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Staphylococcal Enterotoxin C. II. Some Physical, Immunological, and Toxic Properties*

Concordia R. Borja and Merlin S. Bergdoll

ABSTRACT: The enterotoxin C molecule produced by strain 137 undergoes considerable unfolding upon treatment with 5 M guanidine hydrochloride as indicated by a large change in intrinsic viscosity from 3.4 ml/g for the native toxin in 0.05 M sodium phosphate buffer (pH 6.8) to a value of 22.1 ml/g for the expanded configuration. The effect of 8 M urea on the molecular domain is not as dramatic as that produced by 5 M guanidine hydrochloride. On removal of guanidine or urea from enterotoxin C solutions, the resulting viscosity data resemble those given by the untreated enterotoxin. Guanidine-treated and urea-treated enterotoxin C give identical or nearly identical precipitin reactions as that exhibited by the native toxin and also evoke emesis at concentrations identical with that of the untreated toxin. Acetylation of five tyrosyl groups of enterotoxin C does not affect the immunological and toxic properties, which suggests that the "free" tyrosines are not required for serological and emetic activities. Acetylation of all the 21 tyrosyl residues results in an almost total loss of precipitating capacity and ability to induce vomiting in monkeys. The effective average number of determinants per molecule of enterotoxin C which can bind antibody molecules simultaneously was found to be three.

The staphylococcal enterotoxins, namely A, B, and C, which have been identified and purified, are protein in nature. They have also been found to elicit the formation of antibodies when injected to rabbits. Thus, the antigenic property of these toxins has provided a convenient means to identify the type of enterotoxin produced by different strains of *Staphylococcus aureus* under study and has also simplified the technique involved in the detection of new enterotoxins. Of the three enterotoxins that have been purified, only studies on the serological and emetic activities of enterotoxin B have been reported (Dalidowicz *et al.*, 1966), relating these properties of the native toxin to those of the modified (reduced and alkylated) and denatured (use of guanidine hydrochloride) toxin.

The present communication deals with the progressive effect of increasing concentration of alkali on the serological and toxic activities of enterotoxin C. It was also of interest in our laboratory to investigate the changes on the immunological and toxic properties of enterotoxin C that might ensue on treatment of the toxin with denaturing agents (guanidine hydrochloride and urea), a specific chemical modifying agent (*N*-acetylimidazole), and on oxidation with tyrosinase, and to relate these changes to those given by native enterotoxin C. The results are included in this paper.

An attempt has also been made to determine the effective number of determinants per molecule of enterotoxin C which can simultaneously bind with the antibody molecules.

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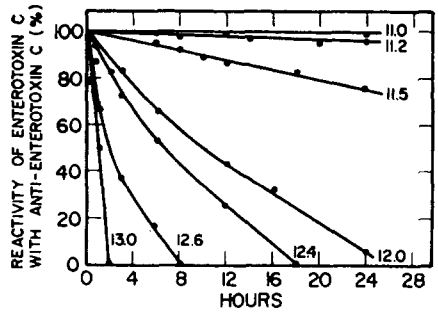

FIGURE 1: Effect of incubation time on reactivity of enterotoxin C with antienterotoxin C at different pH values and at 25°. Single gel diffusion tubes were used to determine the precipitin band movement. The number of each curve refers to pH.

Experimental Section

Materials. Measurement of concentration of purified enterotoxin C used in these experiments and preparation of purified guanidine hydrochloride are essentially the same as in the accompanying paper (Borja, 1969).

Effect of Increasing Concentration of Alkali on the Serological Activity. The percentage reactivity of enterotoxin C with antienterotoxin C at different pH values and at different periods of exposure at a particular pH was determined by adjusting enterotoxin C solutions (1×10^{-5} M) in 0.15 M KCl to the desired pH with carbonate-free 1 or 2 M KOH (prepared so that they were 0.15 M in KCl), kept at 25° for the length of time desired, then back-titrated to pH 7.4 with carbonate-free 1 M HCl containing 0.15 M KCl. For adjustment of pH and back-titration, a Radiometer apparatus was employed which is the same as that used in the accompanying paper (Borja, 1969). The back-titrated solutions were layered carefully on single gel diffusion tubes (Borja and Bergdoll, 1967) at a concentration of 50 μg/ml, the same concentration as that of the control (enterotoxin C in 0.15 M KCl, pH 7.4). The tubes were incubated at 25° and precipitin band movement was measured after 7 days.

Reaction of Enterotoxin C with N-Acetylimidazole and Oxidation by Tyrosinase. The same procedure as that described in the accompanying paper (Borja, 1969) was followed. After oxidation of enterotoxin C with tyrosinase, the mixtures were brought to a pH of 2.0 to denature the enzyme, allowed to stand for about 3 hr, followed by readjustment of pH to 7.4, with the use of a Radiometer titration assembly. Concentrations of 60 and 80 μg/ml of the test solutions and control (enterotoxin C solutions without tyrosinase and subjected to the same conditions as the test solutions) were prepared and layered on single gel diffusion tubes. Precipitin band movement was measured after 7 days of incubation at 25°.

Acetylation and Determination of Free Amino Groups of Enterotoxin C. The reactivity of amino groups of enterotoxin C toward acetylimidazole (360 molar excess) was obtained by determining the degree of amino group substitution as measured by the ninhydrin procedure of Moore and Stein (1954). The unmodified enterotoxin C was used to standardize the scale. The "free" amino groups were determined by the method of Habeeb (1966).

Antiserum to Enterotoxin C. The antiserum to purified enterotoxin C was prepared in rabbits by a method already described (Bergdoll *et al.*, 1965).

Immunodiffusion Tests. A modification of the Ouchterlony double-diffusion technique (Bergdoll *et al.*, 1965) was used to study the reaction of urea-treated and guanidine-treated enterotoxin C with antibodies directed to the native toxin.

Quantitative Precipitin Studies. The analyses of native enterotoxin C, acetylated enterotoxin C, and urea-treated and guanidine-treated enterotoxin C after removal of denaturing agent were carried out as described by Kabat and Mayer (1964). The Lowry modification (Lowry *et al.*, 1951) of the Folin–Ciocalteau method, using reagents standardized against normal rabbit γ-globulin (Nutritional Biochemicals Corp.), was followed to determine the total nitrogen content of the washed specific precipitates. A nitrogen content of 16.2% was taken for enterotoxin C (Borja and Bergdoll, 1967).

Viscosity Studies. Viscosity measurements were made in a thermostated bath at 20° with the use of an Ubbelohde viscosimeter. The efflux time for water at 20° was 160 sec.

Feeding Tests. Solutions of enterotoxin C in 0.15 M KCl were adjusted to pH 11.0, 11.5, and 12.0, allowed to stand for about 24 hr at 5°, back-titrated to pH 7.4, and fed to rhesus monkeys. Dosage levels of 20 μg/monkey were administered orally for both the controls (enterotoxin C in 0.15 M KCl, pH 7.4) and alkaline solutions of the enterotoxin. The monkeys were also challenged with acetylimidazole-treated and tyrosinase-treated enterotoxin C at the same concentration (40 μg/animal) as that of the untreated toxin.

Results

Relation of Serological Activity of Enterotoxin C to Changes in pH and Incubation Time. Figure 1 shows the relation of increasing pH of enterotoxin C solutions in 0.15 M KCl and increasing time of exposure at a specific pH with the reactivity of enterotoxin C with antienterotoxin C. At pH 11.0, there was essentially no loss of immunological activity for periods of incubation even as long as 48 hr (only 24 hr is shown in Figure 1). There appeared to be a very slight loss of serological activity with increasing time of exposure of the enterotoxin at pH 11.2. As soon as the pH was brought to 11.5, there was a significant loss of antigen–antibody precipitin reaction. When incubation lasted 24 hr at this pH, about 25% of the serological activity was lost. At and above pH 12.0, there was a rapid loss of the precipitating capacity of enterotoxin C with increasing time of exposure of enterotoxin C to alkaline solutions. Within 2-hr incubation at pH 13.0, no precipitin reaction was detectable in the enterotoxin C solutions.

Relation of Emetic Activity of Enterotoxin C to Changes in pH. Enterotoxin C solutions that were brought to pH 11.0 and incubated for about 24 hr at 25°, back-titrated to pH 7.4, and fed to rhesus monkeys were found to be

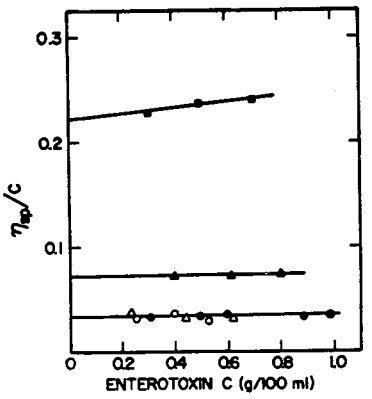

FIGURE 2: Reduced viscosity of enterotoxin C in various configurational states. All solutions contained 0.05 M sodium phosphate. (●) Native enterotoxin C, pH 6.8; (▲) 8 M urea, pH 7.6; (■) 5 M guanidine hydrochloride, pH 5.1; (△) guanidine-treated enterotoxin after removal of denaturant, pH 6.8; (○) urea-treated enterotoxin after removal of urea, pH 6.8.

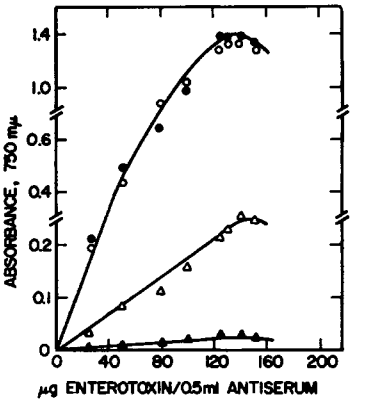

FIGURE 3: Quantitative precipitin curves where antienterotoxin C serum was allowed to react with native enterotoxin (●), enterotoxin C with 5 tyrosyl residues acetylated (○), enterotoxin C with 8–10 tyrosyl groups acetylated (△), enterotoxin C with all the 21 tyrosyl residues acetylated (▲).

toxic at dosage levels equivalent to those of the controls (20 μg/monkey). For solutions incubated at pH 11.5 for about 24 hr at 5°, however, emetic activity was repressed. Solutions of enterotoxin C that were adjusted to pH 12.0 and incubated at 5° for about 24 hr, then back-titrated to pH 7.4 before administering to test animals, were completely devoid of toxic activity.

Effect of Guanidine Hydrochloride and Urea on the Emetic and Serological Activities and Intrinsic Viscosity of Enterotoxin C. The enterotoxin exposed to the action of 5 M guanidine hydrochloride or 8 M urea evoked emesis when fed to rhesus monkeys at the same dosage level as that of the untreated toxin. Quantitative precipitin reactions given by guanidine-treated or urea-treated enterotoxin C after removal of denaturing agent showed similar patterns as that displayed by untreated enterotoxin.

On exposure of enterotoxin C to 5 M guanidine hydrochloride, the reduced viscosity pattern was markedly altered in comparison with that given by untreated enterotoxin (Figure 2). At infinite dilution, the line extrapolates to a value of 22.1 ml/g. A large increase in viscosity in the presence of 6 M guanidine hydrochloride has also been reported for enterotoxin B (Dalidowicz *et al.*, 1966). After removal of the guanidine hydrochloride by dialysis, the viscosity at different concentrations paralleled those exhibited by the native enterotoxin (Figure 2). When enterotoxin C was treated with 8 M urea and allowed to stand for about 45 hr at 5°, the intrinsic viscosity obtained from viscosity studies was 7.2 ml/g (Figure 2), and this value returned to about 3.4 ml/g in 0.05 M sodium phosphate buffer (pH 6.8) (the value given by the enterotoxin when the urea was removed by dialysis and also the intrinsic viscosity of native enterotoxin).

Influence of Acetylation of the Tyrosyl Residues in Enterotoxin C Exposed to Different Treatments on the Precipitin Reaction and Emetic Activity. Figure 3 shows the quantitative curves in which antienterotoxin C serum was allowed to react with the untreated enterotoxin and with enterotoxin exposed to different conditions. When enterotoxin C was treated with *N*-acetylimidazole at pH 7.5, five tyrosyl groups were found to be acetylated (Borja, 1969). The enterotoxin in which five tyrosyl groups were acetylated exhibited a precipitin type of reaction which closely resembles that demonstrated by untreated enterotoxin (Figure 3). Exposure of enterotoxin C to 8 M urea for about 45 hr (pH 7.6) and subsequent treatment with *N*-acetylimidazole at pH 7.5 resulted in the acetylation of 8–10 tyrosyl residues and left 20–25% of the original precipitin reaction. Enterotoxin C which was allowed to stand in 5 M guanidine hydrochloride for about 20 hr, then treated with *N*-acetylimidazole at pH 7.5, was found to contain 21 modified tyrosyl residues and there was an almost total loss of precipitating capacity (Figure 3).

Feeding experiments have demonstrated that the modified enterotoxin C with five tyrosyl groups acetylated was as toxic as the untreated enterotoxin. A similar result was obtained with modified enterotoxin B in which six tyrosyl residues were acetylated.[1] Enterotoxin C with all the 21 tyrosyl groups acetylated was, however, found to be completely devoid of toxic activity.

Effect of Oxidation of Enterotoxin C by Tyrosinase on the Serological and Emetic Properties. Five tyrosyl residues of enterotoxin C were oxidized by tyrosinase (Borja, 1969). The modified enterotoxin C that was allowed to react with antibodies directed toward the native enterotoxin gave the same precipitin band movement as that of the untreated enterotoxin as measured in single gel diffusion tubes. The tyrosinase-treated enterotoxin C was found to evoke emesis in parallel with the native, untreated enterotoxin.

Acetylation of Amino Groups. At a concentration of 360 molar excess of *N*-acetylimidazole, all the "exposed" (six to seven amino) groups of enterotoxin C, determined by the reagent 2,4,6-trinitrobenzenesulfonic acid by the method of Habeeb (1966), were acetylated. In the pres-

[1] F. S. Chu and M. S. Bergdoll, work done at the Food Research Institute but unpublished.

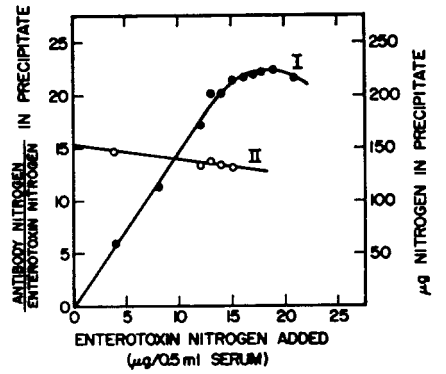

FIGURE 4: Quantitative course of the reaction between enterotoxin C and rabbit antienterotoxin C. Curve I represents the quantitative precipitin reaction of enterotoxin C with antienterotoxin C. Curve II shows the ratio of antibody nitrogen to antigen nitrogen of the specific precipitates plotted atainst the amount of antigen nitrogen added.

ence of urea about 75% of the amino groups were acetylated (38 lysine and 1 free amino group; Huang *et al.*, 1967), and in guanidine hydrochloride, an almost complete acetylation of the amino groups.

Determination of the Number of Antigenic Determinants per Molecule of Enterotoxin C. The quantitative precipitin curve (I, Figure 4), obtained with native enterotoxin C against antiserum, demonstrates a classical precipitin type of curve, showing precipitation throughout the region of antibody excess. Tests of the supernatant solutions revealed distinct zones of antibody excess, equivalence, and antigen excess. Taking a molecular weight of 160,000 for rabbit γ-globulin and 34,100 for enterotoxin C, the molar ratio of antibody to antigen in specific precipitates formed in the region of extreme antibody excess is 3.2. In this region, each antigen molecule is presumably bound to as many antibody molecules as its effective valence permits. The effective average number of determinants per molecule of enterotoxin C which can simultaneously bind antibody molecules is therefore 3.

Discussion

Hydrodynamic data have suggested that enterotoxin C is a relatively compact molecule (Borja and Bergdoll, 1967). On treatment with 5 M guanidine hydrochloride, however, there is a striking increase in intrinsic viscosity, indicating a considerable unfolding of the molecular domain. The data on spectrophotometric titration in guanidine hydrochloride (Borja, 1969) support this contention. The change in intrinsic viscosity of urea-treated enterotoxin C is not as dramatic as that effected by guanidine hydrochloride. This result proves that urea is a much less effective agent in producing denaturation of enterotoxin C than guanidine hydrochloride. Spectrophotometric titration of the enterotoxin (Borja, 1969) and results on the acetylation of enterotoxin C in urea and guanidine hydrochloride support this conclusion. The quantitative reversibility of the guanidine-induced and urea-induced denaturation suggests that the enterotoxin can undergo a spontaneous reassumption of its native structure following a drastic molecular disorganization.

The preservation of the emetic and serological activities on exposing enterotoxin C to solutions of pH 11.0 is of particular interest. It is most likely that the enterotoxin molecule reverts completely to its native configuration on back-titration from pH 11.0 to 7.4, thereby maintaining its biological properties. At pH 11.5, however, there is a rapid significant loss of the precipitin reaction and it is possible to visualize a molecular rearrangement resulting in a modified three-dimensional nature of the exposed antigenic combining site(s). In fact, our studies show that at and above pH 12.0, the leading edge of the precipitin band in single gel diffusion tubes is diffused, very different from that observed in control tubes having bands with sharp leading fronts. Exposure of enterotoxin C for about 2 hr in solutions of pH 13.0 results in a complete loss of serological activity after bringing the pH back to 7.4; this lengthy exposure time at pH 13.0 possibly disorganizes the molecule completely with random rearrangement on back-titration, so that the original character of the antigenic site(s) is not restored. Gerstein *et al.* (1963) have demonstrated that progressive denaturation of pepsin and pepsinogen by alkali reveals and subsequently destroys antigenic combining sites. Maintenance of a folded or tertiary structure has therefore been emphasized (Berson and Yalow, 1961; Givol *et al.*, 1962; Gould *et al.*, 1964; Merigan and Potts, 1966) to play a vital role in the immunological functions of proteins.

The present report indicates a significant decrease in the precipitating capacity of modified enterotoxin C with 8–10 tyrosyl residues acetylated, an almost complete loss of serological activity with all the 21 tyrosyl groups acetylated, and full precipitin reactivity given by the toxin with 5 tyrosyl groups modified. These findings, however, do not permit a solid ground for relating the tyrosyl groups to the antigenic or toxic sites, since the decrease or loss of reactivity might also have resulted from an alteration of the delicate secondary and tertiary structure containing the tyrosyl groups. Furthermore, reactivity of *N*-acetylimidazole is not limited to its interaction with tyrosyl residues, since it could also react with amino and thiol groups and aliphatic hydroxyl groups (Riordan *et al.*, 1965). Enterotoxin C lacks sulfhydryl groups, but our results, however, indicate the acetylation of amino groups. Further work has to be undertaken, therefore, to assess independently the influence of tyrosyl and lysyl residues on the immunological and toxic properties of the enterotoxin before arriving at a definite conclusion. In addition, physicochemical criteria such as viscosity, molecular size, diffusion and sedimentation properties, and other methods available for testing changes should be employed to establish unequivocably the absence of major changes in the protein due to the modification alone. There is some evidence from our studies on the oxidation of enterotoxin C by tyrosinase that the five free tyrosyl groups on the surface are not involved in the preservation of the immunological and emetic properties of the enterotoxin.

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Isolation and Purification of a Tissue Plasminogen Activator and Its Comparison with Urokinase*

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ABSTRACT: A simple procedure is described for the preparation from pregnant hog ovaries of a highly purified tissue plasminogen activator containing 100,000–175,000 tissue activator units/mg of protein. Tissue activator differs from the activator in urine (urokinase). Only within certain limits and under specified conditions is it possible to compare their activities in the same unitage. In fibrin plate assays one tissue activator unit was comparable to 0.1 CTA human urokinase unit. Gel filtration in glycine buffer at pH 2.35 indicated a molecular size of about 60,000 which is larger than the main component observed in urokinase preparations (mol wt 54,000). Though uniform by gel filtration, the tissue activator preparation could be further fractionated by zone electrophoresis. The active component had a mobility close to that of one of the active urokinase components and to that of bovine serum albumin. The properties of the tissue activator make it unlikely that it could be the source of plasminogen activator in urine.

The fibrinolytic activity of animal issues, originally thought to be caused by tissue proteases acting directly on fibrin (Macfarlane and Biggs, 1948), was later (Astrup and Permin, 1947, 1948; Astrup, 1951) found to be an indirect reaction involving the activation of a precursor, plasminogen, present in blood, to the active enzyme, plasmin, a trypsin-like protease (enzyme classification number 3.4.4.14). The tissue plasminogen activator is present in the microsomal fractions of tissues (Tagnon and Petermann, 1949; Lewis and Ferguson, 1950) and in lysosomes (Lack and Ali, 1964). Data on the assay of tissue plasminogen activator, its distribution in tissues, and its possible physiological significance have been reviewed recently (Astrup, 1966). Activators of plasminogen are also present in blood and urine. The human urinary activator (urokinase) has been prepared to a high degree of purity (Lesuk *et al.*, 1965; White *et al.*, 1966).

Purification of tissue plasminogen activator has been hampered because of its strong adherence to particulate cellular material from which it could not be extracted with solutions usually applied for such purposes. The observation that tissue plasminogen activator can be brought into solution by molar thiocyanate (Astrup and Stage, 1952) and separated by acid precipitation (Astrup and Sterndorff, 1956) led to a method for the quantitative assay of tissue plasminogen activator (Astrup and Albrechtsen, 1957). A preparation made from pig heart tissue served as a reference standard. Later, Bachmann and Sherry (1963) observed that

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