The Control of the Swarming of *Proteus vulgaris* by Boric Acid

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SUMMARY: The swarming of *Proteus vulgaris* is inhibited on a heated blood-agar medium containing 0.1 % (w/v) boric acid. This boric acid concentration does not inhibit the growth of many organisms having more exacting metabolic requirements. The medium is equally successful in controlling certain swarming strains of *Pseudomonas pyocyanea*. This inhibition of swarming is possibly due to the formation of a boron-polysaccharide complex, resinous in character, the formation of which is associated with flagellar disintegration.

The swarming of *Proteus vulgaris* in mixed culture is a continual source of trouble to bacteriologists. A method which could control it without affecting the growth of more delicate organisms would be of great value, e.g. in the isolation of gonococci from the urogenital tract. Kramer & Koch (1931) in a summary comparing existing methods indicated chloral hydrate as one of the most successful inhibitors of swarming. Gillespie (1948) used this substance for the isolation of β -haemolytic streptococci on a blood-agar medium in the presence of *P. vulgaris*.

Lominski & Lendrum (1942) proposed the use of surface-active agents to prevent swarming. Those compounds which they described as most effective, together with others not mentioned by them, proved on test in our hands to be incapable of preventing the swarming of *P. vulgaris* when these substances were incorporated in a heated blood-agar medium. Simple inorganic salts were next examined, especially those having some protein-coagulating action, which might be expected to influence either the formation or the action of flagella. Potash alum was inhibitory in a simple nutrient agar medium and in a heated blood-agar medium containing 10 % blood, but was lethal to more delicate organisms at concentrations sufficient to prevent the swarming of *P. vulgaris*. The active part of potash alum proved to be the aluminium. Accordingly, other members of group 3 of the Periodic Table were investigated. Boron compounds and in particular boric acid were found to be peculiarly effective. Boric acid was therefore incorporated in a heated blood-agar medium containing 10 % blood in concentrations ranging from 1 to 0.0001 % (here and throughout % means % (w/v)). The most satisfactory concentration of boric acid for the inhibition of swarming was 0.1 %, and this concentration did not affect the growth of more delicate organisms. At concentrations of 1 % boric acid there was only moderate growth of *Shigella sonnei*, *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Neisseria gonorrhoeae*. Swarming of some of the twelve strains of *Proteus vulgaris* examined began at dilutions lower than 0.05 % boric acid; other strains did not swarm until a dilution of 0.01 % was reached.

In the standard medium used in this department for the isolation of gonococci from the urogenital tract, a heated blood agar containing 20 % blood, it was found advisable to increase the concentration of boric acid to 0.125 %. The suitability of the boric acid medium for isolating gonococci was estimated by comparing the minimum number of gonococci growing on a heated blood-agar medium with and without boric acid. Using six freshly isolated strains of gonococci suspended in 10 % serum broth, and various dilutions of these suspensions, it was found that both media detected the organism at a dilution of 1000 organisms/ml. when the inoculation was made with a standard 3 mm. loop which contained at that dilution *c.* 70 organisms. The medium was tested extensively in the Venereal Diseases Clinic of the General Infirmary at Leeds.

The boric acid medium. The medium was made as follows. A screw-capped bottle of about 550 ml. capacity containing 400 ml. of nutrient agar was placed in the steamer until the agar was melted. The bottle was then transferred to a water-bath at 60° and kept at that temperature for 10 min.; 80 ml. of oxalated horse blood were then added and well mixed. The temperature of the bath was then raised to 75°. When the contents of the bottle had assumed a uniform chocolate colour 12.5 ml. of a 4 % solution of boric acid were added (a 4 % solution of boric acid is self-sterilizing) and well mixed in the medium by rotatory movements of the bottle; plates were then poured. After setting, the plates were kept in the cold-room for use as required; it was found that the medium kept for 4–6 weeks without deterioration.

Technique and results of test

The method adopted for testing the medium was to inoculate first the control plate and then the boric acid plate with the same swab. This procedure was adopted because it tended to load the results against the boric acid plates. In a parallel examination of 350 specimens fifty-six of the control plates were overgrown by *Proteus vulgaris*. Of the boric acid plates none was overgrown by *P. vulgaris*, although in each case that organism was demonstrable in discrete colonies. The successful isolation of gonococci was obtained in thirty-eight instances on the boric acid medium but only in twenty-six on the control medium. The medium therefore shows promise as a diagnostic aid for the detection of the gonococci in the urogenital tract, and may be similarly useful on other occasions where the swarming of *P. vulgaris* impedes the isolation of desired organisms, e.g. in material from burns.

Possible mechanism of action of boric acid

The work of Deuel, Neukom & Weber (1948) suggested a possible explanation for the inhibiting action of the medium on the swarming of *P. vulgaris*. These authors have shown that polyhydroxylic substances, particularly those having a high degree of polymerization, form resinous compounds with aqueous solutions of boric acid even at concentrations low in comparison with those used in the medium described. Electron micrographs of *P. vulgaris* grown on a heated blood-agar medium containing boric acid (Pl. 1, fig. 1) show few

flagella, most of which are broken and detached; cells grown on a similar medium without boric acid show large numbers of undistorted and unbroken flagella. The examination of flagellated bacteria in the electron microscope is complicated by the fact that during mounting and drying the flagella may become detached or broken unless a special technique be adopted. In this case, to avoid damage to the flagella, dilute suspensions were made in filtered distilled water, care being taken to avoid undue shaking and stirring. Small drops of the suspensions were placed on filmed specimen grids. After standing for 5 min. these were drained with a small piece of filter-paper. This method ensured the even distribution of the organisms on the grid, and the rapid drying so obtained minimized distortion and damage to the flagella. The dried grids after washing by flotation on filtered distilled water were shadow-cast with chromium, using the vacuum technique of Williams & Wyckoff (1944). Material in salt solution when placed on the grid is unavoidably accompanied by large amounts of other matter including salt. In order that the structural detail of the material may be clearly seen this extraneous matter must be removed in a way that will not damage the material under examination. Various methods of accomplishing this without affecting the nature and degree of aggregation have been considered by Reed (1946). One of the simplest and most effective is to form a thin layer of a dilute suspension of the material on the filmed specimen grid by using a micro-pipette, and to drain immediately. In such very thin layers the chances of particle aggregation by lateral movement are greatly diminished and the specimens dry rapidly. With most biological material, drying at this stage fixes it in an insoluble state, so that excess of salt can be safely removed by washing before shadow-casting. Figs. 2 and 3 in Pl. 1 were made from material treated in this manner and are clearly free from salt crystals. It was felt that pictures of preparations containing only flagella would prove more instructive for the understanding of the prevention of swarming by boric acid.

Preparation of flagella suspensions

For the preparation of suspensions of flagella the following technique was adopted. A 24 hr. culture of *Proteus vulgaris* which had been inoculated at the junction of the condensation water and the agar slope was used. This ensured that the culture used was actively swarming. The culture was removed by careful washing with 10 ml. of isotonic saline and the resultant suspension divided into two equal parts. To one part boric acid was added to give a final concentration of 0.1 %. Both suspensions were then incubated for 18 hr. at 37° and then 0.1 ml. of 30 % formaldehyde added. The suspensions were then centrifuged at 7000 G for 20 min., and the resultant clear supernatant fluids examined in the electron microscope. The electron micrographs (Pl. 1, figs. 2, 3) show that the boric acid-treated fluid contains the remains of flagella on whose surfaces are numerous small spherical particles and agglomerations of these particles, of which the average diameter is 250 Å. (fig. 3). In the control fluid the flagella appear to be normal and are not clumped (fig. 2).

Possible nature of boric acid-polyhydroxylic complex

It may well be that the particles observable in Pl. 1, fig. 3, represent the protein fraction of the flagella, and that the thinner layer which surrounds them and appears to link them is a complex of the type mentioned by Deuel *et al.* (1948). If this is so then the clumping of the flagella is more easily explicable, as these complexes are known to be of a viscous nature. Deuel (1948) states that the presence of even minute traces of these substances may be detected by viscosimetric measurements. It was found that the fluid treated with boric acid showed a much greater time of flow than the fluid of the control tube, and than negative controls made from uninoculated agar slopes. The same viscosimeter (Ostwald type) was used in each case and was thoroughly cleaned after each reading. The times compared were the mean of ten readings for each specimen; all readings were made at 18°. The increase in time of flow of the specimen treated with boric acid was of the order of 9%.

Deuel (1948) states that the action of acid or fructose destroys these complexes. Fluid obtained, as already mentioned, from a tube which had been treated with boric acid was acidified. To 10 ml. of clear supernatant fluid 1 ml. of 0.1 N-HCl was added, and after 1 hr. specimens were placed on filmed grids. Examination in the electron microscope showed numerous small particles (Pl. 1, fig. 4), slightly smaller than those observable in Pl. 1, fig. 3, which it has been suggested were covered by a thin layer of some less dense material and which is not observable in Pl. 1, fig. 4. These particles are not clumped. This lack of clumping may be due to the destruction by the acid of a complex of the nature already mentioned. Viscosimetric examination showed that the time of flow of the acid-treated specimen approached that of the blank controls.

Effect of copper sulphate and lowering of pH

Other inorganic salts that have been used for inhibiting the swarming of *P. vulgaris* were similarly examined. Copper sulphate when substituted for boric acid at a final concentration of 0.05% had no detectable effect on free flagella. To obviate the possibility that the boric acid phenomenon might be due to a lowering of pH value a specimen was prepared using the same technique but adding 1 ml. of 0.1 N-HCl. In the electron microscope no change from the normal was observed.

DISCUSSION

Although it would be very difficult to prove conclusively that the action of boric acid on the flagella of *Proteus vulgaris* and *Pseudomonas pyocyanea* is due to the formation of a boron complex of the type suggested, the evidence presented points in that direction. It is probable that the differences between the flagella in fig. 1 and fig. 3 of Pl. 1 are due to the very much greater surface area exposed to the action of boric acid in the flagella of fig. 3. Although other interpretations of the electron micrographs are possible the viscosimetric com-

Figs. 1-4

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PLATE 1

parisons favour the theory that a boron complex of the nature described is formed. Assuming this, then the flagella probably consist of aggregates of protein molecules bound and surfaced by a polyhydroxylic substance.

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EXPLANATION OF PLATE

- Fig. 1. Electron micrograph of *Proteus vulgaris* taken from a heated blood-agar medium (10 % blood) containing 0.1 % boric acid after 24 hr. incubation at 37°. Magnification $\times 33,000$.
Fig. 2. Electron micrograph of supernatant fluid from control suspension showing undistorted flagella of *Proteus vulgaris*. Magnification $\times 33,000$.
Fig. 3. Electron micrograph of supernatant fluid from boric acid-treated specimen, showing alteration in appearance of flagella. Magnification $\times 33,000$.
Fig. 4. Electron micrograph of supernatant fluid from specimen treated with boric acid after further treatment with HCl. Magnification $\times 24,000$.

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