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Enhancement of Lysozyme Activity by Anodal Tear Protein* (33951)

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The anodal protein of human tears is a major constituent of the lacrimal secretion. This protein migrates as a "prealbumin" in starch or acrylamide gel electrophoresis and has an s_{w20} value of 2.0 in the analytic ultracentrifuge (1). Human anodal tear protein is antigenic (2) and specific antiserum has been prepared in rabbits. This serum shows no reactivity with any of the protein components of saliva, spinal fluid, urine, or serum when tested by agar diffusion techniques (1). Because lysozyme, a basic protein, is also present in tears, it was postulated that the anodal tear protein is important in either the secretion or action of lysozyme. The enhancement of the action of lysozyme in the lysis of *Micrococcus lysodeikticus* in dilute salt solution by the anodal tear protein is here described.

Methods. Rabbit lacrimal secretions were obtained by placing sterile Schirmer tear test strips in the rabbit conjunctival sac. The papers were permitted to become saturated with conjunctival fluid over a 5-min period. The lacimal protein from two strips was eluted with 0.25 ml of an 0.9% NaCl solution buffered at pH 7.2 with 0.05 *M* phosphate buffer and 100 to 200 such eluates were pooled. After 10-fold concentration by dialysis against polyvinylpyrrolidone, the tear eluates were subjected to Sephadex G200 gel filtration in phosphate buffered saline, pH 7.2. The final peak of the gel filtrate contained lysozyme and the anodal protein, and was concentrated again and dialyzed against phosphate buffer, 0.01 *M* pH 6.7. This protein was passed through a carboxymethyl cellulose column equilibrated with the same buffer. The "fall-through" peak was collected, dialyzed against distilled water and lyophilized. The protein thus obtained was homogeneous by acrylamide gel electrophoresis and had migratory properties in acrylamide gel and immunoelectrophoresis similar to those of human anodal tear protein. Equilibrium ultracentrifugation of rabbit anodal tear protein indicated a molecular weight of 23,000. Assays of lysozyme activity were carried out in a Cary spectrophotometer by the continuous recording of the decrease in optical density of a 0.3 mg/ml suspension of *M. lysodeikticus* (3). The suspension was prepared in 0.01 or 0.1 *M* phosphate buffer, pH 7.0. To a 2.9-ml aliquot of the bacterial suspension was added either 0.1 ml of a solution of rabbit anodal tear protein in distilled water at a concentration of 5 mg/ml or 0.1 ml of distilled water. After a base line reading to

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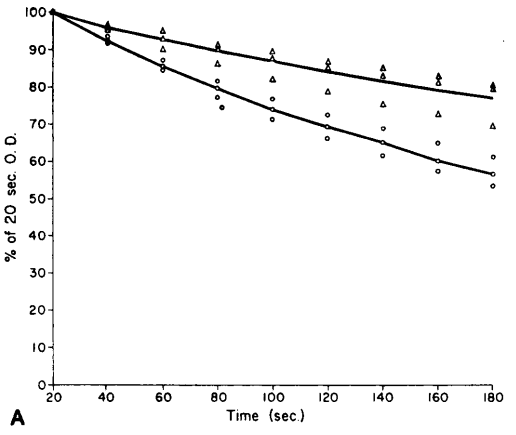
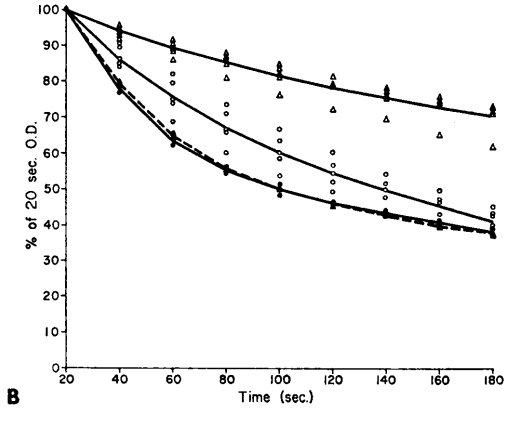


FIG. 1. Clearing of *M. lysodeikticus* suspension: (A), 0.1 ml of a 0.025% solution of egg white lysozyme added to a suspension of bacteria in 0.01 *M* phosphate buffer; (○), prior additon of 0.1 ml of a solution of 5 mg/ml of rabbit anodal tear protein) (Δ), prior addition of distilled water. (B), The same as A except that human lysozyme was used; (open symbols), results in a 0.01 *M* phosphate buffer; (closed symbols), results in a 0.1 *M* phosphate buffer.

exclude lytic activity associated with the tear protein, 0.1 ml of a solution of egg white or human lysozyme was added to the cuvette. The solutions were mixed thoroughly and the optical density of the mixture at 450 mμ was followed for 3 min. Optical densities were expressed as a percentage of the value at 20 sec in order to reduce the errors inherent in the mixing procedure.

Results. Lysis of *M. lysodeikticus* by both egg white and human lysozyme was enhanced by the prior addition of rabbit anodal tear protein. When egg white lysozyme was used (Fig. 1A) prior addition of the anodal tear protein caused a 63% increase in the initial rate of clearing of the bacterial suspension. A 129% increase in the activity of human lysozyme was achieved (Fig. 1B). This enhancement was noted when phosphate buffer in a concentration of 0.01 *M* was employed as the solvent. When 0.1 *M* phosphate buffer was used, enhancement of lysis was not achieved but the activity of lysozyme was greater than in 0.01 *M* phosphate buffer (Fig. 1B). It was postulated that the adjuvant effect of the anodal tear protein was exerted by its electrostatic interaction with the bacteria. Lysozyme, which carries a positive charge at pH 7.0, is unable to interact efficiently with the bacteria, also positively charged, in a suspending medium of low ionic strength. The anodal tear protein, negatively charged at pH 7.0, interacts with bacteria and tends to neutralize their positive surface charge. A similar mechanism has been invoked to explain enhancement of the activity of lysozyme on bacterial cell walls chemically treated in order to introduce carboxyl groups (4). To test this postulate, use was made of egg white lysozyme which had been reacted with the nitro-olefin, 2-nitro-2-butene. The reaction of egg white lysozyme with 2-nitro-2-butene at pH 9, and subsequent purification of the reacted protein by Sephadex G75 gel filtration yielded a product which retained bacteriolytic activity at pH 7.2 in 0.01 *M* phosphate buffer despite an alteration in net charge. This alteration in charge was indicated by a more anodal migration than native egg white lysozyme in cellulose acetate electrophoresis (Fig. 2). If charge neutralization were responsible for the action of anodal tear protein, this lacrimal protein should have little or no effect on the action of lysozyme pretreated with nitro-olefin. The lytic activity of this modified lysozyme was not enhanced by prior addition of anodal tear protein to the *M. lysodeikticus* suspension.



Another acidic protein, human serum albumin, was tested for its ability to enhance

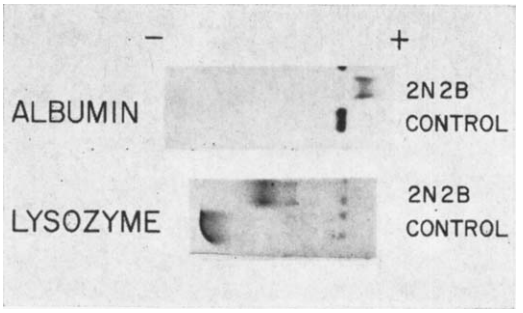


FIG. 2. Cellulose acetate electrophoresis in alkaline buffer of nitro-olefin-treated albumin and lysozyme, labeled 2N2B, and untreated protein, labeled control. Both lysozyme and egg albumin exhibit a more anodal migration after treatment with 2-nitro-2-butene at pH 9.0.

lysozyme activity. The assays were performed in the same manner as with the anodal tear protein, except that 0.1 ml of a 15 mg/ml solution of 4 times recrystallized human serum albumin was added to the suspension of *M. lysodeikticus*. Enhancement of human lysozyme activity was not achieved by prior addition of albumin. Other studies were performed with albumin which had been treated with 2-nitro-2-butene. This treatment rendered the protein more acidic than native human serum albumin (Fig. 2). Nitro-olefin-treated serum albumin also failed to enhance the activity of human lysozyme.

Anodal tear protein has enhanced the lysis of *M. lysodeikticus* by human and egg white lysozyme in dilute salt solutions. This enhancement, although probably related to net charge must be dependent on other specific characteristics of the protein molecule. In studying the activity of an enzymatic protein such as lysozyme, and indeed the activity of all proteins, whatever their physiologic function, the *milieu* in which this action occurs must be considered. Alterations in measured activity of lysozyme in various body fluids may results from alterations in composition or concentration of other proteins in these fluids.

Summary. Anodal tear protein is a constituent of human and rabbit conjunctival fluid. This protein has not been identified in other body fluids. Rabbit anodal tear protein enhances the lysis of *M. lysodeikticus* by human and egg white lysozyme. Neither human serum albumin nor an acidic derivative of serum albumin enhanced the activity of lysozyme. The enhancing action of the acidic tear protein was noted when the assay was performed in 0.01 *M* phosphate buffer, pH 7.0, but was not noted when 0.1 *M* phosphate buffer was used.

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