Production of Neuraminidase by L Forms of *Vibrio cholerae*. (26751)

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Receptor-destroying enzyme (RDE) catalyzes the release of various neuraminic acids from glycoproteins and is therefore a neuraminidase(1). Filtrates with this activity, or the related property of inhibition of hemagglutination by myxoviruses, have been obtained from cultures of *Vibrio cholerae*(2), *Diplococcus pneumoniae*(3), *Clostridium perfringens*(4), and other bacteria(5). An enzyme with similar activity is an integral part of the influenza virus particle(6).

The neuraminidases of *V. cholerae*, *D. pneumoniae*, and influenza PR-8 virus, although possessing similar catalytic specificity, are serologically distinct(7). The present paper reports that the L form of *Vibrio cholerae* also produces a neuraminidase, which is inhibited by antiserum to the enzyme of the parent strain.

Materials and methods. Influenza virus PR-8 strain in the form of infected chorioallantoic fluid was used. The preparation of *V. cholerae* (strain 4 Z) and *D. pneumoniae* (type I-S) neuraminidases, and of antisera to these enzymes and to the influenza virus, has been described previously(7).

L forms of the *Vibrio cholerae* were produced by the method of Dienes(8). Fresh cultures were plated on nutrient agar containing 10% horse serum and penicillin (1000 units per ml). The L forms obtained by this method were transferred on the same type of agar at weekly intervals for 4 months, then induced to grow in broth by layering 10% horse serum-trypticase-soy broth (Baltimore Biological Laboratories) over agar block cultures in Erlenmeyer flasks. The cultures were incubated at 37°C for 72 hours, with periodic shaking. L forms were identified by the method of Dienes(8). Attempts to induce reconversion to the original bacterial form were unsuccesful, indicating that the L forms were stable. The broth filtrate was centrifuged at 25,000 G for 2 hours in the cold and the supernatant fluid studied for neuraminidase activity.

Virus, neuraminidase and anti-neuraminidase titrations were performed by the hemagglutination methods previously described(7). Specific neuraminidase activity was examined by incubation of the Vibrio L filtrate with highly purified α-1 glycoprotein* at 37°C for one hour and determination of free N-acetylneuraminic acid by the method of Warren (9). Total neuraminic acid was determined using the resorcinol method(10).

Results. Agglutination of chicken erythrocytes by influenza PR-8 virus was inhibited to a 1:32 dilution by Vibrio L broth filtrate. Eight-fold concentration of the filtrate by pressure ultrafiltration(7) resulted in inhibitory titers of 1:128-1:256.

Concentrated Vibrio L filtrate was dialyzed for 48 hours against 0.05M NaCl and titered for inhibitory activity in .155M saline-cacodylate buffer pH 6.8. The hemagglutination titer was reduced to 1:4. Titration of this material in calcium-borate buffer (11) gave titers of 1:64-1:128, indicating the need of the L filtrate enzyme for calcium ions, similar to that of the enzyme of the parent organism. Similarly, addition of 5% sodium citrate or 0.2M disodium ethylenediaminetetracetic acid resulted in sharp disappearance of inhibitory activity.

Antiserum to the neuraminidase of the parent strain of *V. cholerae*, neutralized hemagglutination-inhibition by Vibrio L neuraminidase to nearly identical levels, 1:128-1:256, when titrated simultaneously against 5 hemagglutination-inhibition units of the parent and L form neuraminidase. Antisera to pneumococcal neuraminidase and to influenza PR-8 virus exhibited no inhibitory effect. No attempt was made to produce antiserum to the neuraminidase of the L form.

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Neuraminic acid was released quantitatively from α-1 glycoprotein by incubation with Vibrio L form neuraminidase. Reaction mixtures containing 1000 μg of α-1 glycoprotein yielded 104 μg of neuraminic acid when determined by the resorcinol method and 114 μg when aliquots were measured by the Warren reaction after enzyme action. These results are in close agreement with the expected value of 110 μg, since N-acetylneuraminic acid constitutes 11% of the α-1 glycoprotein. Neuraminic acid was not detected in the Vibrio L filtrate by the resorcinol method.

Discussion. The retention of various properties of the parent strain by L forms of bacteria has been noted previously. Scheibel and Assandri(12) were able to isolate L forms of *Cl. tetani* toxigenic for mice, similar to the findings of Tulasne and Lavillaureix (13) who studied a water-vibrio EZ-5. Kandler and Kandler(14) demonstrated retention of various biochemical functions by L forms of *B. proteus* and *V. cholerae,* although specific enzymatic activities were not investigated.

In the present study, the neuraminidase of *Vibrio cholerae* L form was found to be identical in all respects with the same enzyme of the parent strain. Inhibition of hemagglutination by influenza virus was indistinguishable from that produced by the parent strain and specific release of neuraminic acid from a suitable substrate was easily demonstrable. The neuraminidases of both the parent and L form exhibited the same dependence upon calcium ions for activation. Further evidence of the identical nature of the 2 enzymes is provided by the neutralization studies. Specific antiserum to neuraminidase of the parent strain suppressed the activity of the parent and L form enzymes to exactly the same degree. Absorption studies were not done. It is unlikely that they would have yielded additional information. Scheibel and Assandri(12) noted neutralization of the toxin of *Cl. tetani* L forms by tetanus antitoxin.

Summary. A stable L form of *Vibrio cholerae* (strain 4Z) has been found to produce a neuraminidase capable of inhibiting hemagglutination by influenza virus and of liberating neuraminic acid from purified α-1 glycoprotein. The enzyme activity was neutralized by antiserum to the neuraminidase of the parent *Vibrio cholerae* strain.

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Reversal of Experimental Endotoxin Shock with a Combination of Aldosterone and Metaraminol. (26752)

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A combination of hydrocortisone and metaraminol reversed endotoxin shock in the dog, which was not accomplished with either drug alone(1,2). This report is concerned with the remarkable activity of aldosterone and metaraminol under similar experimental conditions.

Materials and methods. After equilibra-