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Some Factors affecting the Viability of Faecal Bacteria in Water

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SUMMARY: The effect of various conditions on the rate of death of *Bacterium coli* and of *Streptococcus faecalis* in dilute buffer solutions was followed by making colony counts at intervals during prolonged periods of incubation and then constructing mortality curves. The form of these curves was characteristic for each organism. Of the pH values investigated (approximately 5, 6, 7 and 8) both organisms were least viable in the range 6 to 7. *Strep. faecalis* was not affected by the degree of oxygenation of the water but *Bact. coli* died much more rapidly under anaerobic than under aerobic conditions.

Growth of *Bact. coli* was observed with as little as 0.28 part per million organic matter in solution. A much higher concentration of nutriment was required for growth of *Strep. faecalis*; with a concentration slightly below that required for growth the viable population remained virtually stable for a long period. The rate of death depended on the age of the cells at the time of immersion in water.

Factors affecting the longevity in water of organisms used as indices of pollution are of importance in the sanitary survey of rivers or in assessing the suitability of water for drinking purposes. Reports in the literature suggest that the period of survival may be conditioned by a number of factors but the conclusions drawn are often open to doubt owing to failure to arrange for conditions to be the same in comparable experiments. Moreover, in earlier work there is often some doubt of the precise characters of the organism referred to as *Bacterium coli*. Very few observations appear to have been made on the longevity of *Streptococcus faecalis*.

METHODS

The factors studied were pH value, oxygen tension, concentration of nutrient material, and age of culture at time of immersion in water. In the investigation of any one factor, precautions were taken to ensure that other conditions remained constant. The strains of bacteria used had all been isolated from sewage or river water and before use in this work were tested for characteristic reactions. These were: *Bact. coli* (type I): Gram negative, non-sporing short rod, indole +, MR +, VP -, citrate -, acid and gas in MacConkey broth at 44°; *Strep. faecalis*: Gram positive coccus in pairs and short chains, acid clot in litmus milk, growth at 45°, growth in broth containing 6.5 % sodium chloride, survives heating for 30 min. at 60°, growth at pH 9.6. Incubation was at 20° unless otherwise stated.

Very dilute phosphate buffer solution (0.003M) was used as the suspending fluid, a concentration sufficient to maintain the pH value virtually constant throughout the course of an experiment. The solution was sterilized by auto-

claving either in hard glass bottles fitted with stoppers or in conical flasks covered with beakers; cotton wool or other organic material was not allowed in contact with the buffer or with the inside of a flask. Unless anaerobic conditions were being studied the solution was aerated with sterile diffused air for 1 hr. before use.

Colony counts were made either in Petri dishes or by the spinning bottle technique described by Proom & Hemmons (1949). Nutrient agar was used as the medium for *Bact. coli*, glucose yeast extract agar for *Strep. faecalis*; inoculated plates and bottles of both media were incubated at 37°.

RESULTS

Form of death curve

When numbers of surviving bacteria, expressed as percentages, were plotted against the periods for which the culture had been suspended in buffer, the form of the curve was usually sigmoid, as in Fig. 1. Such curves approached the horizontal axis so gradually that an exact period of survival could not be estimated. When, however, the percentages of bacteria surviving were replaced by the logarithms of the percentages, the curves approached the horizontal axis much more steeply, as in Fig. 2. The period of survival was therefore conveniently expressed as the time corresponding to the point of intersection of such a curve with the axis, i.e. the time required for destruction of 99 % of the cells originally present.

The curves for *Bact. coli* and *Strep. faecalis* were characteristically of different shapes, and corresponded to the two principal types of deviation from the logarithmic form discussed by Hinshelwood (1951).

With *Bact. coli* a large section of the 'log-survivors' curve sometimes approximated to a straight line, as in Fig. 2 (*Bact. coli*), but often the final stages of decline might be abnormally protracted so that the curve turned outwards at the lower end, as in Fig. 3 and in curve *A*, Fig. 5. This could be explained by the presence of cells of abnormally high resistance, which Hinshelwood suggests may arise from a previous mutation. Some support for this view was obtained in one set of experiments where the deviation was particularly marked and plate cultures of the declining population showed the presence of both smooth and rough forms of colony, both of which when tested gave characteristic reactions for *Bact. coli*. This variation was, however, only transient for, on preparing cultures from the two types of colony and comparing their rates of decline when suspended in dilute buffer solution, both the 'log-survivors' curves were found to approximate closely to straight lines and to show very similar rates of decline. Platings from both these suspensions now showed the presence of only smooth colonies.

With *Strep. faecalis* some time elapsed before the lethal action of the water took full effect but then it acted comparatively rapidly. The 'log-survivors' curve sloped very gently at first and then quite steeply. In some tests there was no appreciable diminution in count for several days and then in a further similar period of time the suspension was virtually totally destroyed.

Effect of pH value

Rates of death were determined at pH values approximately 5, 6, 7 and 8, in buffer solutions containing 5 parts per million (p.p.m.) of an 18 hr. nutrient broth culture of *Bact. coli* or 3 p.p.m. of a 72 hr. culture of *Strep. faecalis* in glucose-yeast extract broth. Fourfold colony counts were made on the contents of duplicate flasks at intervals during incubation. Rates of death in duplicate flasks were found to be of the same order, but there were profound

Fig. 1

Fig. 1. Rate of death of *Bact. coli* and of *Strep. faecalis* in 0.003M phosphate buffer at different pH values.

Fig. 2

Fig. 2. Rate of death of *Bact. coli* and of *Strep. faecalis* in 0.003M phosphate buffer at different pH values (logarithmic scale).

differences between average rates of death at different pH values. Fig. 1, in which the averages of the eight values are plotted, shows the same trend for both organisms; viability is greatest at pH 5.0-5.2, least at pH 6.1-7.1 and intermediate between these extremes at pH 7.6-7.7.

Effect of oxygen tension

Rates of death of each organism under aerobic and anaerobic conditions were compared. For several hours before use a current of sterile nitrogen was passed through the liquid in the bottles to be used for anaerobic conditions, anaerobiosis being confirmed by testing for dissolved oxygen by the micro method of Fox & Wingfield (1938). The buffer for aerobic experiments was

aerated with a current of sterile air. Four bottles (two aerobic and two anaerobic) were each inoculated with 5 p.p.m. of an 18 hr. broth culture of the test organism. Before and immediately after each sampling a current of air or nitrogen was passed through the suspensions and the bottles were then re-sealed. In all aerobic bottles the liquid remained virtually saturated throughout the experiments. In the anaerobic bottles containing *Bact. coli* the dissolved oxygen remained between 1.5 and 4.0 % of saturation for the first 3 days and between 9 and 18 % for the next 6 days; in those inoculated with *Strep. faecalis* it was between 1 and 6 % of saturation during the first 6 days of incubation, after which no further values were obtained.

Fig. 3. Rate of death of *Bact. coli* in 0.003M phosphate buffer at pH 6.0 under aerobic and anaerobic conditions.

Results (expressed graphically in Figs. 3 and 4) show that *Bact. coli* died much more rapidly under anaerobic than under aerobic conditions, a finding similar to that observed by Whipple & Mayer (1906) for *Salmonella typhi*. With *Strep. faecalis*, on the other hand, there was no significant difference in the rates of mortality under the two conditions.

Effect of small concentrations of nutrient material

Reports in the literature suggest that growth of bacteria in water containing only traces of nutrient material may depend on a number of conditions. Savage & Wood (1917) found that, whereas streptococci did not multiply in sterile tap water, *Bact. coli* showed an increase in every test. Butterfield (1929) recorded growth of *Bact. aerogenes* in media containing 0.5 p.p.m. glucose and 0.5 p.p.m. peptone, ZoBell & Grant (1943) of heterotrophic marine bacteria in mineral solutions containing as little as 0.1 p.p.m. peptone or glucose, and Burke & Taschner (1936) of *Bact. coli* in water containing sufficient potato extract to give a figure for 'oxygen consumed' of 0.51 p.p.m. or above. Heukelekian & Heller (1940) found that by increasing the effective surface (for example by adding glass beads) appreciable growth of *Bact. coli* was

obtained at much lower concentrations of nutrient material than normally. This effect of surface was thought by ZoBell (1943) to be largely due to concentration of nutrient material on it. Bigger (1937) observed that one strain of *Bact. coli* attained numbers of several hundred thousand/ml. in sterilized tap water and over a prolonged period of storage the population showed pronounced rises and falls. Further experiments (Bigger & Nelson, 1941, 1943) showed that, provided ammonia and carbon dioxide were present, *Bact. coli* would grow in distilled water containing talc or one of a number of other inorganic materials in a finely divided or porous condition.

Fig. 4. Rate of death of *Strep. faecalis* in 0.003M phosphate buffer at pH 6.0 under aerobic and anaerobic conditions.

Observations on the effect of small concentrations of nutrient material on viability were made with suspensions of organisms which had been freed as far as possible from organic matter by repeatedly centrifuging and resuspending in buffer solution. Measured quantities of appropriate dilutions of glucose-yeast extract broth (0.3 % Yeastrel, 2 % peptone, 0.5 % glucose, and 1 % Lemco) were added to separate bottles of the bacterial suspension to give concentrations ranging from nil to several hundred p.p.m.

Two sets of experiments were carried out with each species of organism, the first with a pure culture of a single strain, the second with a mixture of several strains. Freedom from contamination was established periodically throughout the experiments by picking colonies from the plates and testing their characters.

The average counts recorded in these experiments are shown in Tables 1 and 2. Growth of the mixed strains of *Bact. coli* occurred at much lower concentrations of nutrient material than with the single strain and the level of population eventually maintained increased with the concentration of nutrient. The lowest concentration of broth in which substantial growth occurred

Table 1. *Effect of small concentrations of nutrient material on viability of Bact. coli in 0.003M phosphate buffer at pH 6.0.*

(Surviving bacteria as colony counts/ml. of suspension, each figure being the average of three counts.)

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)					
	0	2.5	10	25	100	250
	Colony counts/ml. suspension					
Exp. I. Pure culture of single strain:						
0	2,340	2,280	—	2,390	—	2,080
23	900	299	—	290	—	> 20,000
44	374	101	—	457	—	> 20,000
71	193	81	—	585	—	> 20,000
95	131	61	—	548	—	6,200,000
143	81	46	—	356	—	100,000,000
240	34	26	—	37	—	7,400,000
336	27	14	—	9	—	2,980,000
432	20	8	—	4	—	3,550,000
504	9	2	—	2	—	2,740,000
583	4	0	—	0	—	2,790,000
672	1	0	—	0	—	3,810,000
744	0	0	—	0	—	4,010,000
Exp. II. Mixed culture of five strains:						
0	1,720	1,880	1,260	1,580	1,950	1,900
22	401	151	279	520	72,000	850,000
47	274	80	278	1,370	1,860,000	8,800,000
71	129	69	584	6,430	49,600,000	522,000,000
94	89	105	2,000	40,000	4,000,000,000	4,000,000,000
166	86	57	> 5,000	107,000	1,220,000	3,290,000
214	97	57	67,000	160,000	1,190,000	6,700,000
264	65	42	56,000	79,000	800,000	2,800,000
336	55	42	44,000	83,000	850,000	2,850,000
415	58	39	50,000	83,000	680,000	2,610,000
504	61	56	53,000	104,000	950,000	2,690,000
576	52	39	49,000	70,000	660,000	2,730,000

was 10 p.p.m., corresponding to a content of 0.03 p.p.m. Yeastrel, 0.2 p.p.m. peptone, 0.05 p.p.m. glucose, and 0.1 p.p.m. Lemco. The 5-day biochemical oxygen demand (at 18.3°) of this dilute broth was 0.26 p.p.m. and the oxygen absorbed from acid permanganate in 4 hr. at 26.7° was 0.08 p.p.m.

With streptococci no growth was observed in any of the concentrations of broth up to 375 p.p.m. though the period of survival tended to be prolonged as the concentration increased. With 375 p.p.m. broth, survival was so prolonged that there was virtually no diminution in numbers over the test period of 34 days. When the concentration of nutriment rose to 500 p.p.m. slight but definite growth took place. This was 50 times the lowest concentration in which growth of *Bact. coli* had been observed.

The presence of a minute quantity of organic matter (less than 1 p.p.m.) may induce a profound departure from the true form of the death curve for *Bact. coli* (Fig. 5).

Table 2. *Effect of small concentrations of nutrient material on viability of Strep. faecalis in 0.003M phosphate buffer at pH 6.0*

(Surviving bacteria as colony counts/ml. of suspension.)

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)			
	0	3.75	37.5	375
	Colony count/ml. suspension			
Exp. I. Pure culture of single strain (average results of eight counts):				
0	3240	3190	3360	3230
5	3530	3520	3390	3530
24	3050	3300	3380	3370
75	—	—	3160	3560
97	286	1770	—	—
120	173	426	2990	3590
147	39	59	1770	3400
168	—	—	893	3600
172	3	7	—	—
190	—	—	501	3170
243	—	—	177	3350
268	—	—	72	—
292	—	—	47	3370
34 days	—	—	—	2880

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)					
	0	10	25	100	250	500
	Colony count/ml. suspension					
Exp. II. Mixed culture of six strains (average results of four counts):						
0	460	427	457	453	557	440
24	214	229	200	237	356	334
72	49	57	50	86	203	319
96	32	33	41	49	164	460
124	19	29	20	32	133	700
163	9	15	13	20	99	640
192	4	10	9	16	81	593
244	3	7	6	15	79	623
264	—	—	—	—	77	1573

Fig. 5

Fig. 5. Effect of trace of nutrient material on rate of death of *Bact. coli* in 0.003M phosphate buffer.

Fig. 6

Fig. 6. Periods of survival in 0.003M phosphate buffer at pH 6.0 of cultures of *Bact. coli* and *Strep. faecalis* of different ages.

Effect of age of cells at time of immersion

At intervals during incubation (at 37°) of broth cultures of the test organisms dilute suspensions were prepared in phosphate buffer at pH 6·0, each suspension containing approximately 5 p.p.m. of culture.

The periods of immersion required to destroy 99 % of bacteria are shown for each pair of duplicate bottles in Fig. 6. It is evident that age affected the two species differently, a young culture of *Bact. coli* being more resistant, and a young culture of *Strep. faecalis* more sensitive, than older cultures. The period of survival of both organisms tended to increase as the culture aged between 24 and 96 hr. With *Strep. faecalis* this tendency was accompanied by fluctuations in resistance; these were less marked with *Bact. coli*.

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