Beta-propiolactone as an agent for enzyme sterilization

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SYNOPSIS A chemical method for the sterilization of pancreatin and trypsin is given. Evidence is presented to show that this treatment does not diminish the specific activity of the enzymes and has some advantages in microbiological techniques.

Beta-propiolactone (BPL) is of value as a sterilizing agent because it causes negligible alteration to the antigenic structure of microorganisms (Sever, Castellano, Pelon, Huebner, and Wolman, 1964), and, since it is rapidly hydrolysed to beta-hydroxypropionic and beta-chloropionic acids, needs no subsequent removal (Peck, 1960). It therefore seemed reasonable to expect that enzyme solutions could also be sterilized by this agent.

Pancreatin and trypsin solutions used in microbiology are usually prepared by an aseptic technique or sterilized by membrane filtration. This investigation was undertaken to see if BPL affected specific enzyme activity and if the hydrolytic breakdown products of BPL resulted in inhibition of microorganisms or tissue cells after they had been treated by the enzymes.

Materials and Methods

Beta-propiolactone (British Drug Houses) was stored at -20°C . Crystalline trypsin (Novo Industries) was stored at $+4^{\circ}\text{C}$. Buffered pancreatin tablets (Oxoid) were stored at room temperature. A glass electrode Beckman pH meter was used for pH estimation. Virus titres were estimated by the method of Reed and Muench (1938) using four tubes per dilution.

Two enzyme solutions in common use are buffered pancreatin/trypsin (P/T) for the liquefaction of sputa, as described by Rawlins (1968), and trypsin 0.25% in balanced salt solution for the disaggregation of cells in tissue culture work. These solutions and a solution of buffered pancreatin (Rawlins, 1955) were prepared in 50 ml amounts. One ml of a 1/15 v/v solution of BPL in cold deionized water was added

to 50 ml of enzyme solution prewarmed to 37°C . The requisite amount of caustic soda determined by preliminary trials was added and the mixture shaken for two minutes at room temperature. The solutions were decanted into fresh sterile bottles to avoid subsequent contamination of the treated solution with untreated enzyme present round the necks of the bottles. Treatment continued for 90 minutes in a 37°C water bath, with shaking at 15-minute intervals. After treatment storage was at 4°C .

Proteolytic Activity

This was estimated by Harrison's modification of Kniskopf's method (Harrison, 1943). Serial twofold dilutions of treated and untreated enzymes were made in normal saline. One drop of each dilution was placed onto a section of exposed photographic film, which was incubated at 37°C for 15 minutes in a humidified chamber. The film was flooded with cold water and enzyme activity was shown by a clearly punched hole in the emulsion. The results are shown in Table I. In numerous trials no differences were detected.

Sterilizing Ability of BPL 1/750 v/v

Five common laboratory contaminants, viz, *Micrococcus* sp., *Alkaligenes faecalis*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and a *Candida* sp. recently isolated from routine cultures, were grown overnight on blood agar. The growth was scraped into quarter strength Ringer's solution and a viable count carried out. Aliquots of each suspension, containing 5×10^8 viable organisms per millilitre, were mixed and 0.5 ml of the mixture added to 50 ml lots of untreated enzyme. To each bottle 0.5 ml of a suspension of *Bacillus subtilis* (NCTC 10073)

Enzyme	Aseptically Prepared		BPL Sterilized				
			Before pH Adjustment		After pH Adjustment		
	pH	Titre	pH	Titre	N/ NaOH (ml)	pH	Titre
Pancreatin	7.5	1/64	5.3	1/32	1.0	7.6	1/64
Trypsin	7.4	1/64	5.0	1/32	1.1	7.6	1/64
Pancreatin/trypsin	7.4	1/128	5.2	1/64	1.05	7.6	1/128

Table I *Proteolytic activity and pH of treated and untreated enzymes*

spores, containing 4.8×10^7 spores per millilitre was also added. Each millilitre of the untreated enzyme solutions therefore contained 1×10^6 of the five vegetative bacterial species and 4.8×10^6 spores. One bottle of each contaminated enzyme was retained as a control while the remainder were treated with BPL. After incubation at 37°C for 24 hours viable counts were made from each bottle on blood agar and Sabouraud's agar plates. No growth of any organism was obtained from any of the treated bottles whereas recovery of the six bacteria from the control bottles was virtually 100%.

Shelf Life at 4°C

Beta-propiolactone-treated and aseptically prepared pancreatin/trypsin were stored in the refrigerator for one month and checked at two-day intervals for sterility and their ability to liquefy purulent sputa. There was no difference in activity, but four out of

six unopened bottles of aseptically prepared pancreatin/trypsin were contaminated after two weeks' storage. All six bottles of BPL-treated pancreatin/trypsin were sterile after four weeks' storage.

Bacterial Inhibition by Breakdown Products

One hundred and ninety two random sputa were liquefied, half with aseptically prepared P/T and half with BPL sterilized pancreatin/trypsin. The organisms isolated from the sputa are shown in Table II. The isolation rates showed no significant differences at any level when tested by the χ^2 distribution.

Tissue and Viral Inhibition by Breakdown Products

Hela and monkey kidney cell monolayers, disaggregated with membrane-filtered trypsin solution and with BPL-sterilized trypsin, were seeded into tubes

Isolation	Mucoid and Salivary Sputum		Mucopurulent Sputum		Purulent Sputum	
	Standard	BPL	Standard	BPL	Standard	BPL
<i>Haemophilus influenzae</i>	5 (8.6%)	2 (3.8%)	4 (12.9%)	6 (18.7%)	3 (17.6%)	5 (19.3%)
<i>Streptococcus pneumoniae</i>	3 (5.1%)	4 (7.5%)	2 (6.5%)	8 (25.0%)	2 (11.8%)	2 (7.6%)
<i>Staphylococcus aureus</i>	2 (3.5%)	1 (1.9%)	5 (16.1%)	1 (3.1%)	2 (11.8%)	3 (11.6%)
<i>Streptococcus pyogenes</i>	—	1 (1.9%)	—	2 (6.3%)	—	—
<i>Coliform</i>	9 (15.5%)	6 (11.3%)	2 (6.5%)	2 (6.3%)	2 (11.8%)	5 (19.3%)
<i>Proteus</i> sp.	3 (5.1%)	4 (7.5%)	3 (9.7%)	1 (3.1%)	—	—
<i>Pseudomonas aeruginosa</i>	—	3 (5.7%)	2 (6.5%)	1 (3.1%)	1 (5.9%)	2 (7.6%)
<i>Candida</i> sp.	9 (15.5%)	6 (11.3%)	5 (16.1%)	3 (9.4%)	3 (17.6%)	5 (19.3%)
Normal flora	27 (46.7%)	26 (49.1%)	8 (25.7%)	8 (25.0%)	4 (23.5%)	4 (15.3%)
Totals	58 (100%)	53 (100%)	31 (100%)	32 (100%)	17 (100%)	26 (100%)

Table II *Isolations from 192 graded sputa treated with standard and BPL-treated pancreatin/trypsin¹*

¹Total isolates more than total sputa due to some multiple isolations.

	Monkey Kidney Cells		Hela Cells	
	Filtered Trypsin	BPL-treated Trypsin	Filtered Trypsin	BPL-treated Trypsin
Time for confluence	4.5 days	4 days	4.5 days	4.5 days
Duration of maintenance	15 days	16.5 days	14 days	14 days
Virus titre after 4 days' growth	4.1×10^4	4.3×10^4	2.5×10^4	2.3×10^4

Table III *Growth of cells and virus with cells treated with filtered and BPL-treated trypsin¹*

¹Each tube seeded with 10^4 cells, virus inoculum 1×10^3 TCID₅₀, each result the mean of two trials.

at identical levels. Growth medium was added and stationary incubation carried out at 37°C. Time taken for the monolayer to reach confluence, duration of maintenance, and ability to support the growth of influenza virus A2/Hong Kong/68 variant were compared. No significant differences were observed, as shown in Table III.

Discussion

The concentration of BPL chosen was 1/750 v/v as being halfway between the 1/1,000 v/v concentration used for virus vaccines and the 1/500 concentration used for preparation of inactivated bacterial antigens. This is a simple technique requiring few facilities and ensures sterility where moderate levels of contamination are involved. There is clear evidence that bacterial growth is not inhibited and more limited evidence that tissue and viral growth is unaffected. The effect of BPL on other enzymes, active at different pH levels, was not investigated. The two clear advantages that emerge are certain sterility, even in the hands of inexperienced technicians, due to the lack of aseptic dispensing at any stage, and the resulting prolonged storage life. No attempt has been made to weigh any other advantages or disadvantages of this method over filtration or aseptic preparation, as these may vary according to the circumstances of each particular laboratory. These results do suggest that a wider application may be possible.

Beta-propiolactone irritates the eyes, can be a

vesicant on the skin, and is carcinogenic in rats and mice (Roe and Salaman, 1958; Dickens and Jones, 1961). The last two workers investigating the carcinogenic activity of lactones and related structures in rats found that penicillin G was also productive of transplantable neoplasms, though less active than beta-propiolactone. It is therefore recommended that suitable precautions be observed with the pure liquid, and that manipulations should be made with due care.

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