minutes, temperature 37 C.

9. Curran, H. R. and Evans, F. R. 1938, Sensitizing bacterial spores to heat by exposing them to ultraviolet light. *J. Bact.* **36**: 455-465.

10. Smolens, J. and Stokes, J. 1954, Combined use of ultraviolet irradiation and beta-propiolactone sterilization of sera infected with a virus. *Proc. Soc. Exper. Biol. & Med.* **86**: 538-539.

DISCUSSION

The present experiments have shown that resistant bacterial spores of varied species may be rapidly killed by BPL in organic as well as inorganic substrates. Published reports have indicated low residual toxicity for animals^{3,4,5,7,13} and apparent minimal change in the constituent proteins.^{5,12,13} In keeping with these findings, we observed that lactone-treated milk, when neutralized, supports active bacterial multiplication and spore germination. The acid formed by the hydrolysis of BPL may preclude its use for the sterilization of certain delicately balanced systems, even with compensatory adjustment of the pH. The effectiveness and apparent utility of BPL for the sterilization of blood and plasma have been indicated,^{2,3,4,7} and there is evidence that this lactone may be used to sterilize arterial homografts without impairing their function;^{11,14,15} in one study the transplants were inoculated with spores (Trafas et al).¹⁴

The activity of BPL against fungi and bacteria led Bernheim and Gale¹⁶

to suggest its use as a skin disinfectant. This application would appear to be unpromising, since the undegraded compound applied to the skin of rats has been reported to produce local sarcomata.¹⁷ The possible use of BPL in the sterilization of surgical instruments might be considered. Perkins¹⁸ has stated that "the commonly used methods of sterilization of surgical instruments in boiling water or immersion in chemical solution are not adequate for the destruction of infectious hepatitis virus in the presence of organic matter." BPL, in sterilizing concentrations, did not corrode test instruments when the period was not prolonged.

The exact mechanism of action of BPL is not known. According to Trafas et al¹⁴ the compound splits on either side of the oxygen ring and attaches to all the free reacting groups of protein materials. In its reaction with wool (a non-aqueous system) BPL attaches to various amino acid side-chain groups, which is followed by an "anchored" polymerization at these sites (Jones and Lundgren).¹⁹ Studies on fungi (Bernheim and Gale¹⁶ and Gale²⁰) suggest that the cell surface or membrane is a primary site of action; this is reflected in the oxygen uptake and deamination of amino acids by intact and by ground cells following treatment.

11. Lo Grippo, G. A., Overhulse, P. R. and Szilagyi, D. E. 1954, Procedure for the sterilization of contaminated arterial grafts with beta-propiolactone. *Bact. Proc.* pp. 61-62.
12. Lo Grippo, G. A. and Hartman, F. W. 1954, Antigenicity of beta-propiolactone inactivated virus. *Federation Proc.* **13**: 503.
13. Basinski, D. H. and Remp, D. G. 1955, Effect of beta-propiolactone on physicochemical properties of some plasma proteins. *Federation Proc.* **14**: 178.
14. Trafas, P. C., Carlson, R. E., Lo Grippo, G. A. and Lam, C. R. 1954, Chemical sterilization of arterial homografts. *Arch. Surg.* **69**: 415-424.
15. Szilagyi, D. E., Overhulse, P. R. and Lo Grippo, G. A. 1954, The use of chemically sterilized human arterial homografts. *Clin. Res. Proc.* **2**: 108.
16. Bernheim, F. and Gale, G. R. 1952, Effect of β -propiolactone on metabolism of *Pseudomonas aeruginosa* and growth of certain fungi. *Proc. Soc. Exper. Biol. & Med.* **80**: 162-164.
17. Walpole, A. L., Roberts, D. C., Rose, F. L., Hendry, J. A. and Homer, R. F. 1954, Cytotoxic agents: IV. The carcinogenic actions of some monofunctional ethyleneimine derivatives. *Brit. J. Pharmacol.* **9**: 306-323.
18. Perkins, J. J., 1954, Antiseptics, disinfectants, fungicides and sterilization. Edited by C. F. Reddish, Lee and Febiger Co., p. 662.
19. Jones, H. W. and Lundgren, H. P. 1951, Modification of wool with beta-propiolactone. Part I: The chemistry of the reaction. *J. Text. Res.* **21**: 620-9.
20. Gale, G. R. 1953, The effects of β -propiolactone on the metabolism of *Blastomyces dermatitides*. *J. Bact.* **65**: 505-508.

SUMMARY

The effects of β -propiolactone (BPL) on the viability of bacterial spores were studied. Under suitable conditions, BPL rapidly killed resistant bacterial spores in water, milk, and nutrient broth; a small proportion of the population was more resistant than the majority and survived low or medium concentrations of the drug. Relatively high concen-

trations of BPL (1.0 to 1.5%) rapidly sterilized nutrient broth containing spores (1×10^6 /ml). Sporicidal activity of BPL was greatly enhanced by increases in temperature from 3 to 45 C. In aqueous substrates at room temperature and above, BPL was rapidly converted into compounds which had no sporicidal activity. Some possible applications of the drug are discussed.