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Bacteriostatic Effects of Horse Sera and Serum Fractions on *Clostridium welchii* Type A, and the Abolition of Bacteriostasis by Iron Salts

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Summary. Under a variety of conditions of concentration, Eh, and pH, horse anti-*Clostridium welchii* serum and normal horse serum exerted similar bacteriostatic effects against *Cl. welchii* Type A. Ferric iron abolished the bacteriostatic effect when added during the first 2 hours of incubation at Eh+60 mV. Ferrous iron abolished the bacteriostatic effect when added after 3 hours. Ferric iron abolished the bacteriostatic effect at −140 mV. A mixture consisting of horse β_2- and γ-globulins together with human transferrin exerted a bacteriostatic effect similar to that of whole serum. This system responded in the same way as whole serum to the addition of iron. A mixture of horse β_2- and γ-globulins exerted an immediate bactericidal effect which could not be prevented by ferric iron.

INTRODUCTION

Previous work showed that antiserum normally exerted a powerful inhibitory effect on the growth of *Clostridium welchii* type A in the allantoic cavity of embryonated hen eggs (Bullen, Wilson and Cordiner, 1961). After killing the embryo by refrigeration, subsequent incubation allowed the bacteria to multiply rapidly; this was considered to be related to the fall in redox potential (Eh) which accompanies the death of the embryo. Further studies under controlled conditions *in vitro* (Bullen, Dobson and Wilson, 1964) showed that marked inhibition of bacterial growth occurred in the presence of 12 per cent v/v antiserum at pH 7·5 and Eh+60 mV but as the pH and Eh were lowered, the inhibitory power of the serum decreased. The growth pattern of the bacterium in passively immunized animals appeared similar to that observed *in vitro* (Bullen and Cushnie, 1962). This paper attempts to compare the antibacterial effects of normal and immune sera *in vitro* and describes the effect of iron on whole sera and certain serum fractions which may be responsible for the antibacterial effect.

MATERIALS AND METHODS

Organism

Clostridium welchii type A, strain CN 2726 was obtained from the Wellcome Research Laboratories, Beckenham, Kent and used throughout this work. Inocula were prepared as described by Bullen *et al.* (1964) and viable counts were made according to the method of Bullen *et al.* (1961).

Preparation of culture medium

The medium was based on the synthetic medium 199 of Morgan, Morton and Parker (1950); it contained inorganic salts, amino acids, glutathione, glucose and adenine; but no ferric nitrate, vitamins or other growth factors were added. Adenine appears to be essential for the growth of *Cl. welchii* (Fuchs and Bonde, 1957). 'Analar' reagents were used if available; the amino acids were chromatographically homogeneous (The British Drug Houses Ltd, Poole, Dorset). The medium was prepared at ten times the concentration used by Morgan *et al.* (1950). In order to prevent the formation of a precipitate, five separate solutions were prepared and then mixed in numerical order. Solution (1) L-cystine (20 mg), L-tyrosine (40 mg), DL-tryptophane (20 mg) and adenine (5 mg) were dissolved in warm water (5 ml) by the addition of about 0·5 ml of N-hydrochloric acid. Solution (2) (15 ml) contained ten times the quantities of the remaining amino acids used by Morgan *et al.* (1950) together with reduced glutathione (0·5 mg) and L-glutamine (100 mg). Solution (3) (40 ml) contained NaCl (6·8 g), KCl (0·4 g), $NaOAc.3H_2O$ (84 mg) and glucose (1 g). Solutions (4), (5) and (6) (10 ml each) contained respectively, $NaH_2PO_4.2H_2O$ (0·15 g), $CaCl_2.6H_2O$ (0·4 g) and $MgSO_2.7H_2O$ (0·2 g). The final volume was 100 ml. After sterilizing by membrane filtration, the medium was dispensed in 20 ml amounts into new screw cap bottles and then stored at $-20°$.

The vitamins of medium 199 (Morgan *et al.*, 1950) were replaced by a 1 per cent w/v aqueous solution of liver extract ('Panmede' manufactured by Paines and Byrne Ltd, Pabyrn Laboratories, Greenford, Middlesex) which was prepared freshly each week and stored at 4°. The solid was stored in small portions at $-20°$. Sodium bicarbonate solution (6 per cent w/v) was sterilized by autoclaving (15 minutes at 15 lb/in^2) in tightly capped bottles and then stored at 4°. Solutions of the redox dyes (2·0 mM) were prepared freshly each week and sterilized by heating in boiling water for 10 minutes.

The iron concentration in the basic medium was found to be 2·6 μM (Collins, Diehl and Smith, 1959). When allowance was made for the iron content of the liver extract (0·04 per cent) the total iron concentration in the medium when in use was about 0·27 μM.

Anaerobic culture apparatus

Essentially the apparatus consisted of a small scale version of that described by Dobson and Bullen (1964). Glassware was made by Quickfit and Quartz Ltd, Stone, Staffs. The organisms were grown at 37° in a two-necked, jacketed culture vessel (A) (Fig. 1) in which the contents were stirred magnetically. The head (B) when fitted into the B19/26 socket of the culture vessel, allowed the N_2/CO_2 gas mixture to enter the vessel through the B7/16 side arm and the central vertical tube. The vertical B10/19 socket carried the platinum electrode assembly. Exhaust gases left the apparatus through the narrow outlet tube which also served as an inoculating and sampling point. The electrode assembly consisted of 3×20 cm lengths of 30 S.W.G. platinum wire each of which had a 7 cm length of 20 S.W.G. copper wire soldered to its upper end. Where necessary, the wires were coated with an epoxy resin ('Araldite', Ciba Ltd, Duxford, Cambridgeshire) to provide electrical insulation. Each platinum wire was then sealed into a soft glass capillary tube leaving 2 mm of wire protruding at the lower end. The three tubes were then sealed together into a $0·3 \times 17·5$ cm soft glass tube and the whole assembly fitted into a B10/19 gas inlet. The two pieces of glass were connected by means of a silicone rubber sleeve. The three copper wires finally passed through a No. 9 silicone rubber stopper at the top of the gas inlet. The electrodes were connected to the millivoltmeter through a multi-position

switch. When in use, the B10/19 socket of the culture vessel was fitted with a No. 9 silicone rubber stopper which carried three lengths of polyethylene tubing (1 mm internal diameter). One tube contained 3·5 M potassium chloride in 3 per cent agar for connection to the 3·5 M KCl calomel reference electrode. The second and third tubes were connected to separate magnetic valves (Radiometer, Copenhagen, type MNV1). One valve was used to admit expired air from a balloon to raise the Eh as required whilst the other was connected to a small reservoir of freshly prepared 0·5 per cent sodium dithionite in 0·001 N sodium hydroxide (Michaelis, 1946). The Eh of the medium was controlled by means of a Radiometer titrator type TTT1, operating through the electrodes and valves already described.

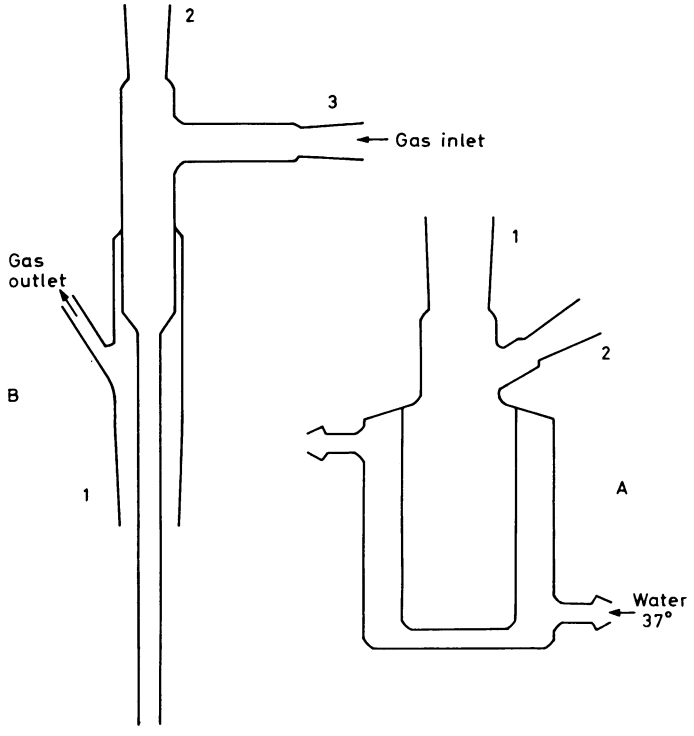

FIG. 1. Anaerobic apparatus. A, Jacketed culture vessel: (1) B19/26 socket for head B; (2) B10/19 socket for initial gassing and connections to magnetic valves. B, Head: (1) B19/26 cone; (2) B10/19 socket for platinum electrode assembly; (3) B7/16 socket.

The vessel was cleaned by soaking in a 1:1 mixture of 'Analar' nitric and sulphuric acids and then rinsed in de-ionized water; all other glassware was soaked in a 1 per cent solution of 'Pyroneg' (Diversey (UK) Ltd, Cockfosters, Hertfordshire) followed by N-hydrochloric acid before rinsing in distilled water.

Operation

The sterile vessel was charged with synthetic medium (1·5 ml), 1 per cent w/v 'Panmede' (0·3 ml), dye solution (0·23 ml, final concentration 30 μM) and sufficient water to give a final volume of 15·0 ml, after allowing for the sodium bicarbonate, serum and inoculum which were to be added later. The culture vessel was evacuated in an anaerobic jar to 15 mm pressure in order to remove dissolved air. After placing the vessel on a magnetic

stirrer, water at 37° was pumped through the jacket and a sterile oxygen free, CO_2/N_2 mixture was passed into the medium through the B10/19 socket. A standardized combined electrode (Radiometer, type GK2021C) was used to measure the pH of the solution which was raised to 7·0–7·2 by the addition of 6 per cent w/v sodium bicarbonate solution. After adding the serum sample, the pH was carefully raised to 7·50 (0·60–0·65 ml, 6 per cent w/v $NaHCO_3$ required). After 20 minutes the head carrying the platinum electrodes was fitted and gas was then admitted through the B7/16 side arm. After adjusting the Eh of the medium to the required value, the inoculum (0·15 ml in 10 per cent v/v broth–saline) was added.

Experiments at pH 7·5 were carried out under an atmosphere of 95 per cent N_2+5 per cent CO_2 whilst at pH 7, 75 per cent N_2+25 per cent CO_2 was used. The pH of the culture fluid at the end of an experiment in which inhibition of growth occurred was never found to be more than 0·05 units higher than the initial value.

The behaviour of the system was very similar to that described by Dobson and Bullen (1964) and although the millivoltmeter used in the present work was less sensitive, the Eh could normally be maintained within ±2 mV of the required value.

Sera

The preparation of antiserum P10 has been described previously (Bullen *et al.*, 1964); the antitoxin content in units per 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 normal horse serum (EX 3366, The Wellcome Research Laboratories) was obtained from an unimmunized horse and selected for its low antitoxin content which was less than 0·01 α-antitoxin units and less than 0·5 θ-antitoxin units per ml. Serum samples were sterilized by filtration through a cellulose ester membrane, pore size 0·1–0·5 μ (Oxo Ltd, Queen Street Place, London, E.C.4) and stored at −20°.

Purification of reagents

Deionized water was obtained by passing glass-distilled water over a mixed bed ion exchange resin. Twice distilled, constant boiling hydrochloric acid was prepared from a 1:1 mixture of 'Analar' hydrochloric acid and deionized water; after standardization, it was diluted to give a 1·0 N solution.

Anhydrous 'Analar' disodium hydrogen phosphate was twice re-crystallized from deionized water and stored as the dodecahydrate. 'Analar' sodium chloride was recrystallized by dissolving in water at 50° and after filtering, slowly adding an equal volume of ethanol to the cold solution.

Dialysis

Phosphate buffered saline (pH 7·4) was prepared from NaCl (9·93 g), $Na_2HPO_2.12H_2O$ (5·77 g) and 1·0 N HCl (2·2 ml) made up to 1·0 l. Whole sera and fractions (10 ml) were dialysed against the buffer at 2° by the method of Craig and King (1955) using 5 × 100 ml volumes changed at 30 minute intervals followed by 500 ml overnight.

Reduction of ferric iron at −140 mV

Culture medium (15 ml) containing 50 μM ferric iron (ferric ammonium sulphate) was maintained at Eh −140 mV, pH 7·5 and 37° for 1 hour after which a sample (2·5 ml) was withdrawn and added to 4 N-acetate buffer pH 5·2 (0·1 ml). To the remaining medium was added a ten-fold excess of 2,2′-dipyridyl in ethanol (0·12 ml). After 5 minutes a

sample (2·5 ml) was withdrawn and mixed with acetate buffer (0·1 ml). This solution, together with an authentic sample of the 2,2′-dipyridyl complex of ferrous iron, had λ max = 522 mμ. From the extinction (0·326) at 522 mμ the concentration of ferrous iron in the medium appeared to be 40 μM.

Fractionation of horse serum

Horse serum No. 3 (The Wellcome Laboratories), after dilution 1:2 in 0·05 M Tris–acetate, pH 8·3, was dialysed against the same buffer and then subjected to continuous electrophoresis employing a potential gradient of 1700 V (Hannig, 1961). Fractions having mobilities lower than albumin were designated β_1-, β_2- and γ-globulins.

Isolation of human transferrin

Pooled human serum was fractionated by the rivanol procedure of Sutton and Karp (1965) at 2°. No iron was added initially to the serum and it proved unnecessary to absorb the rivanol on starch as it was not retained by DEAE-Sephadex A-50. The transferrin from 200 ml of human serum was eluted from a 7×4 cm bed of DEAE-Sephadex in 150 ml 5 mM Tris–hydrochloride, pH 8·8 containing 0·5 M sodium chloride.

After concentration, the solution (20 ml) was dialysed against 0·05 M Tris–hydrochloride, pH 8 and then applied to a 2·0×50 cm column of DEAE-Sephadex (A-50, fine) equilibrated at 4° with 0·1 M Tris–hydrochloride, pH 8. Linear gradient elution to 0·18 M Tris–hydrochloride, pH 8 (400 ml in each vessel) was carried out with a flow rate of 60–70 ml/hour, each fraction being approximately 5 ml. Protein in the effluent was detected by the extinction at 280 mμ. The recovery of transferrin from tubes 110–140 approached 90 per cent as estimated from the total iron binding capacity of the sample.

Iron estimations

Serum iron was estimated by the method of Wooton (1964) but using, in place of tripyridyl, 2,4,6-tripyridyl-1,3,5-triazine (12 mg) dissolved in 80 per cent ethanol (12 ml) containing ascorbic acid (160 mg). For the determination of the unsaturated iron binding capacity, serum (2·4 ml) was mixed with 5 per cent w/v $NaCHO_3$ solution (0·05 ml). The extinction at 470 mμ (Aasa, Malmström, Saltman and Vänngård, 1963) against a serum blank, was recorded 15 minutes after each addition of aliquots (0·05 ml) of a 1·5 mM ferric nitrate solution. The unsaturated iron binding capacity was then calculated by the method of Schade and Caroline (1946).

RESULTS

MEDIUM

Using freshly prepared commercial samples of medium 199, it was found that the initial growth phase observed in the presence of specific immune antiserum was followed by a period of bacteriostasis (up to 4 hours). After the medium had been stored for 1 month at 4° considerable destruction of the bacteria occurred following the initial growth phase. After allowing for experimental error, results obtained after storing the semi-synthetic medium and solid liver extract at −20° for 3 months, were identical with the results obtained initially. Growth of the organism in this medium was similar to that observed in medium 199, the generation time being 20 minutes at pH 7·5 and Eh +60 mV.

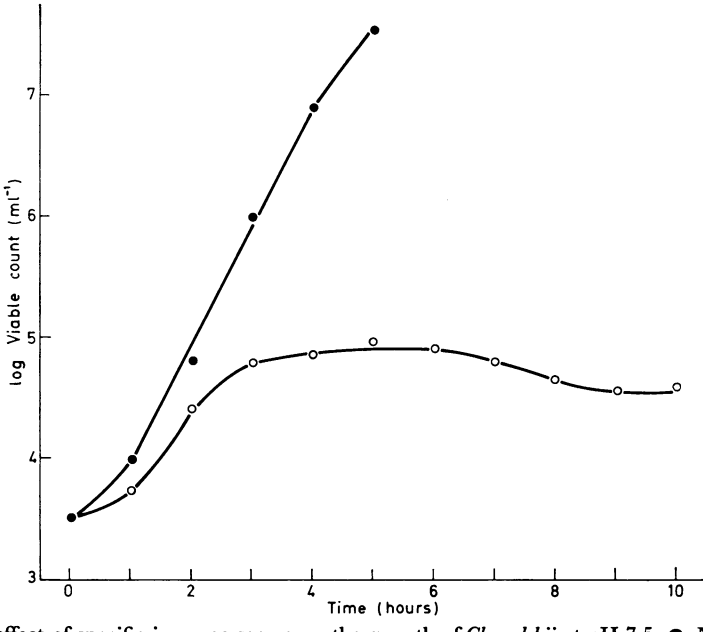

FIG. 2. The effect of specific immune serum on the growth of *Cl. welchii* at pH 7·5. ●, No serum, Eh +60 mV; ○, 12 per cent v/v immune serum, Eh 0 mV.

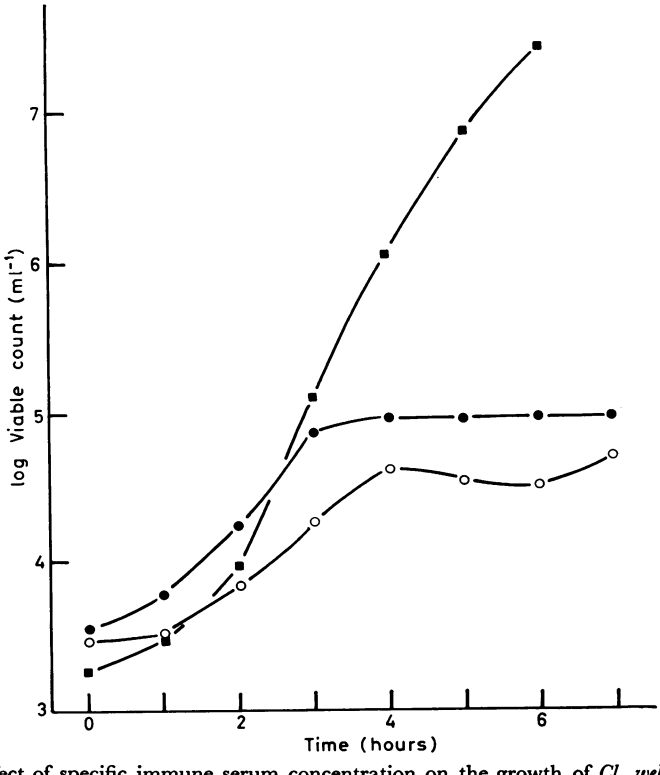

FIG. 3. The effect of specific immune serum concentration on the growth of *Cl. welchii* at pH 7·5. ●, 12 per cent v/v, Eh 0 mV; ○, 6 per cent v/v, Eh 0 mV; ■, 3 per cent v/v, Eh +60 mV.

A population of 5 to 10×10^7 per ml was reached at 5 hours (Fig. 2). In the presence of 12 per cent v/v antiserum at 0 mV the initial bacterial growth was followed by bacteriostasis for at least 7 hours (Fig. 2).

THE EFFECT OF NORMAL AND SPECIFIC IMMUNE SERUM

Figs. 3 and 4 show the antibacterial effect of increasing concentration of specific immune serum and normal serum respectively; no inhibition occurred in the presence of 3 per cent v/v serum. The fact that Bullen *et al.* (1964) found no inhibition with 6 per

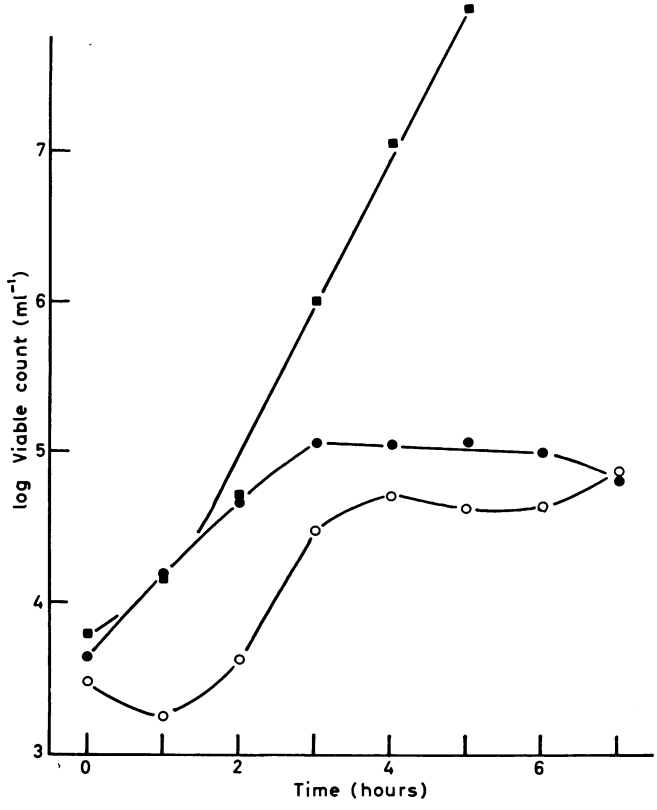

FIG. 4. The effect of normal serum concentration on the growth of *Cl. welchii* at pH 7·5. ●, 12 per cent v/v, Eh 0 mV; ○, 6 per cent v/v, Eh 0 mV; ■, 3 per cent v/v, Eh +60 mV.

cent v/v specific immune serum at 0 mV, whilst inhibition was observed in this experiment, probably results from some difference in the media. These conditions might be expected to favour the detection of differences in the antibacterial power of the two sera but in fact no significant difference was observed.

THE EFFECT OF NORMAL AND SPECIFIC IMMUNE SERUM AT Eh −140 mV

The results obtained at −140 mV and pH 7·5 (Fig. 5) suggested that the normal serum exerted a slightly more powerful inhibitory effect under these conditions.

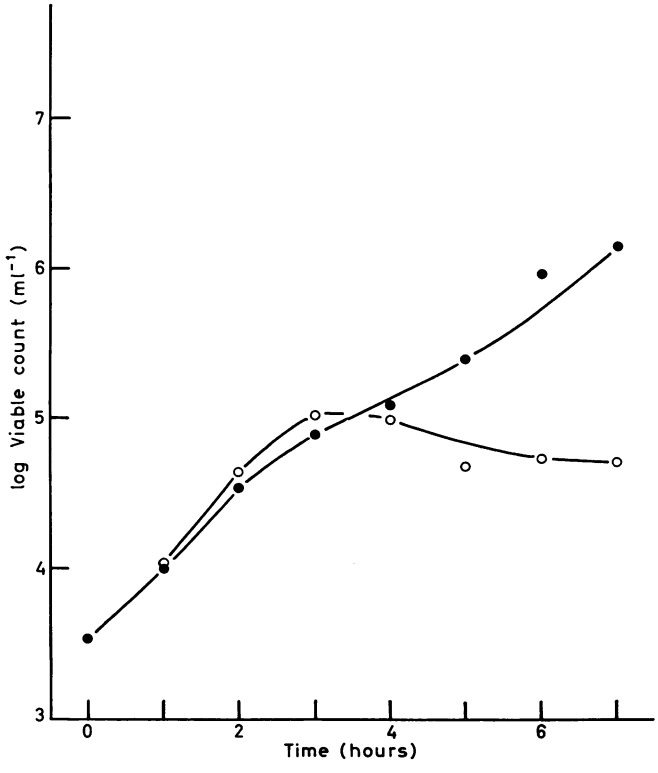

FIG. 5. The effect of 12 per cent v/v immune serum (●) and normal serum (○) at Eh −140 mV, pH 7·5.

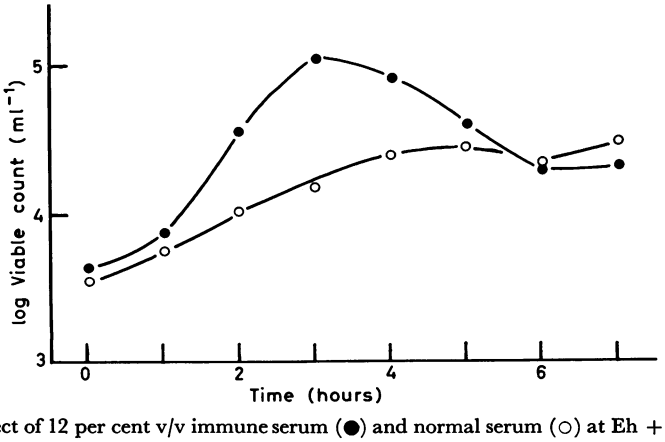

FIG. 6. The effect of 12 per cent v/v immune serum (●) and normal serum (○) at Eh +60 mV, pH 7·0.

THE EFFECT OF NORMAL AND SPECIFIC IMMUNE SERUM AT pH 7

Bullen *et al.* (1964) found that the antibacterial effect of specific immune serum decreased as the pH was lowered. At pH 7, Eh +60 mV (Fig. 6) it appeared that the immune serum was less inhibitory than the normal serum for the first 4 hours.

THE EFFECT OF IRON ON BACTERIOSTASIS

It was found that some samples of serum lost their antibacterial effect after dialysis against isotonic phosphate buffer, pH 7·4, containing a mixture of disodium hydrogen phosphate and potassium dihydrogen phosphate. Bornside, Merritt and Weil (1964) showed that ferric iron abolished the antirespiratory activity of rabbit serum on *Bacillus subtilis*. Since tests showed that the 'Analar' potassium dihydrogen phosphate was contaminated with iron, it appeared worthwhile to examine the effect of iron on horse serum. Fig. 7 shows the effect of adding ferric iron (final concentration 10 μM as ferric ammonium

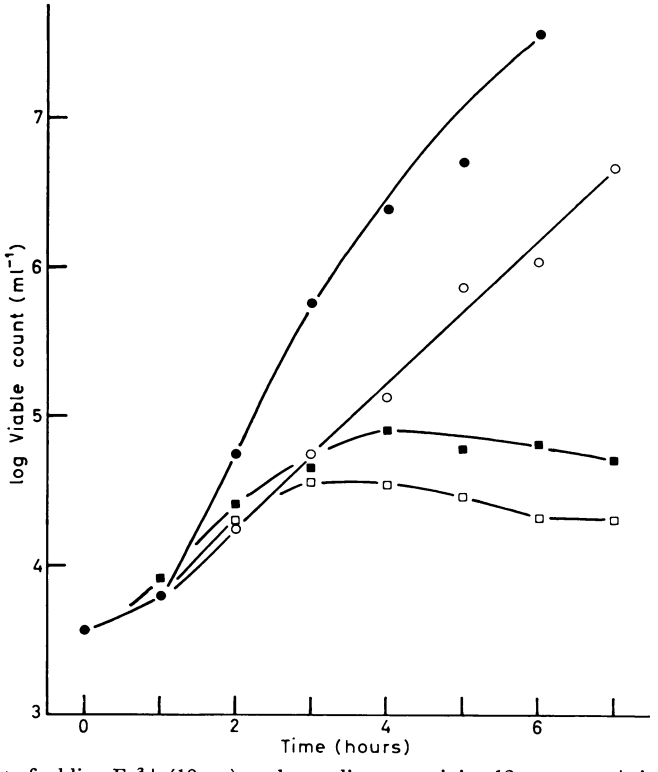

FIG. 7. The effect of adding Fe^{3+} (10 μM) to the medium containing 12 per cent v/v immune serum at Eh +60 mV, pH 7·5. Iron added at 0 (●), 1 (○), 2 (■) and 3 (□) hours.

sulphate) at various times to the system containing 12 per cent v/v immune serum at +60 mV. When added at 0 hours, iron completely abolished the antibacterial power of the serum but was progressively less effective after this time, there being no detectable effect at 3 hours. Ferric iron (10 μM) also failed to abolish the antibacterial effect of normal serum when added at 3 hours (Fig. 8).

In the case of ferrous iron (10 μM as ferrous ammonium sulphate), rapid abolition of the inhibition at +60 mV occurred when the salt was added at 3 hours (Fig. 8). At 0 mV, 1 μM ferrous iron added at 3 hours caused rapid growth of the organisms. These effects seemed to depend upon the oxidation state of the iron rather than the Eh of the medium, since ferric iron, added at 3 hours to the system maintained at 0 mV, did not abolish the inhibition. These observations suggested that the ferrous–ferric system was not in true

equilibrium under these conditions although the E_0' would be expected to lie at about −100 mV (Michaelis and Friedheim, 1931). It was found that the half life for the aerial oxidation of a 30 μM ferrous iron solution in 0·15 M Tris–hydrochloride, pH 7·4, was 13 minutes at 20°, hence ferrous iron should be relatively stable in the culture fluid at neutral pH. At Eh −140 mV however, ferric iron (10 μM) rapidly abolished the inhibitory effect of normal serum when added at 3 hours (Fig. 8). In a control experiment ferrous

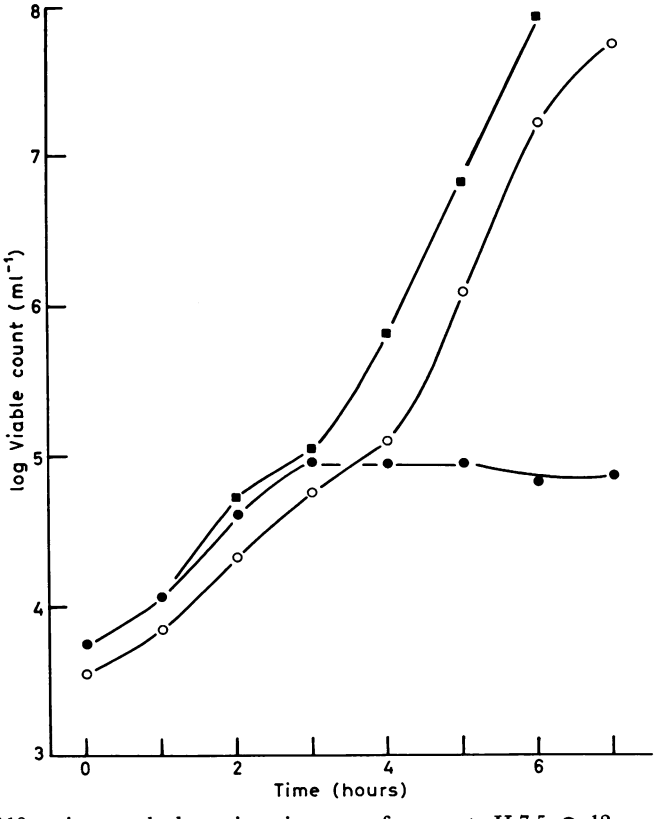

FIG. 8. Effect of 10 μM iron on the bacteriostatic power of serum at pH 7·5. ●, 12 per cent v/v normal serum, Eh +60 mV, Fe^{3+} added at 3 hours; ○, 12 per cent v/v immune serum, Eh +60 mV, Fe^{2+} added at 3 hours; ■, 12 per cent v/v normal serum Eh −140 mV, Fe^{3+} added at 3 hours.

iron was detected spectroscopically as the 2,2′-dipyridyl complex 1 hour after adding ferric iron to the medium maintained at −140 mV. From these observations it would appear that at Eh values considerably below 0 mV ferric iron was reduced to the ferrous form which then abolished the inhibitory effect of the serum.

The above results could indicate that serum produces some derangement of the bacterial iron metabolism. Cells grown in broth gave a positive test for iron when treated with 2,2′-dipyridyl. Pappenheimer and Shaskan (1944) made a similar observation with *Cl. welchii* PB6K. When grown in media having iron concentrations less than 9 μM, the bacteria carried out a homolactic type of fermentation which may be related to the failure to degrade pyruvate (Wolfe and O'Kane, 1953). Cobalt can replace the iron required for the degradation of pyruvate which normally serves as the ultimate source of electrons for

hydrogen production. Glycolysis in clostridia requires iron as a prosthetic group for aldolase but again cobalt can replace iron (Bard and Gunsalus, 1950).

The Eh of actively growing cultures of *Cl. welchii* CN 2726 approached that of the hydrogen electrode at neutral pH (−420 mV) and it may be assumed therefore that the cells contain hydrogenase. Iron appears to be involved in electron transport in clostridia both as a component of hydrogenase (Peck and Gest, 1956) and ferredoxin (Sobel and Lovenberg, 1966); the functions of the latter can be carried out by the dye, methyl viologen ($E_0' = -440$ mV) (Valentine, Mortensen and Carnhan, 1963). It was found that cobalt (10 μM cobaltous chloride), methyl viologen (10 μM) and pyruvate (10 mM) when added separately or together at 0 hours to the medium containing 12 per cent v/v antiserum at 0 mV, did not abolish the inhibition.

ANTIBACTERIAL EFFECTS OF SERUM FRACTIONS

As several workers (Schade, 1960, 1963; Bornside *et al.*, 1964; Martin, 1962) have attempted to define a possible role for the serum iron binding protein, transferrin, in non specific resistance, on the basis of its potential ability to render iron unavailable to pathogens it was of some interest to examine the role of transferrin, together with the immune globulins as possible inhibitory agents for *Cl. welchii*. Attempts to isolate horse transferrin by the rivanol procedure were not successful as the product finally isolated by ion exchange chromatography contained some β_2-globulin. Human transferrin which was 23 per cent saturated with iron and gave a single arc on immunoelectrophoresis, was isolated as described in the experimental section. The saturated and unsaturated iron-binding capacities (in moles iron per litre) of the serum samples are shown in Table 1; the human transferrin was assayed at a concentration corresponding to that of the horse antiserum.

TABLE 1

IRON-BINDING CAPACITY OF HORSE SERA AND HUMAN TRANSFERRIN

	Saturated iron binding (μM)	Unsaturated iron binding (μM)	Saturation (%)
Horse antiserum (P10)	23	104	18
Normal horse serum (Ex 336)	11	136	7·5
Human transferrin	36	121	23

Horse β_2- and γ-globulins were isolated by continuous electrophoresis and after concentration and dialysis were diluted to the same volume as the serum sample from which they were isolated. Samples taken for assay therefore corresponded approximately in globulin content to whole serum. Immunoelectrophoresis of the two globulins produced somewhat diffuse arcs with the expected electrophoretic mobilities.

When tested separately at 0 mV and at a level corresponding to 12 per cent v/v serum the transferrin caused some increase in the lag phase but the β_2- and γ-globulins had no effect on the bacterial growth pattern (Fig. 9). When transferrin was tested with either each globulin separately, or both globulins together, the growth curve was in each case similar to that obtained in the presence of whole serum (Fig. 10).

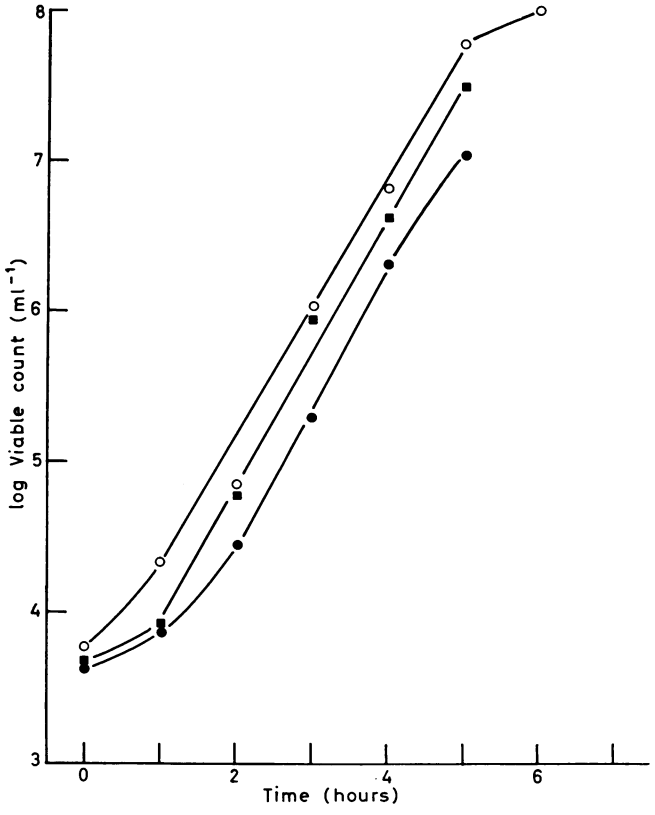

FIG. 9. Growth of *Cl. welchii* in the presence of fractions corresponding to 12 per cent v/v serum at Eh 0 mV, pH 7·5. ●, Human transferrin; ○, horse β_2-globulin; ■, horse γ-globulin.

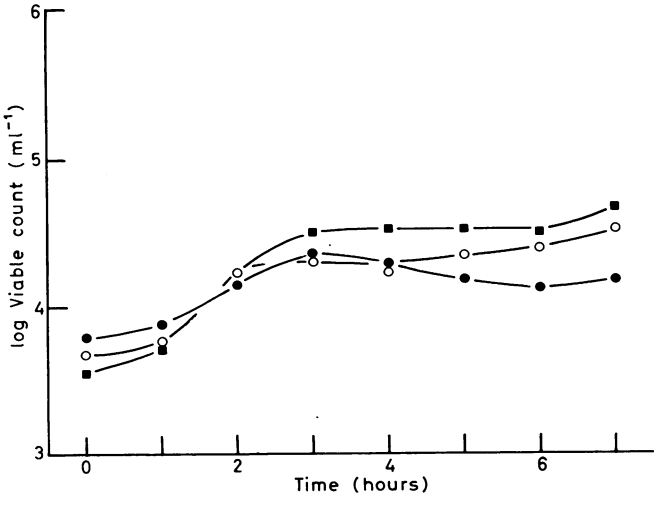

FIG. 10. The effect of mixed fractions equivalent to 12 per cent v/v serum, on the growth of *Cl. welchii* at Eh 0 mV, pH 7·5. ●, Human transferrin and horse β_2-globulin; ○, human transferrin and horse γ-globulin; ■, human transferrin, horse β_2- and γ-globulins.

A mixture of both globulin solutions (12 per cent v/v β_2- plus 12 per cent v/v γ-globulin) had an immediate bactericidal effect in the medium at 0 mV as the viable count taken 5 minutes after inoculating $4{\cdot}7 \times 10^3$ organisms ml corresponded to less than 10^2/ml and no bacteria could be detected at 5 hours in 0·1 ml of culture fluid spread over the surface of a blood agar plate. When the globulin solutions were tested together at 6 and 3 per cent v/v normal growth of the bacteria occurred.

THE EFFECT OF IRON ON SERUM FRACTIONS

An examination of the effect of iron on the serum fractions should indicate how closely this system resembles whole serum. Comparison of Fig. 11 with Fig. 7 shows that bacterial growth in the presence of the three serum fractions and ferric iron was somewhat slower than that found with whole serum and ferric iron. When added at 3 hours to the mixture

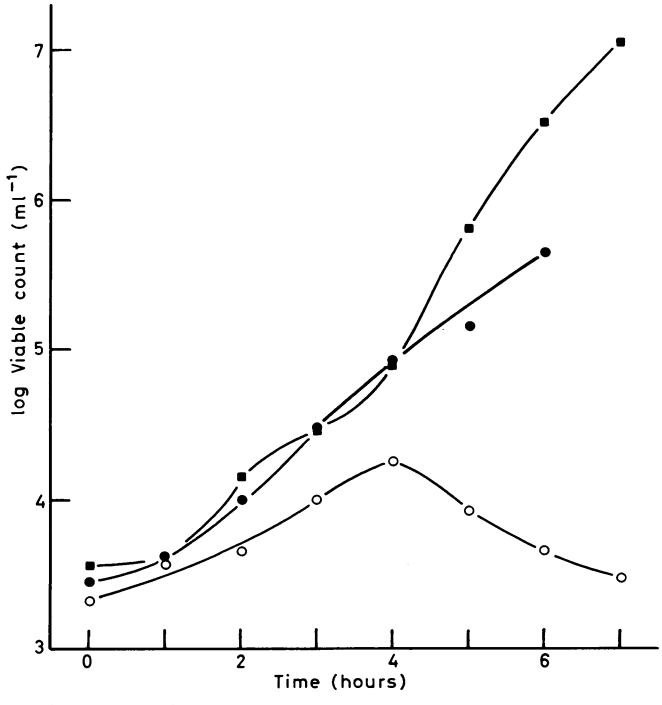

FIG. 11. The effect of iron at pH 7·5 on the bacteriostatic power of the mixed fractions equivalent to 12 per cent v/v serum. ●, 10 μM Fe^{3+}, added at 0 hours, Eh +60 mV; ○, 10 μM Fe^{3+}, added at 3 hours, Eh 0 mV; ■, 1 μM Fe^{2+}, added at 3 hours, Eh 0 mV.

maintained at 0 mV, ferric iron had no effect but ferrous iron abolished the bacteriostatic effect (see Figs. 11 and 8). Ferric iron when added at 0 hours to the medium at 0 mV containing 12 per cent v/v of each globulin solution, failed to prevent the bactericidal effect described above. The viable count did however remain at about 10^2/ml for 3 hours and then increased to $8{\cdot}3 \times 10^3$ by 6 hours.

DISCUSSION

In order to obtain reliable information concerning the antibacterial properties of serum and its component proteins it is essential that the bacterial culture medium should give a

reproducible pattern of growth. Since commercial samples of TC medium 199 proved to be unsatisfactory in this respect, the semi-synthetic medium was developed. It is interesting to note that the inhibitory effect of serum upon the growth of *Cl. welchii* CN 2726 at +60 mV, has now been observed with four different media namely, allantoic fluid, Bacto-casitone medium supplemented with yeast extract, commercial samples of tissue culture medium 199 (Bullen and Dobson, 1962; Bullen *et al.*, 1964) and the semi-synthetic medium used in the present work. The inhibitory power of the serum does depend to some extent upon the medium used since the bacterial destruction and regrowth observed after 3 hours in commercial samples of medium 199 is not seen with either Bactocasitone (unpublished observations) or the semi-synthetic medium where the bacterial count remains practically constant after 3 hours. The initial observations on the ability of iron salts to abolish the inhibition were made whilst using medium 199.

Comparison of the antibacterial effects of normal and specific immune serum at varying concentrations and under different conditions of Eh and pH suggests that they exert similar antibacterial effects and since ferric iron is unable to abolish the inhibitory effect of either serum when added at 3 hours, they may produce the same biochemical lesion. Using different serum samples, Bullen and Dobson (1962) also noted some similarities in the antibacterial effects of normal and immune sera *in vitro*.

Twelve per cent of the normal and immune serum corresponds respectively to an α-antitoxin content of 0·0012 and 15·6 units/ml of medium. Since these sera appear to exert very similar antibacterial effects it would seem that the α-antitoxin plays little or no part in the inhibitory process *in vitro*. In the case of the embryonated egg, normal serum affords protection only against small numbers of bacteria whilst specific immune serum protects against much larger numbers (Bullen *et al.*, 1961). In this case the protective effect may be related to the level of α-antitoxin required to prevent damage to the chorio-allantoic membranes.

In the absence of added ferric iron, the transferrin present in medium containing 12 per cent v/v antiserum would be 18 per cent saturated, whilst in a similar solution containing iron at a concentration of 10 μM it would be 83 per cent saturated. The growth curves obtained under these conditions show some resemblance to those obtained with staphylococci growing in the presence of egg white which contains the iron binding protein, conalbumin (Theodore and Schade, 1965). This suggests that the growth of *Cl. welchii* CN 2726 in the presence of horse serum may be related to the degree of saturation of the transferrin. The fact that the concentration of ferrous iron (1 μM) required to abolish the inhibition of 12 per cent v/v serum, is insufficient to saturate the transferrin (12 μM in unsaturated iron binding capacity) and also bears no obvious stoichiometric relationship to the 7S globulin content (approximately 20 μM) suggests that the metal interacts directly with the bacteria.

The progressive lack of response to added ferric iron at Eh +60 mV indicates that the serum produces some change in the bacterial metabolism which eventually results either in the failure to take up iron from the transferrin complex, or failure of the cell to reduce ferric iron to the ferrous form which appears to be essential for both glycolysis and electron transport (Mortensen, 1963). Although it has not been possible to interfere with the inhibitory process by the addition of substances which might be expected to replace iron, the lesion induced by the serum may well be relatively specific since the cells remain viable for several hours although multiplication is prevented.

The shape of the growth curve obtained in the presence of transferrin together with

β_2- and γ-globulin suggests that these fractions together may represent the active components of whole serum and this idea is supported by the fact that the system responds to the addition of iron in a manner similar to that of whole serum. Since transferrin is an extremely powerful iron binding agent (Aasa *et al.*, 1963), it was surprising to find that it was unable to prevent growth of the bacteria. Schade (1960) suggested that it may be difficult to isolate transferrin capable of inhibiting bacterial growth because of the ease with which it appears to become physiologically denatured. Bornside *et al.* (1964) found that a purified preparation of rabbit transferrin containing a relatively large amount of iron (70 per cent saturated) actually stimulated the respiration of *B. subtilis*; in the present work the iron content of the transferrin was relatively low (23 per cent saturated). Although no quantitative test has been applied to test for denaturation of the transferrin preparation this would seem to be unlikely in view of the close resemblance of the bacterial growth curves obtained in the presence of the fractions to those obtained with whole serum.

The bactericidal effect of β_2- and γ-globulins remains unexplained but it is interesting to note that ferric iron was unable to prevent this although when added to a mixture of the three fractions, ferric iron allowed relatively rapid growth to proceed. This observation is at variance with that of Bornside *et al.* (1964) which suggested that in the case of rabbit serum, iron inactivated the 7S globulins which normally exerted an antirespiratory effect on *B. subtilis*.

Examination of the result obtained with *Cl. welchii* CN 2726 suggests that the behaviour of transferrin presents something of a paradox in that although it forms a bacteriostatic system with either β_2- or γ-globulin, it also appears to prevent the rapid lethal effect exerted by β_2- and γ-globulin together.

The results presented above indicate that when the organism is exposed to the simultaneous action of transferrin and β_2- or γ-globulin, some change in the bacterial iron metabolism takes place. β_2- and γ-globulins do not appear to be specific iron binding proteins (Itzhaki, 1961). It may be that the β_2- and γ-globulin fractions interact with the bacterial cell in a way which impairs its normal capacity to remove iron from the transferrin complex, the growth observed during the first 3 hours then proceeds at the expense of endogenous iron until this reaches a level below which further multiplication becomes impossible. At present no information is available concerning the nature of the bactericidal effect of the immune globulins or the way in which transferrin prevents this.

The pH and Eh dependence of the antibacterial effect of serum (Bullen *et al.*, 1964) could be related to the fact that the dissociation constant of the transferrin complex increases as the pH is lowered (Fiala, 1949) whilst ferrous iron dissociates more readily than ferric iron from conalbumin (Schade and Caroline, 1944). The small differences in the activities or normal and immune sera at low pH and Eh may be related to their different iron contents (Table 1).

When considering the antibacterial properties of serum, with respect to Gram-positive bacteria it is essential to distinguish relatively prolonged bacteriostatic effects from immediate bactericidal effects. In the present case the action is bacteriostatic and does not depend upon the complement system (Bullen *et al.*, 1964). It is probably unrelated to β-lysin which is bactericidal, adsorbed by Seitz filters (Donaldson, Ellsworth and Matheson, 1964) and appears to arise from disintegrating platelets (Hirsch, 1960; Jago and Jacox, 1961). Horse serum exerts its bacteriostatic effect after membrane or Seitz filtration (Bullen, personal communication).

The present results are in agreement with those of other workers on the role of iron

in abolishing the antibacterial effects of serum (Jackson and Morris, 1961; Schade, 1963). Weinberg (1966) has recently reviewed this topic and indicated, as did Schade (1960), that transferrin may be of importance in non-specific resistance. Martin (1962) showed that the unsaturated iron binding capacity and antirespiratory activity to *B. subtilis in vitro* were both increased in hypogammaglobulinaemia; this increase however appears to be unable to compensate for the low immune globulin concentration *in vivo* as these patients are susceptible to repeated Gram-positive infections (Wood, Burgess and Morginson, 1961). The observations presented here indicate that transferrin together with β_2- and γ-globulin are required to form a bacteriostatic system for *Cl. welchii* type A *in vitro*. Results to be presented in the following paper (Bullen, Cushnie and Rogers, 1967) indicate that these observations may be of some relevance to resistance *in vivo*.

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