Bacteriostatic Effects of Specific Antiserum on *Clostridium welchii* type A. The Role of E_h and pH of the Medium

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SUMMARY

The bacteriostatic effect of specific antiserum on *Clostridium welchii* type A was profoundly influenced by the pH and E_h of the medium. With suitable concentrations of specific antiserum relatively high pH and E_h values led to a well-marked inhibition of growth accompanied by destruction of the bacteria. A relatively low pH or E_h led to a decrease or abolition of the inhibitory power of the specific antiserum. Neither complement nor properdin appeared to be involved in the bacteriostatic effect.

INTRODUCTION

Specific antiserum provides complete protection against many lethal doses of *Clostridium welchii* type A introduced into the allantoic cavity of embryonated eggs. The protective effect appears to reside in the ability of the antiserum to inhibit the growth of the bacteria. However, antiserum does not inhibit bacterial growth in killed eggs (Bullen, Wilson & Cordiner, 1961). Since dead tissue is generally considered to be more reducing and more acid than living tissue we decided to investigate the effects of E_h and pH on the bacteriostatic properties of *C. welchii* antiserum. Preliminary experiments with allantoic fluid *in vitro* showed quite clearly that the degree of bacteriostasis was profoundly influenced by the E_h of the medium (Bullen & Dobson, 1962). Allantoic fluid was considered unsuitable for a more detailed investigation, since its composition is variable and not entirely known, (Needham, 1931; Festenstein, 1957). The experiments described in this paper were done with a chemically defined tissue culture medium in which *C. welchii* grew very well.

METHODS

Organisms. *Clostridium welchii* type A strain CN2726 was used for all the experiments *in vitro*. Strain CN2726 and type A strain CN1491 were both used for immunization in preparing the antiserum. Both strains were obtained from the Wellcome Research Laboratories (Beckenham, Kent).

Specific Clostridium welchii antiserum. One antiserum (P 10) was used for all the experiments; this was obtained from a pony after eight courses of immunization against *Clostridium welchii* type A. Formalized whole cultures of strain CN2726 were used for the first seven courses; for the eighth course the pony was injected intramuscularly with a mixture of concentrated toxin from strains CN2726 and CN1491, and subcutaneously with washed and formalized organisms of strain CN2726. The antitoxin content of the antiserum in units/ml. was as follows;

α -antitoxin 130 (international units); θ -antitoxin 90 (provisional units); κ -antitoxin 330 (provisional units); μ -antitoxin 11 (provisional units). The antiserum was stored at -20° .

Culture medium. Sufficient tissue culture medium TC199 (without phenol red; Difco) was used to give a final volume of 70 ml. after the addition of poisoning agent, bacterial inoculum and antiserum. The bicarbonate concentration was increased by adding 0.1–0.15 g. dry NaHCO_3 sterilized by heating to 160° for 2 hr.

Viable counts. Viable counts were made on blood agar plates (Bullen *et al.* 1961).

Control of E_h and pH. As described by Dobson & Bullen (1964).

Broth saline. 10 % (v/v) of papain digest broth in 0.85 % (w/v) NaCl.

Experimental procedure. TC199 medium was placed in the culture vessel. The stirrer was switched on and after a gas mixture of 5 % (v/v) CO_2 + 95 % (v/v) N_2 had bubbled through the medium for about 15 min., the NaHCO_3 was added. The glass electrode, after standardization in phosphate buffer, together with the four platinum E_h electrodes, was inserted in the culture vessel. The KCl agar bridge was cut at both ends with sterile scissors and one end was placed in the medium and the other in the calomel electrode. After adding 0.5–1.0 ml. of a solution of the appropriate redox dye to give a final concentration of $30 \mu\text{M}$ the pH value was adjusted by varying the concentration of CO_2 in the gas bubbling through the medium. When the pH value was approximately correct the gas stream was switched to pass over the surface of the medium. When required, warmed sterile antiserum was added at this stage. The E_h was adjusted by cautious addition of sterile 5 % (w/v) $\text{Na}_2\text{S}_2\text{O}_4$.

An actively growing culture of *Clostridium welchii* (1½ hr old) in papain digest broth, was centrifuged and the supernatant fluid removed. The bacteria were re-suspended in broth saline and adjusted to an opacity corresponding to tube No. 8 on the Brown's scale (Burroughs Wellcome and Co.). Suitable dilutions were prepared in broth saline and the bacteria added to the medium in a volume of 0.6 ml. After adding the bacteria the culture was well mixed with the aid of the syringe, and a sample withdrawn for a viable count. Final adjustments of pH and E_h were made as quickly as possible. When antiserum was used, all the samples, after the first hour, were homogenized in an MSE (London) (5 ml.) homogenizer for 8 min. before the viable counts were made; this broke up the chains of organisms which invariably formed in the presence of antiserum.

RESULTS

In the absence of antiserum the organisms grew well (Figs. 1–4; Table 1). The lag phase varied from approximately 1 hr at $E_h +60 \text{ mV}$, pH 7.5, to 30 min. at $E_h -140 \text{ mV}$, pH 7.5. The culture consisted largely of well separated individual bacteria (Pl. 1, fig. 1).

The effect of E_h and pH on the generation time. At pH 7.5 faster growth in the logarithmic phase occurred under more reduced conditions. The effect of changing from pH 7.5 to 6.5 was slight (Table 1).

The effect of different concentrations of antiserum on bacterial growth. In the five experiments shown in Fig. 1 the E_h was controlled at $+60 \text{ mV}$ and the pH value at 7.5. The presence of 3 % (v/v) *Clostridium welchii* antiserum (P 10) had a stimulating effect on bacterial growth. The lag phase was decreased to 30 min. and the

Table 1. *Effect of E_h and pH on generation time of C. welchii during the logarithmic phase of growth without serum*

Redox dye	pH	E_h	Time controlled (hr)	Generation time (min.)
thionine	7.48 ± 0.007	$+59 \pm 1.1$	3.5	27
1-naphthol-2-sodium sulphionate-indo-8'5'-dichlorophenol	7.49 ± 0.003	$+60 \pm 2.1$	3.5	27
methylene blue	7.51 ± 0.005	0 ± 0.7	3.0	21
indigo carmine	7.51 ± 0.014	-141 ± 1.0	3.0	19.5
thionine	6.51 ± 0.006	$+60 \pm 1.5$	3.5	24

Fig. 1. The effect of specific antiserum concentration on the growth of *C. welchii* at pH 7.5; $E_h + 60$ mV.

Symbol	Antiserum P 10 (%, v/v)	pH (mean $\pm$ s.d.)	E_h (mV) (mean $\pm$ s.d.)	Time controlled (hr)
●	0	7.48 ± 0.007	$+59 \pm 1.1$	3.5
■	3	7.50 ± 0.005	$+60 \pm 1.7$	3.25
△	6	7.50 ± 0.010	$+58 \pm 1.6$	8.0 +
○	12	7.50 ± 0.004	$+59 \pm 2.0$	8.0 +
□	24	7.52 ± 0.012	$+61 \pm 0.5$	8.0 +

organisms grew slightly faster than in the absence of specific antiserum. Antiserum at 6 % (v/v) had a well-marked bacteriostatic effect. The organisms grew well for the first 2 hr, but by 3 hr multiplication had stopped. This was followed by a steady decline in the number of viable organisms until the 6th hr. Thereafter the number of viable organisms slowly increased. Similar patterns of growth and destruction occurred with 12 % (v/v) and 24 % (v/v) antiserum.

The effect of changes in E_h . Figure 2 shows the effect of changes in E_h on the inhibitory power of 12 % (v/v) antiserum P 10 when kept at pH 7.5. At E_h +60 mV the number of viable organisms declined rapidly between the 3rd and 6th hr; at E_h 0 mV the peak of bacterial growth was slightly greater than at +60 mV and there was less destruction between the 3rd and 6th hr; at E_h -140 mV there was no apparent destruction but the growth rate was decreased by 7-8 times compared with the control. Figure 3 shows similar experiments with 6 % (v/v) P 10 antiserum. Good inhibition of bacterial growth occurred at 60 mV; at 0 mV and -140 mV, there was no inhibitory effect.

Fig. 2

Fig. 2. The effect of E_h on the growth of *C. welchii* with 12 % (v/v) antiserum P 10 at pH 7.5.

Symbol	Antiserum (%, v/v)	pH (mean $\pm$ s.d.)	E_h (mV) (mean $\pm$ s.d.)	Time controlled (hr)
●	0	7.51 $\pm$ 0.014	-141 $\pm$ 1.0	3.0
△	12	7.51 $\pm$ 0.011	-141 $\pm$ 2.3	8.0 +
□	12	7.52 $\pm$ 0.003	-3 $\pm$ 1.9	8.0 +
○	12	7.50 $\pm$ 0.004	+59 $\pm$ 2.0	8.0 +

Fig. 3

Fig. 3. The effect of E_h on the growth of *C. welchii* with 6 % (v/v) antiserum P 10 at pH 7.5.

Symbol	Antiserum (%, v/v)	pH value (mean $\pm$ s.d.)	E_h (mV) (mean $\pm$ s.d.)	Time controlled (hr)
●	0	7.51 $\pm$ 0.014	-141 $\pm$ 1.0	3.0
□	6	7.52 $\pm$ 0.011	-142 $\pm$ 1.0	3.0
○	6	7.51 $\pm$ 0.005	0 $\pm$ 1.0	3.0
△	6	7.50 $\pm$ 0.010	+58 $\pm$ 1.6	8.0 +

The effect of changes in pH value. During the experiments shown in Fig. 4 the E_h was controlled at +60 mV. With 12 % (v/v) antiserum P 10 at pH 7.5 there was well-marked inhibition of bacterial growth. At pH 6.8 the degree of inhibition was

far less than at pH 7.5; at pH 6.5 there was no inhibition, and growth was identical to that observed in the absence of antiserum. When the antiserum concentration was decreased to 6% (v/v) the inhibitory effect though well marked at pH 7.5 (Fig. 1) was completely abolished at pH 6.8.

The effect of changes in pH and E_h . The inhibition produced by 12% (v/v) antiserum P 10 at pH 6.8, E_h 0 mV was so slight that it was doubtful whether it was within the experimental error of the method. A slight decrease in growth rate was noted after the 3rd hr, but E_h control was lost after 3 hr 45 min., after which growth was somewhat faster. This can be compared with the definite inhibition obtained with 12% (v/v) antiserum P 10 at pH 6.8, E_h 60 mV (Fig. 4) and pH 7.5 E_h 0 mV (Fig. 2).

The effect of heating antiserum P 10. The antiserum was heated to 56° for 30 min. in a water bath. This had no effect on the bacteriostatic effect (Fig. 5).

Fig. 4

Fig. 5

Fig. 4. The effect of pH value on the growth of *C. welchii* with 12% (v/v) antiserum P 10 at E_h + 60 mV.

Symbol	Antiserum (%, v/v)	pH value (mean $\pm$ s.d.)	E_h (mV) (mean $\pm$ s.d.)	Time controlled (hr)
●	0	6.51 $\pm$ 0.006	+60 $\pm$ 1.5	3.5
□	12	6.50 $\pm$ 0.008	+59 $\pm$ 1.6	3.5
△	12	6.80 $\pm$ 0.008	+59 $\pm$ 1.8	8.0 +
○	12	7.50 $\pm$ 0.004	+59 $\pm$ 2.0	8.0 +

Fig. 5. The effect of heated antiserum P 10 on the growth of *C. welchii* at pH 7.5; E_h + 45 mV; 12% (v/v) antiserum.

Symbol	Antiserum treatment	pH value (mean $\pm$ s.d.)	E_h (mV) (mean $\pm$ s.d.)	Time controlled (hr)
○	Unheated	7.51 $\pm$ 0.005	45 $\pm$ 1.7	8.0 +
●	Heated	7.52 $\pm$ 0.007	46 $\pm$ 1.2	8.0 +

Morphology of Clostridium welchii. Actively growing organisms in tissue culture medium without specific antiserum P 10 consisted of well separated Gram-positive rods, about $1-3\ \mu$ long and $0.5-0.8\ \mu$ wide (Pl. 1, fig. 1). A similar morphology was seen under widely different E_h and pH values. In the presence of specific antiserum P 10 when conditions were unsuitable for inhibition of growth the organisms occurred in long chains, sometimes of 50 individuals or more. All these organisms were Gram-positive and were much larger than those seen in control cultures (Pl. 1, fig. 2).

In the presence of specific antiserum P 10 when conditions were suitable for inhibition of growth the organisms were much larger than those seen in control cultures and occurred in short chains. The first visible signs of damage consisted of small Gram-negative patches in otherwise intact bacteria. Some individuals became entirely Gram-negative and others showed various degrees of disintegration, leading eventually to complete lysis (Pl. 1, figs. 3-10). All stages of damage, from apparently normal individuals, to the faint outlines of 'ghosts' were sometimes seen in a single chain of bacteria. Visible signs of damage were most numerous after incubation for 5-6 hr.

DISCUSSION

In the absence of specific antiserum *Clostridium welchii* type A grew well in the tissue culture medium. At the highest E_h tested, +60 mV, pH 7.5, the organisms grew logarithmically, with loss of E_h control after 3 hr and a viable count of several million organisms/ml. after 5 hr.

The presence of 3 % (v/v) antiserum P 10 slightly stimulated growth; under the same conditions 6 % (v/v) antiserum inhibited growth. After 6 hr the viable count was similar to the initial count and E_h control was maintained for at least 8 hr. Rather to our surprise, no greater inhibition occurred when the concentration of the antiserum was increased to 24 % (v/v).

In every case where inhibition occurred there was an initial period of vigorous growth similar to or faster than the rate of growth in control cultures. This was usually followed by a rapid decrease in the viable count, accompanied by the appearance of Gram-negative organisms some of which appeared to be lysing (Pl. 1, figs. 3-10). The decrease in viable count must be attributed to death of the bacteria and not to the formation of chains, since all the samples which showed chain formation were homogenized before counting. This could be seen to break up the chains satisfactorily. However, the most striking evidence that chain formation did not affect the viable count was provided by experiments with specific antiserum where conditions were suitable for chain formation but unsuitable for inhibition of growth. In these circumstances the growth curves were identical with the controls (Fig. 4; Pl. 1, fig. 2).

The inhibition of growth produced by antiserum depends profoundly on the E_h and pH values of the medium. A decrease in E_h or pH value or in antiserum concentration decreased or abolished the inhibitory effect. However, these three variables interacted, and it was therefore not possible to define values where the inhibitory effect was lost. Thus at pH 7.5 there was still some inhibitory effect with 12 % (v/v) antiserum P 10 at E_h -140 mV, but with 6 % (v/v) antiserum there was no inhibition at 0 mV or below. Similarly, at E_h +60 mV and pH 6.8, inhibition

occurred with 12 % (v/v) antiserum but not with 6 %. This can be contrasted with the results obtained at $E_h + 60$ mV and pH 7.5 where good inhibition was obtained with both 6 % and 12 % (v/v) of antiserum. Following the period of bacterial destruction the viable count started to increase again, although much more slowly than at the beginning of the experiment. The reasons for this are unknown.

Comparatively little is known of the systems involved in the bacteriostatic effect. It seems clear that neither properdin nor complement are necessary since the antiserum can be heated to 56° without loss of activity (Fig. 5). It is suspected that proteins are involved as the antisera can be dialysed without loss of activity in embryonated eggs and *in vitro* (Bullen *et al.* 1961; Bullen & Dobson, 1962). There is also evidence that substances present in normal sera may be concerned since these sera have similar but not such powerful effects (Bullen & Dobson, 1962). It seems quite possible that the bacteriostatic effects of antisera observed *in vitro* have some relevance to the problem of immunity to *Clostridium welchii* infections. It is known that protective antisera have a bacteriostatic effect in the allantoic cavity of living embryonated eggs. In dead eggs, where the conditions should be more reducing, antisera do not inhibit growth (Bullen *et al.* 1961). In severely damaged muscle of mice and guinea-pigs where both the E_h and pH are known to be low, the presence of specific antiserum has no effect on the growth of *C. welchii*. However, in relatively undamaged muscle where the E_h and pH would be expected to be high, the presence of specific antiserum leads to an abrupt cessation of bacterial growth 4 hr after infection (Bullen & Cushnie, 1962). Whether this is due directly to bacteriostatic effects of the specific antisera is a question that cannot be answered at the moment. Nevertheless, there are no reasons for supposing that this could not be the explanation, and it is encouraging that the pattern of growth and inhibition in embryonated eggs, mice or guinea-pigs, is similar to that seen *in vitro*.

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REFERENCES

- BULLEN, J. J., WILSON, A. B. & CORDINER, K. (1961). The effect of normal and immune sera on the growth of *Clostridium welchii* type A in the allantoic cavity of embryonated hen eggs. *J. Path. Bact.* **82**, 383.
- BULLEN, J. J. & CUSHNIE, G. H. (1962). Experimental gas gangrene: the effect of antiserum on the growth of *Clostridium welchii* type A. *J. Path. Bact.* **84**, 177.
- BULLEN, J. J. & DOBSON, A. (1962). Bacteriostatic properties of sera and immunity to *Clostridium welchii* type A. *Colston Pap.* **13**, 127.
- DOBSON, A. & BULLEN, J. J. (1964). A method for the control of E_h and pH during bacterial growth. *J. gen. Microbiol.* **35**, 2.
- FESTENSTEIN, G. N. (1957). Reducing substances in the allantoic fluid of the chick embryo. *Biochem. Biophys. Acta*, **26**, 645.
- NEEDHAM, J. (1931). *Chemical Embryology*, pp. 1094–99 and 1538. Cambridge: The University Press.

EXPLANATION OF PLATE 1

Clostridium welchii type A strain CN2726. Growth in tissue culture medium. Gram stain. The magnification of all figures is 1450.

Fig. 1. Without specific antiserum. pH 7.2. E_h uncontrolled.

Fig. 2. 12 % (v/v) antiserum P10. pH 6.5. $E_h + 59$ mV. No inhibition of growth.

Figs. 3-10. 12 % (v/v) antiserum P10. pH 7.5, $E_h + 59$ mV. Inhibition of growth. Note Gram-negative patches and disintegrated rods.