# Reactivation of Papain-Treated Endotoxin. (30112)

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(Introduced by John J. Munoz)
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It has been reported that purified endotoxin from *Salmonella enteritidis* containing only 0.2% nitrogen, all of which was accounted for as hexosamine nitrogen, was as potent as endotoxins containing large portions of protein or peptide components(1). Kim and Watson(2) demonstrated that the pyrogenicity of this endotoxin could be diminished by incubation with papain. This inactivation was interpreted either as being a result of hydrolytic action (proteolytic or peptidolytic) upon a minute protein or peptide constituent of the endotoxin, or as due to an esterase activity of papain upon long-chain fatty acids retained in the preparation. However, the exact nature of the papain-endotoxin interaction remained obscure.

Recently, Rudbach and Johnson(3) described a method by which the biological activity of Boivin-type endotoxin from *Salmonella typhosa,* diminished as a result of incubation with human plasma, could be restored by treatment of the inactive reaction mixture with a proteolytic enzyme from *Streptomyces griseus* (pronase) followed by precipitation with ethanol. Such results were interpreted as indicating a binding of the endotoxin by a plasma protein. In view of this finding, together with the consistent lack of correlation between potency of endotoxins and content of proteins, peptides, or fatty acids (1), we wished to examine whether or not the activity of a purified endotoxin, diminished by treatment with papain, could be restored by treatment with pronase and ethanol.

*Materials and methods. Endotoxins.* An aluminum citrate-endotoxin complex(4) of a highly refined endotoxin from *Salmonella enteritidis* and a conventional aqueous ether extract from whole cells of the same organism were used in this study. These preparations have been more fully described by Ribi *et al*(1).

*Inactivation of endotoxin by papain.* The protocol outlined by Kim and Watson(2) was employed as follows. The endotoxin (10 mg) was dissolved in distilled water (1 ml) and heated in a boiling water bath for 2 minutes. To this was added 9 ml of phosphate buffered saline (0.05 *M* phosphate and 0.1 *M* NaCl at pH 7.0). One ml of this endotoxin solution (containing 1 mg) was added to 9 ml of reduced buffer (1 ml of 0.4 *M* acetate, pH 4.0; 1 ml of 1.5 *M* NaCl; 1 ml of 0.05 *M* L-cysteine-HCl; 1 ml of 0.01 *M* tetrasodium ethylenediamine tetraacetic acid (EDTA); 5 ml of distilled water) followed by 0.1 ml of papain (twice recrystallized, Nutritional Biochemicals Corp., Lot 5261 containing 20 mg of protein per ml). Control solutions were prepared as follows: 1 ml of endotoxin in 9 ml of reduced buffer, plus 0.1 ml water; 1 ml of endotoxin in 9 ml of reduced buffer, which lacked EDTA, plus 0.1 ml papain; and 1 ml water in 9 ml reduced buffer, plus 0.1 ml papain. All samples were incubated at 65°C for 16 hours, and then immersed in a boiling water bath for 2 minutes to inactivate the residual papain. The acetate buffer was then neutralized with sodium hydroxide, and 0.1 ml of 1% merthiolate was added to each tube.

*Restoration of papain-inactivated endotoxin.* Samples of papain-inactivated endotoxin, the endotoxin control in the reduced buffer, and the endotoxin which had been incubated with papain in the absence of EDTA were stored at 4°C. A 10 ml sample of the papain-inactivated endotoxin and a 10 ml sample of the papain which had been incubated in reduced buffer were carried through the procedure of Rudbach and Johnson(3) for restoration of endotoxic activity. Briefly, 4 mg of pronase (Pronase P, B grade, Cali-

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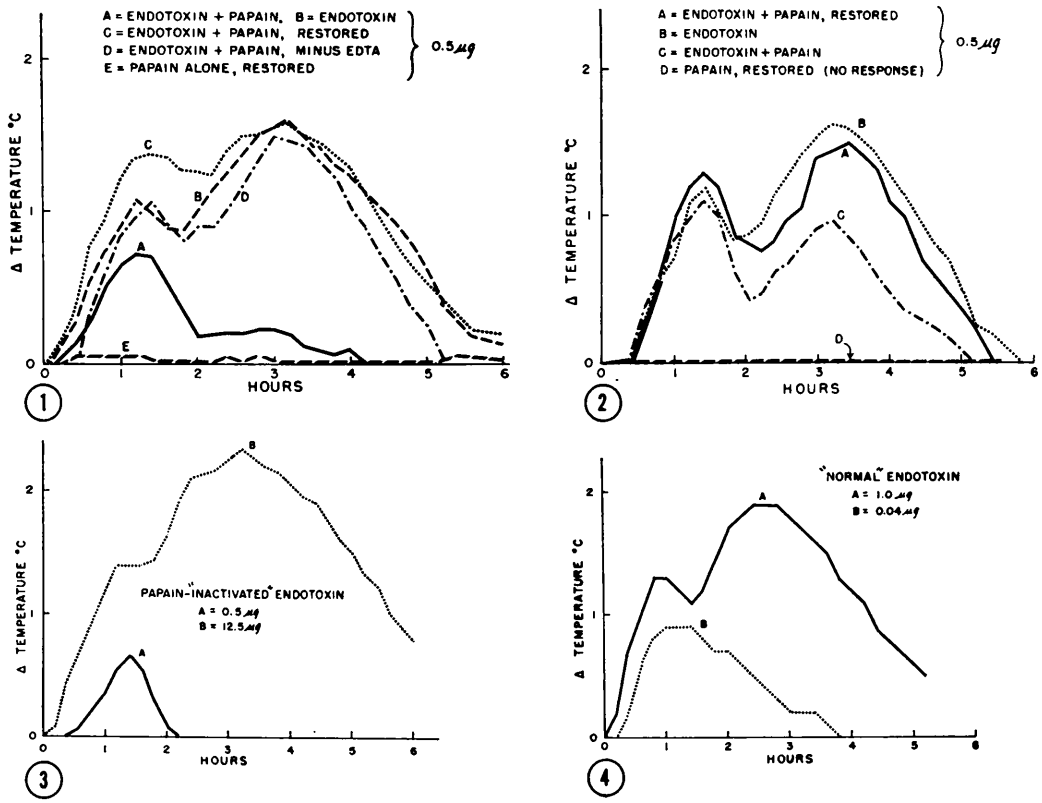


FIG. 1. Pyrogenic responses of rabbits to similar dilutions of papain-inactivated peptide-free endotoxin, papain-inactivated endotoxin restored with pronase and ethanol, and control solutions. Curves A, B, C and E represent average temperature response of 6 rabbits. Curve D is average response of 3 rabbits.

FIG. 2. Pyrogenic responses of rabbits to similar dilutions of papain-inactivated conventional aqueous ether endotoxin and control solutions. Each curve represents average temperature response of 3 rabbits.

FIG. 3. Pyrogenic responses of rabbits to 0.5 μg and 12.5 μg of papain "inactivated" peptide-free endotoxin. Each curve represents average temperature response of 3 rabbits.

FIG. 4. Pyrogenic responses of rabbits to 0.04 and 1.0 μg of "normal" peptide-free endotoxin. Each curve represents average temperature response of 3 rabbits.

fornia Biochemical Corp., Lot #109080) were added, and the samples were incubated at 37°C for 18 hours. The digests were then precipitated with 6 volumes of absolute ethanol, and the precipitates were collected and reconstituted to 10 ml with water.

*Pyrogenicity determinations.* A description of the method has been published(5). The representative fever curves reported in Fig. 1-4 each represents the average temperature responses of 3 to 6 rabbits to the amount of material indicated in the text.

*Results.* Kim and Watson(2) showed that papain would mitigate the pyrogenicity of endotoxin when the incubation was performed either at pH 4.0 or 7.0, but greater inactivation occurred at pH 4.0. However, a single attempt to inactivate endotoxin by incubation with papain at pH 7.0 was unsuccessful; therefore, all subsequent incubations were performed at pH 4.0. The fever curves in Fig. 1 demonstrate that papain, in the presence of cysteine and EDTA, could diminish the pyrogenicity of endotoxin, primarily by abolition of the second fever peak (Fig. 1, curve A). That the papain was causing the inactivation was demonstrated by a typical febrile response to endotoxin incubated in reduced buffer without papain (Fig. 1, curve B). A second control showed that omitting EDTA resulted in a mixture which was unable to inactivate endotoxin (Fig. 1, curve

D). Fig. 1, curve C shows that a quantitatively and qualitatively normal pyrogenic response could be obtained from papain-inactivated endotoxin which was subjected to the pronase and ethanol treatment. The lack of pyrogenicity in the negative control of the incubation mixture containing papain but no endotoxin and carried through the incubation and restoration procedures shows that the pyrogenicity which was restored to the papain-inactivated endotoxin was indeed due to reactivated endotoxin and not to the reagents employed for the restoration (Fig. 1, curve E, no response).

In our laboratory it was noted that different lots of papain were unequal in their capacity to reduce the pyrogenicity of endotoxin. The effectiveness of several preparations of papain varied from almost complete reduction of the pyrogenic properties of endotoxin to no observable effect. Similarly, a papain which markedly diminished the pyrogenicity of one endotoxin, such as that shown in Fig. 1, was relatively inactive on another endotoxin (Fig. 2). In that latter case, unequivocal reactivation of the papain-treated endotoxin is more difficult to demonstrate (Fig. 2, curve A), owing to the lesser difference in magnitude between the pyrogenic response elicited by the endotoxin control (Fig. 2, curve B) and that of the papain-treated endotoxin (Fig. 2, curve C). However, reactivation of the pyrogenicity of the papain-treated endotoxin is evinced.

The demonstration that the pyrogenicity of papain-inactivated endotoxin could be restored, contradicted the theory that a hypersensitivity factor, enhancing the 3-hour peak of a biphasic fever curve, had been separated from that portion of the endotoxin which was assumed to be responsible for the primary toxicity (the early fever peak). However, it remained to be shown whether or not an allergenic group, putatively responsible for enhancement of the second fever peak, might be specifically blocked by a presumptive papain-endotoxin complex. Fig. 3 shows the result of a 25-fold increase in the dose of papain-inactivated endotoxin given to rabbits. This response suggested that the monophasic fever curve of papain-inactivated endotoxin was due to a general reduction of pyrogenicity of the molecule and was only a manifestation of the fever response to a low dose of endotoxin. The fever curves in Fig. 4 show that such could indeed be the case. An endotoxin preparation eliciting a typical biphasic fever at 1.0 $\mu$g (Fig. 4, curve A), when decreased to one-twenty-fifth that amount (0.04 $\mu$g), induced a monophasic febrile response in rabbits (Fig. 4, curve B) which resembled that given by papain-inactivated endotoxin. Similar curves have been published by Watson and Kim(6).

*Discussion.* If the inactivation of the endotoxin by papain was due to hydrolytic cleavage of a portion of the molecule by a peptidase or esterase activity, then effective reactivation would be unlikely. However, if the papain was binding to the endotoxin with concomitant blocking of portions of the molecule which were responsible for biological activity, then removal of the papain should result in restoration of the endotoxic activity. The data presented here support the latter hypothesis. The papain-inactivated endotoxin could be restored to apparently normal pyrogenicity by procedures previously shown to restore biological activity to endotoxin which had been inactivated by human plasma(3). Whether pronase reactivates the endotoxin by proteolytic action or by an ionic displacement (7) is not known.

An explanation is also offered for the apparent abolition of only the second fever peak in papain-inactivated endotoxin, based on the response of rabbits to varying doses of endotoxin. It was shown that the second fever peak could be regained when the dose of papain-inactivated endotoxin was increased 25-fold. Furthermore, the "normal" endotoxin, which induced a biphasic febrile response, produced only a monophasic response when the dose was correspondingly reduced. Thus, the apparent abolition of the second fever peak in the papain-inactivated endotoxin could be explained as a dose-response phenomenon, resulting from a general reduction of the pyrogenicity of the preparation rather than a specific destruction or blocking of a polypeptide.

The inactivation of a purified *S. enteritidis*

endotoxin by papain does not appear, from these findings, to conflict with previous observations on the lack of correlation of biological potency of the endotoxin with its content of protein, peptide, or fatty acid. However, all endotoxins may not be affected by papain in a like manner; therefore we do not attempt to generalize beyond the data presented here.

*Summary.* The pyrogenicity of endotoxin which had been diminished by incubation with papain was restored by treatment of the inactive complex with pronase and ethanol, indicating that a hydrolytic cleavage of the endotoxin molecule had not occurred. In addition, it was shown that the reduction of the second fever peak in papain-inactivated endotoxin could be explained on the basis of a normal dose-response phenomenon. These findings suggest that there is no disagreement between the inactivation of a purified endotoxin by papain and the observed correlation of the biological activity of endotoxin with the polysaccharide portion of the molecule.

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## Vinblastine Effect on Nucleic Acids and Ribonuclease of Lymphoid Tissue.* (30113)

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Vinblastine, one of the periwinkle alkaloids, has found limited clinical usefulness in the management of certain tumors. Studies on the mechanism of action of the drug have revealed that it inhibits mitosis in early metaphase in tissues studied(1,2,3). Biochemical studies do not reveal any effect of the agent on cellular respiration, glycolysis, protein or nucleic acid synthesis(4). Beer(5) investigated the effect of the drug on the incorporation of $^{14}C$-labeled formate into the nucleic acids of the liver of intact and partially hepatectomized rats. In both cases he found that vinblastine treated animals incorporated more of the label into RNA than did controls. Enhanced liver RNA synthesis has also been obtained with corticosteroids by Lowe(6), and by Feigelson *et al*(7). These studies, and the fact that vinblastine is clinically most useful in the management of tumors of lymphoid origin make it desirable to know the influence of the drug on RNA and ribonuclease activity in normal and neoplastic lymphoid tissue, parameters which we have recently shown to be affected by corticosteroids(8,9).

*Materials and methods.* Sprague-Dawley rats weighing approximately 125 g and obtained from Camm Research Institute were injected subcutaneously with 1.0 mg vinblastine sulfate (Velban†) and sacrificed by decapitation 22 hour later. The thymus was removed and assayed for RNA and DNA content by the methods of Ceriotti(10) and Seibert(11), respectively. RNase in 10% thymus homogenates was assayed at pH 5.9 by a method previously reported by us(8).

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