Toxic Factors in Enzymes Used in Laundry Products

Abstract. *Autolyzates of* Bacillus subtilis *used as enzymes in laundry products contain several potentially toxic substances.*

Enzyme preparations have been widely used for laundering purposes, either in the form of presoaks or mixed with detergents. According to information released by the manufacturers, these preparations are derived from cultures of *Bacillus subtilis*, and consist essentially of bacterial autolyzates which have been concentrated but not significantly purified. In addition to a multiplicity of different enzymes, these autolyzates contain various cellular constituents and metabolic products of the bacterial culture used for their preparation.

As could have been predicted, exposure to such crude bacterial products has elicited a variety of allergic reactions ranging from dermatitis to respiratory ailments (*1*). Such reactions have been particularly numerous and violent among factory workers engaged in the handling and packaging of products containing the so-called enzymes. But it can be expected that they will increase in severity and prevalence among the general public if the use of enzyme products becomes more widespread. The present study, however, is not concerned with this aspect of the problem. It is focused rather on the fact that certain of the bacterial constituents and metabolites which accompany the enzymes have biological activities that render them potentially dangerous.

The tests reported below were carried out with 22 different enzyme preparations, free of detergents; 19 of them had been obtained from the manufacturers through the courtesy of Dr. David Steinman of the Federal Trade Commission; the other three were presoak preparations bought on the open market, two in New York and one in Switzerland. Five different kinds of tests were carried out on most of these preparations.

1 and 2) Hemolytic and hemagglutinating activity was determined in vitro against sheep erythrocytes resuspended in barbital buffer at *p*H 7.0, with or without 0.5 percent bovine serum albumin.

3) Antibacterial activity was tested in vitro against various strains of staphylococci, enterococci, and coliform bacilli, both in nutrient broth and on agar media.

4) Toxic activity in vivo was determined by injecting into the peritoneal cavity of specific pathogen-free mice various amounts of enzyme (from 0.2 to 2000 μg) resuspended in 0.2 ml of mixed phosphate buffer at *p*H 7.0. The animals were weighed at 10 a.m. for several consecutive days before and after injection of the material.

5) The effect of the enzyme preparations on susceptibility to infection was tested by infecting mice with 0.2 $\times 10^{-4}$ ml of broth culture of *Klebsiella pneumoniae* at 10 a.m. At various intervals of time before or after infection, the animals received various amounts of enzyme preparation as indicated in the preceding paragraph.

The results (Tables 1 and 2) can be summarized as follows. Many of the preparations tested, but not all, exhibited one or several of the following activities: hemolysis, hemagglutination, inhibition of bacterial growth. When present, these activities were not decreased by heating the enzyme preparation at 100°C or by adding serum albumin to the test system. Antibacterial activity was the strongest against staphylococci, weaker against enterococci and coliform bacilli. Inhibition of bacterial growth on agar media was less pronounced than in liquid media.

The hemolytic and antibacterial activities were associated with the soluble fractions of the enzymatic materials, whether heated or unheated. In contrast, the hemagglutinating activity was removed by filtration or high-speed centrifugation, but could be recovered in the particulate matter thus separated from the materials.

In the test systems used, these activities in vitro could be detected with concentrations of the unheated, uncleared materials ranging from 0.01 to 0.1 percent. Since the activity persisted after heat coagulation of the material at boiling temperature, and removal of the precipitate by centrifugation and filtration, it follows that the active material is effective at much smaller concentrations than these figures indicate.

Several of the preparations caused death of a large percentage of animals within a few hours when injected into the peritoneal cavity in doses exceeding 0.2 mg, but the results were not sufficiently consistent to justify calculation of the LD_{50}.

At smaller doses, most preparations caused a marked loss of weight of the animals, usually within less than 24 hours after injection. The loss was of the order of 1 g per 20 g mouse and could be produced by injecting 0.2 mg of the untreated enzyme preparation; smaller doses (0.02 mg or less) also had a weight-depressing effect. All animals regained their initial body weight within 48 hours after injection.

At doses of 0.1 mg per mouse, all preparations aggravated infection with *Klebsiella pneumoniae*, as measured by the time of death after infection. In fact, aggravation of infection was detected even with doses as small as 0.002 mg (2 μg) or even smaller.

With regard to both weight depression and enhancement of infection, the material retained all its activity after heating at boiling temperature and after removing the insoluble material by centrifugation and filtration.

Initial tests have shown that enhancement of staphylococcus infection can be achieved with doses of enzyme preparation—either unheated and unfiltered, or heated and filtered—of the same order as those that aggravate infection with *Klebsiella pneumoniae*.

The various biological activities in vitro and in vivo differed quantitatively from one enzymatic preparation to the other. Furthermore, their association appeared to be random; some prep-

Table 1. Comparative in vitro activities of eight different enzyme preparations.

Preparation*	Hemolysis (supernatant)	Hemagglutination (precipitate)	Antibacterial activity		
			Staphylococci	Enterococci	Coliform
A	No	Yes	+	+	−
B	No	No	+++++	++++	++
C	Yes	No	++++	+++++	−
D	Yes	No	+++++	+++++	+
E	No	No	−	−	−
F	No	Yes	−	−	−
G	No	Yes	+++	+++	−
H	No	No	−	−	−

* Results obtained with 0.03 percent concentration of enzyme preparations.

Table 2. Infection-enhancing effect of an enzyme preparation. The enzyme was injected intraperitoneally 6 hours after infection with 0.2×10^{-4} ml of *Klebsiella* culture.

Enzyme injected (μg)	Percentage of deaths (cumulative) at indicated days after infection*			
	1	2	3	4
400	24	49	93	100
40	13	37	68	91
4	12	23	28	31
0	0	0	0	7

* The total numbers of animals (from 12 different experiments) exceeded 100 for each group.

arations were hemagglutinating but not hemolytic or antibacterial, and vice versa. Some preparations did not exhibit any in vitro activity in our tests, even though they were extremely active in depressing the body weight of mice and in aggravating infections. There seemed to be an association between the latter two activities.

Table 1 presents results with eight different enzyme preparations, selected to illustrate the wide range of differences in their activities in vitro.

The experiments dealing with the enhancement of *Klebsiella* infection in mice have been carried out under a wide range of conditions with each of the enzyme preparations available. Table 2 includes the results of 12 different tests with one single preparation over a period of 8 months.

Conclusions. All preparations of *Bacillus subttilis* autolyzates (so-called enzymes) tested were capable of causing a rapid loss of body weight when injected by the peritoneal route into specific pathogen-free mice; they also aggravated experimental infection with *Klebsiella pneumoniae* and staphylococcus. Some of the preparations, but not all, caused hemagglutination, hemolysis, and other cytotoxic effects. These various in vivo and in vitro activities differed qualitatively and quantitatively from preparation to preparation.

In all cases, the in vivo and in vitro activities persisted unaltered after the enzyme preparation had been heated at boiling temperature for 15 minutes, then clarified by centrifugation and filtration.

Although no attempt has been made to compare the immunological activities of the enzymatic preparations, the present findings may have a bearing on this problem. The profound differences observed among the various preparations probably have their origin in the characteristics of the bacterial strains of *Bacillus subtilis* from which they were prepared. It is not unlikely therefore that the various preparations also differ in immunological specificity and sensitizing ability—a fact which would greatly complicate the formulation of safety controls in their manufacture and use.

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Reference

1. L. M. Lichtenstein *et al.*, *J. Allergy* **47**, 53 (1971).

24 May 1971

Antiviral Activity of Polyribocytidylic Acid in Cells Primed with Polyriboinosinic Acid

Abstract. *Separate administration of polyribocytidylic acid [poly(rC)] and polyriboinosinic acid [poly(rI)] to cell cultures in vitro resulted in an antiviral activity identical to or greater than that resulting from addition of the poly(rI) • poly(rC) complex. Priming of cells with poly(rI), followed by treatment with poly(rC), gave a consistently greater antiviral activity than poly(rI) • poly(rC) itself. This priming effect was obtained in several cell cultures challenged with different viruses. In vivo, the antiviral activity of poly(rI) • poly(rC) was only partially restored if poly(rI) and poly(rC) were injected separately; prior injection of poly(rI) proved superior in restoring this antiviral activity as compared to prior injection of poly(rC).*

Homopolynucleotide complexes such as poly(rI) • poly(rC) (homopolymer pair of polyriboinosinic acid and polyribocytidylic acid) are highly active in inducing interferon and cellular resistance to virus infection, both in vitro and in vivo, as originally described by Field *et al.* (*1*). The antiviral activity of poly(rI) • poly(rC) and other polynucleotide complexes has been related to their stable, highly ordered, double helical structure and to their resistance against premature enzymatic degradation (*2–5*). In these and several other studies on the antiviral and antitumor activity, and toxic properties of poly(rI) • poly(rC), the two constituent homopolymers were always administered in the form of the double-stranded complex but never separately, one after the other. In a study on the toxicity of poly(rI) • poly(rC) in adrenalectomized rats, separate injection of the individual homopolymers in rapid succession proved as lethal as injection of the complex (*6*).

Separate administration of poly(rC) and poly(rI) to cell cultures in vitro resulted in an identical or greater antiviral activity than addition of the poly(rI) • poly(rC) complex itself. These findings may open new avenues in deciphering the mechanism by which (double-stranded) polynucleotide complexes trigger the production of interferon and other host cell responses.

The homopolynucleotides poly(rI) and poly(rC) (*7*) were dissolved in phosphate-buffered saline (PBS) and stored at −20°C. Concentrations of the polymers were determined spectrophotometrically (*2*). The homopolymer pair poly(rI) • poly(rC) was prepared by annealing the individual homopolymers (*2*). Before use, poly(rI), poly(rC), and poly(rI) • poly(rC) were diluted in Eagle's minimum essential medium (MEM) to the appropriate concentrations. As described previously (*4*), poly(rI) • poly(rC) was heated at 37°C in MEM before exposure to the cells.

Cellular resistance to bovine vesicular stomatitis virus (VSV) (Indiana strain) was determined in seven different cell cultures [five continuous cell lines: HSF (human skin fibroblasts), RK 13 (rabbit kidney) cells, mouse L 929 cells, HeLa cells, HK (human kidney) cells; and in two primary cell cultures: MEF (mouse embryo fibroblasts), PRK (primary rabbit kidney) cells] by inhibition of virus plaque formation or viral cytopathogenicity. Cellular resistance to vaccinia virus was determined in RK 13 cells by inhibition of virus plaque formation. Twenty-four hours after exposure of the cell cultures to the polymers (the second polymer, if the individual homopolymers were administered in succession), virus was added, and virus plaque formation (or cytopathogenicity) was recorded 2 to 3 days later. Interferon production was measured in vivo (young NMRI mice, 12 to 14 g) after intravenous or intraperitoneal injection of the polymers; serum interferon titers were determined in a plaque inhibition