Fractionation of Staphylococcal Enterotoxin A by Isoelectric Focusing

The isoelectric point of the antigenically distinct staphylococcal enterotoxins A and C_2 was determined to be 6.8 and 7.0 respectively[1,2]. However, we observed that enterotoxin A was eluted from QAE-Sephadex with 0.033 *M* ethylene diamine (pH 6.4), conditions under which enterotoxin C_2 was till adsorbed (manuscript in preparation). This behaviour on ion-exchange chromatography suggested a pI value for enterotoxin A appreciably greater than that for enterotoxin C_2. The isoelectric point of enterotoxin A was re-examined in the present study by the method of isoelectric focusing[3].

Materials and methods. Purified enterotoxin A was subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis[4] and ultracentrifugation using a Spinco Model E.

The electrofocusing apparatus (LKB 8102/400 ml) was used and the operation performed according to the instructions given by the manufacturer[5]. The absorbance of fractions (3.5 ml) at 277 nm was determined using the Hitachi model 139 spectrophotometer and 1-cm cells. The pH of the fractions was measured at 25 °C with a Radiometer PHM 26 meter. When the electrode was standardized at the temperature of isoelectric focusing the pH of the fractions (at 4 °C) was increased by 0.46 pH unit. Fractions containing enterotoxin A were located and their titers determined by slide-gel double-diffusion tests[6].

Ampholines were separated from the isoelectric focused components by Gel filtration through a column (2.5 × 95 cm) of Sephadex G-100. The eluted components

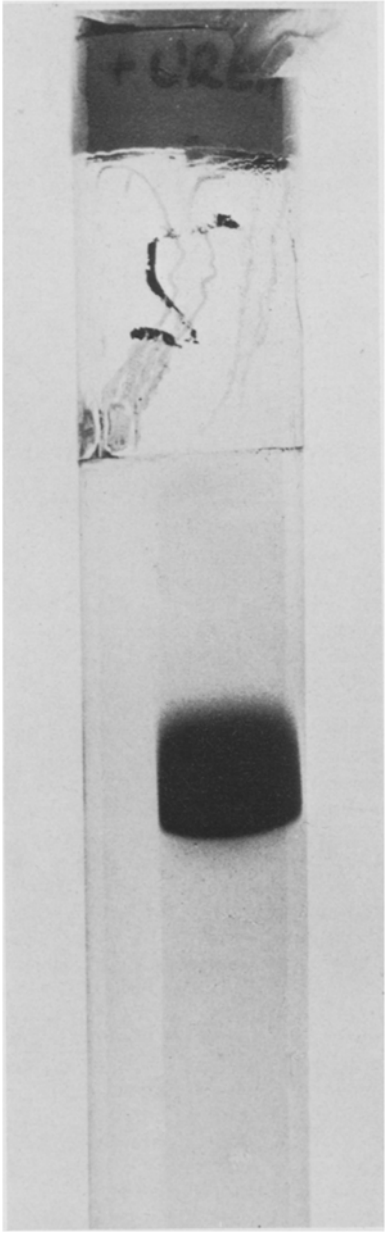

Fig. 1. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis of purified enterotoxin A. Protein was incubated in 1% SDS, 1% β-ME and 4 *M* urea.

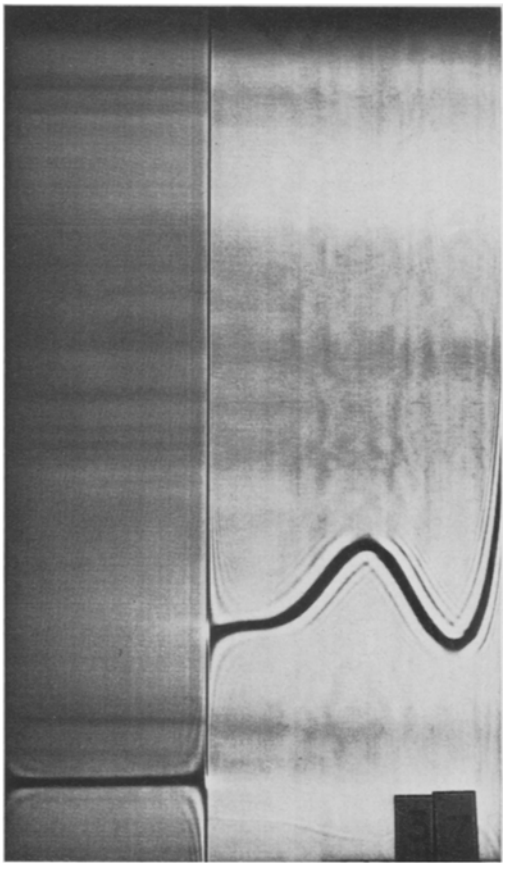

Fig. 2. Sedimentation pattern of enterotoxin A in Spinco Model E analytical centrifuge at concentration of 10 mg/ml in 0.075 sodium phosphate buffer pH 6.5. The picture was taken 96 min after reaching full speed of 56,000 rpm, at a bar angle of 60°, the temperature was 20°C.

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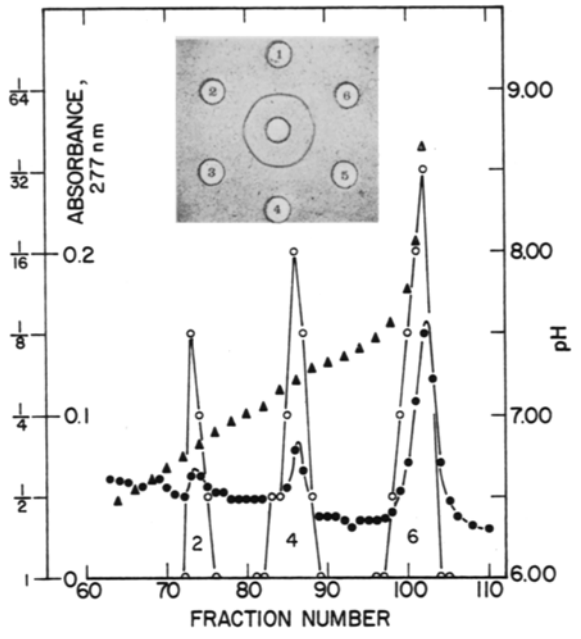

Fig. 3. Isoelectric focusing of enterotoxin A. Electrofocusing was performed at 600V for 72 h with a pH 5–8 ampholyte system. Absorbance at 277 nm (●); doubling-dilution titer of toxin, (0); pH at 25 °C, (▲). Insert: Immunodiffusion of isoelectric components wells 1,3,5; unfractionated enterotoxin A wells 2,4,6.

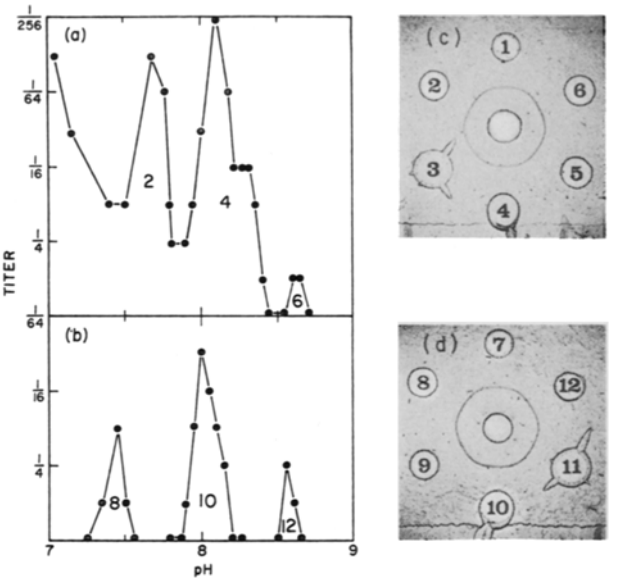

Fig. 4. Isoelectric focusing and immunodiffusion of enterotoxin A. Electrofocusing was performed at 600V for 72 h with a pH 7–10 ampholyte system. Doubling-dilution titer of toxin is plotted against the pH. a). Purified enterotoxin A. b) Reference enterotoxin A (A530P100). c) Focused components wells 1,3,5; unfractionated enterotoxin A, wells 2,4,6. d) Focused components wells 7,9,11; unfractionated enterotoxin A wells 8,10,12.

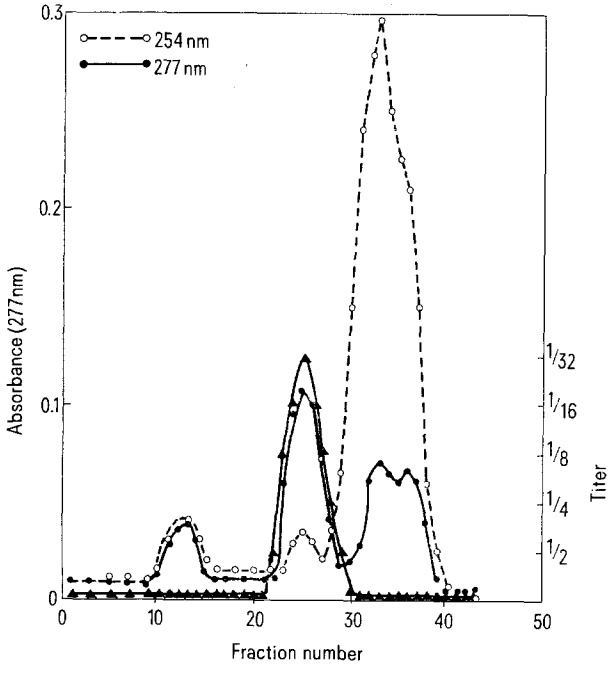

Fig. 5. Gel chromatography on Sephadex G-100 of enterotoxin A components separated by isoelectric focusing. Electrofocused fractions with pI values of 6.78, 7.21, 7.60, 8.10, 8.30, and 8.60 were each pooled into separate tube and 10 ml of each fraction was chromatographed on a 2,5×40 cm column on Sephadex G-100 and eluted with the equilibrating buffer (0.1 *M* Ammonium bicarbonate pH-8.1). The absorbance of fractions (3 ml) was measured at 277 nm and at 354 nm. Absorbance at 277 nm (●); absorbance at 254 nm (0); doubling-dilution titer of toxin (x).

were checked for immunological activity by gel-immunodiffusion[6].

Results and discussion. Homogeneity of the purified enterotoxin A prior to electrofocusing was shown by polyacrylamide gel electrophoresis (Figure 1) and by ultracentrifugation (Figure 2).

Results of an electrofocusing experiment in the region between pH 5–8 as shown in Figure 3 indicate the presence of 2 components with pI values of 6.78 and 7.21. An immunologically reactive zone was also found at the cathode. When the same sample was electrofocused in the region between pH 7 and 10, four components with pI values of 7.60, 8.10, and 8.60 were distinguished (Figure 4a). The component present in greatest concentration focused at pH 8.10. An immunologically reactive zone, presumably consisting of the pI 6.78 and 7.21 components (Figure 3) was found at the anode. The value for the titer was proportional to the protein concentration and the titer 'peaks' coincided with maximum protein concentration of the fractions.

In gel chromatography similar elution volumes (elution volume/void volume about 2.22) were obtained when the enterotoxin A components separated by isoelectric focusing were eluted from Sephadex G-100. A typical elution pattern obtained from gel chromatography is shown in Figure 5.

From the results presented above, it seems evident that enterotoxin A is heterogeneous. It is unlikely that this heterogeneity could be due to the preparation since analysis of reference enterotoxin A (A530P100) also revealed heterogeneity and the presence of a major component with pI–8.00 (Figure 4b).

Although the enterotoxin A fractions differ in charge as demonstrated by isoelectric focusing they are identical by serology and have apparently identical molecular weights. The heterogeneity could be due mainly to different

amounts of amide groups that can alter the net charge of the protein molecule.

SPERO et al.[7] have shown that partial deamidation was responsible for the multiple forms of enterotoxin B. If this explanation is applicable to enterotoxin A, the pI 8.60 component would correspond to the most fully amidated molecule; this would be converted to the more acid fractions of the toxin by deamidation.

The apparent discrepancy in the pI value found by CHU et al.[1] for enterotoxin A (pI-6.8) and in the results obtained in this laboratory (pI-8.1 major peak) can be explained by assuming that the more basic components were not eluted off the CM-cellulose column used for the purification of enterotoxin A by CHU et al.[1].

While this work was in progress SCHANTZ et al.[8] reported heterogeneity of enterotoxin A from *S. aureus* strain 13N-2909. By isoelectric focusing they separated 4 components with pI values of 6.64, 7.26, 7.68, and 8.10 (at 4 °C). The component present in greatest concentration focused at pH 7.26.

Résumé. L'entérotoxine A a été divisée en six composants par focalisation isoélectrique. Les points isoélectriques (pI) variaient entre 6.78 et 8.60 à 25C; le composant prédominant se focalisait à un pH de 8.10. Les caractéristiques immunologiques et le poids moléculaire des 6 composants furent identiques.

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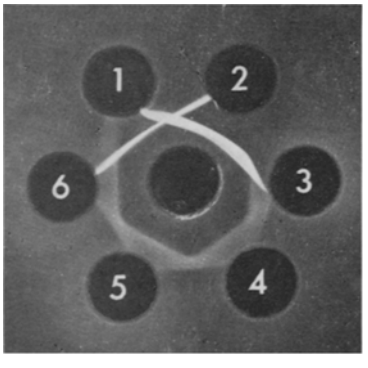
[9] We thank Professor M. S. BERGDOLL, University of Wisconsin for kindly supplying *S. aureus* strain 11N-165 reference toxin (A530-P100) and antitoxin A serum. We also thank R. GAGNÉ, Y. YANO and D. JEFFREY for excellent technical assistance.

Effects of Splenectomy on Amphibian Antibody Responses

In mammals, splenectomy has repeatedly been shown to impair antibody responses to various antigens, particularly those introduced into the circulation[1–4]. Similar effects have been observed in birds[5,6] and in reptiles[7]. Conversely, splenectomized teleosts (*Lutjanus griseus*) and sharks (*Ginglymostoma cirratum* and *Negaprion brevirostris*) have produced antibodies in titres comparable with intact animals, irrespective of the route of antigen administration[8], whilst good antibody production to both soluble and particulate circulating antigens has been demonstrated in splenectomized amphibians (*Xenopus laevis*)[9]. There is thus some indication that extra-splenic sites play a more significant immunological role in the lower vertebrates than in the amniotes. Information on the poikilothermic species is relatively sparse at present, however, and restricted to a few antigens.



Double diffusion in agar gel, demonstrating similar antigen persistence in the sera of intact and splenectomized toads at 4 weeks after injection of whole human serum (WHS). Central well, 1:5 anti-WHS produced in goat; well 1, HGG (1 mg/ml); well 2, HSA (1 mg/ml); wells 3 and 4, experimental sera from splenectomized toads; wells 5 and 6, experimental sera from sham-operated toads. All 4 experimental sera have a single line of persisting antigen which shows identity with HSA (but no identity with HGG).

The present study is designed to investigate further the immune capabilities of splenectomized *Xenopus*, and to establish whether their ability to elicit a response depends on the antigen presented. A complex antigen, whole human serum (WHS), has been used as a convenient means of testing the animals' ability to respond to a variety of protein antigens.

Materials and methods. *Xenopus* used in these experiments were all mature adult males weighing between 33 and 65 g. They were maintained in tap water at 20 °C and fed weekly on minced meat. Splenectomies were performed as described previously[9]. Sham-splenectomized animals were subjected to the same operative trauma as the splenectomized group, but leaving the spleen intact. All animals were immunized 6 days after the operation.

Antigen consisted of 10 mg/ml freeze-dried WHS (Hyland, California) in saline, emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit). This emulsion was injected into the dorsal lymph sac at a rate of 5 µl/g body weight.

Toads were divided into 3 groups, each consisting of 3 splenectomized and 3 sham-operated animals. Those of groups I and II were given a single injection of antigen and killed at week 4 and week 8 respectively; group III was given a second injection at week 4 and killed at week 8. Blood was collected from the heart, allowed to clot, and

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