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I. A DIFFERENTIAL RESPONSE OF VIRULENT AND AVIRULENT CELLS

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(14) EFFECTS OF BICARBONATE ON GROWTH OF PASTEURELLA PESTIS.

I. A DIFFERENTIAL RESPONSE OF VIRULENT AND AVIRULENT CELLS

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ABSTRACT

The observation of Delwiche et al that the virulence of broth cultures of Pasteurella pestis aerated at 37°C can be maintained by addition of bicarbonate ~~has been~~ WAS confirmed and extended. A differential growth response by virulent and avirulent cells to added bicarbonate under certain conditions ~~has been~~ WAS demonstrated. The effect of supplemental bicarbonate on initiation of growth from a virulent inoculum was found to be (a) stimulation of both virulent and avirulent cells at low levels of added bicarbonate, (b) depression of both virulent and avirulent cells at higher initial concentrations, or (c) depression of avirulent mutants with simultaneous stimulation of virulent cells at a narrow range of intermediate levels.

I. INTRODUCTION

A striking reduction of virulence results when virulent strains of Pasteurella pestis are cultured in certain broth media with aeration at 37°C.[1] This loss of virulence, which does not occur at 26°C, was shown to be due to conditions selective for avirulent mutants present in the virulent inoculum or arising during growth. Higuchi and Carlin,[2] working with defined media, suggested that the growth advantage of the avirulent mutants was due to their ability to initiate growth immediately while the virulent cultures showed a prolonged lag phase at 37°C. This temperature-dependent population shift resulting in a predominance of avirulent mutants can be prevented (a) by addition of calcium, strontium, or zinc;[3] (b) by adjustment of the initial pH to 7.8; (c) by addition of certain spent culture media;[4] or (d) by addition of bicarbonate.[5]

In finding that addition of bicarbonate nullified the growth advantage of avirulent mutants, Delwiche et al[5] demonstrated a suppression of avirulent cells and postulated a differential inhibitory mechanism. This report presents a direct demonstration of a differential growth response by virulent and avirulent cells to bicarbonate.

II. MATERIALS AND METHODS

The virulent Alexander strain (ASC 1) used by Delwiche,[5] was grown for 48 hours at 26°C on blood agar base (Difco) slants, suspended and diluted in potassium phosphate buffer (0.06 M, pH 7.2 to 7.3) for inoculation of broth cultures.

The medium used for broth cultures was brain heart infusion broth (BHI) purchased from Difco without added glucose, so that various energy sources could be tested. Additions to the autoclaved broth (15 ml in 250-ml Erlenmeyer flasks) were made immediately from fresh sterile solutions of glucose (25 per cent), $NaHCO_3$ (10 per cent in phosphate buffer sterilized by filtration), $CaCl_2$ (0.2 M), $MgSO_4$ (0.244 M). Inoculated flasks, closed with diSPo plastic foam stoppers (Scientific Products Co.), were incubated at 37°C on a reciprocal shaker with a 2 3/4-inch stroke at 91 excursions per minute.

Total viable bacterial counts were determined by pour plates in blood agar base (Difco) supplemented with 0.1 per cent glucose and 0.04 per cent sodium sulfite (BAB), and incubated at 37°C for 48 hours. Separate viable counts of avirulent mutants were made as described by Higuchi and Smith[6] in the selective agar containing magnesium and oxalate (MGOX), which permits only the avirulent cells to form colonies. Nonpigmented variants, the P^- of Jackson and Burrows,[7] were detected by spreading 0.1 ml on the hemin agar developed by these authors, and incubating at 26°C for 4 days.

Virulence for the mouse* was determined by the intraperitoneal route as described earlier.[5]

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

III. RESULTS

The ability to make separate viable counts of avirulent mutants in a virulent inoculum made it possible to observe the effects of bicarbonate on growth of both types of cells. Although addition of 0.1 per cent sodium bicarbonate to a culture always caused it to remain virulent, the effect on initiation of growth was variable; sometimes the flasks became turbid earlier and sometimes later than controls. Titration of added bicarbonate showed that addition of smaller amounts of bicarbonate consistently stimulated early growth during the first 24 hours of both virulent and avirulent cells, but larger amounts inhibited growth or resulted in killing of part of the inoculum. Intermediate amounts in a narrow initial concentration range evoked a differential growth response, stimulating virulent cells and retarding avirulent cells. In the representative experiment recorded in Figure 1, addition of 0.075 per cent sodium bicarbonate stimulated and retarded the initial growth of the virulent and avirulent populations, respectively, as compared with the culture containing no added bicarbonate. Although this amount of bicarbonate maintained a predominance of virulent cells in the culture for two days, larger amounts, which initially depress both virulent and avirulent growth, were usually more effective in preventing a large increase in proportion of avirulent cells in older cultures.

Figure 1 shows that the differential early growth response (within 24 hours) occurs at an intermediate concentration of added bicarbonate, in this case 0.075 per cent, with 0.05 per cent stimulating both virulent and avirulent cells and 0.1 per cent depressing both. This intermediate range of added bicarbonate concentrations, which appears to be a combination of favorable and toxic effects, varies somewhat from one experiment to another for reasons that are not readily apparent. Variation in gas exchange would be expected to affect the results by change in oxygen and carbon dioxide availability. Covering a flask with aluminum foil was found repeatedly to result in a growth stimulation of virulent cells by 0.1 per cent bicarbonate, which caused initially toxic effects (Figure 1) in control flasks stoppered with cotton or plastic foam. As discussed by Delwiche,[5] initial pH of cultures were changed no more than 0.2 unit at neutrality, and the pH data presented in Table I provide no explanation of the results.

Figure 2 and Table II present representative data showing maintenance of virulence by addition of either bicarbonate or calcium, and acceleration of virulence loss by added magnesium. The results with calcium are typical, showing good maintenance of high virulence for the mouse, high virulent to avirulent cell ratios, and a tendency toward an early death phase. The LD_{50} values of 24, <2, 2, and 60, at 0, 24, 48, and 72 hours respectively illustrate another point that is often observed and in need of explanation (95 per cent confidence limits equal 14 to 40, ca 1 to 3, 1 to 3, and

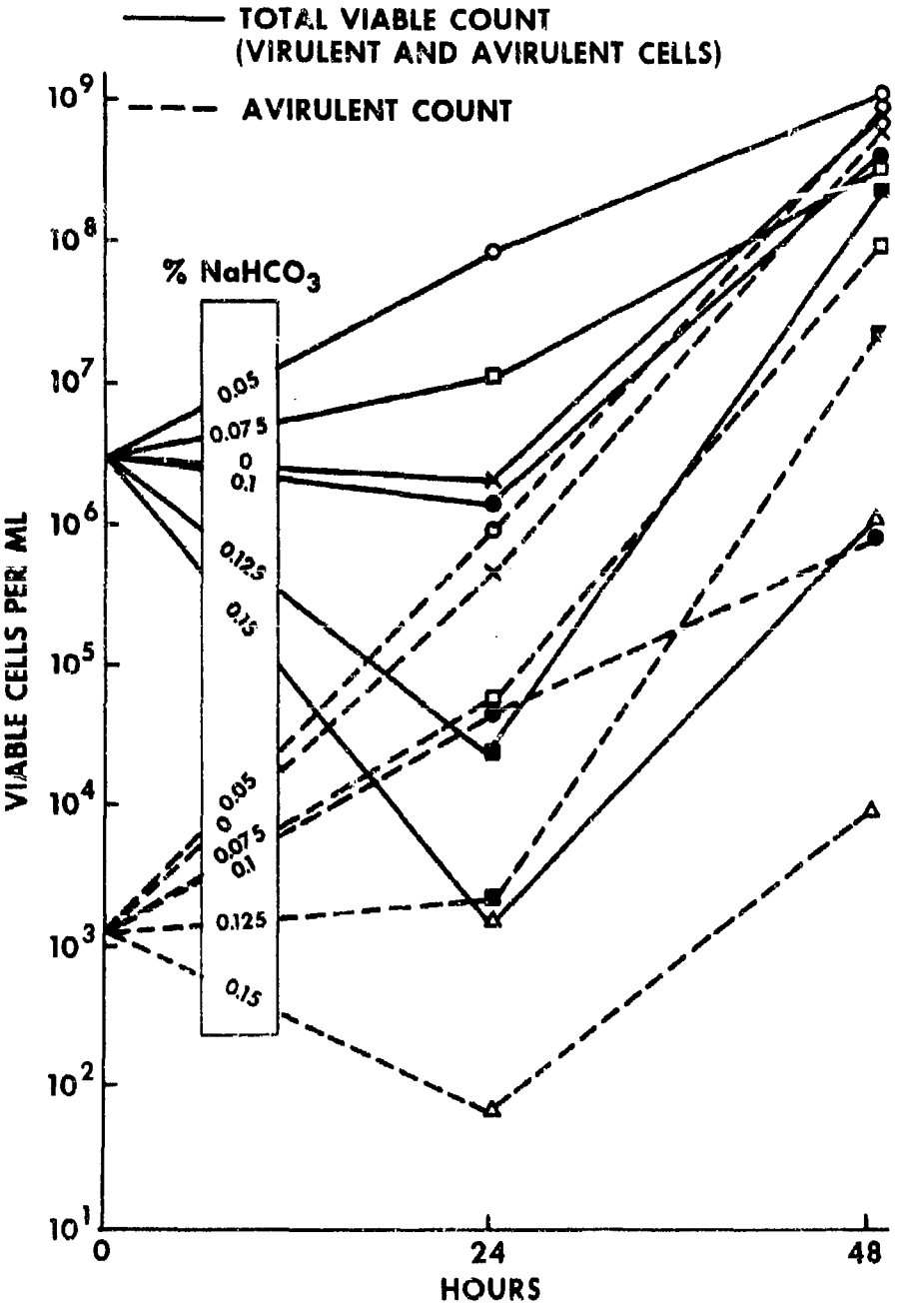

Figure 1. Growth Response of P. pestis to Bicarbonate. All flasks contained 0.2 per cent glucose and were closed with plastic foam stoppers.

TABLE I. pH VALUES OF CULTURES IN FIGURE 1

Per Cent $NaHCO_3$	pH		
	0 hr	24 hr	48 hr
0.0	6.8	6.8	7.1
0.05	6.85	7.0	6.8
0.075	6.9	7.3	6.8
0.1	6.95	7.5	7.1
0.125	7.0	7.7	6.8
0.15	7.0	>8.0	>8.0

TABLE II. pH VALUES OF CULTURES IN FIGURE 2

Additive	pH			
	0 hr	24 hr	48 hr	72 hr
None	6.6	6.6	6.4	>8.0
$NaHCO_3$	6.8	7.0	6.6	7.0
$CaCl_2$	6.6	6.2	>8.0	>8.0
$MgSO_4$	6.6	6.4	6.4	>8.0

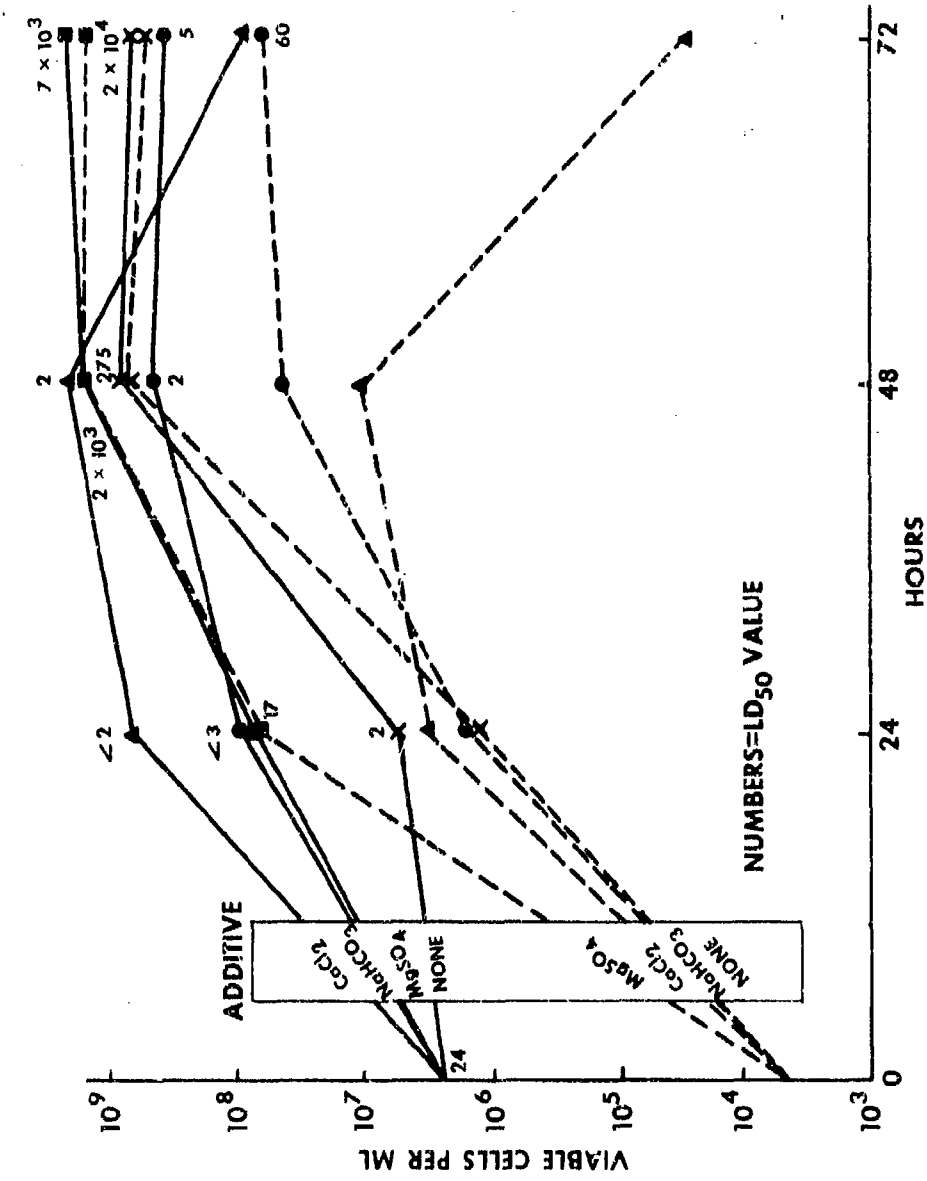

Figure 2. Maintenance of Virulent Populations by Calcium or Bicarbonate and Accelerated Selection of Avirulent Populations by Magnesium. Calcium, 2mM; bicarbonate, 0.1 per cent or 11.9mM; magnesium, 2.4mM. All flasks contained 0.2 per cent glucose, and were closed with cotton stoppers covered with paper. Solid lines represent total viable counts (virulent and avirulent cells); broken lines represent avirulent count.

33 to 115, respectively). The slight depression of virulence seen in such aging cultures probably has a physiological basis, since it cannot be accounted for by predominance of other known less virulent genetic types such as nonpigmented mutants. The culture containing 0.1 per cent bicarbonate remained virulent as usual, with an LD_{50} of 5 at 72 hours; however, at this time the percentage of avirulent mutants had risen from about 0.2 per cent to about 10 per cent. The results upon addition of magnesium afford an example of the great complexity and often deceptive character of growth curves. Total viable counts alone at 24 hours would indicate that the virulent inoculum had grown to about the same extent upon addition of either magnesium or bicarbonate. However, the differential count shows clearly that magnesium stimulates the growth of avirulent mutants; practically all of the total growth in this culture is accounted for by these avirulent cells; there is actually little or no growth of the virulent cells (our experience would predict that of 10 colonies tested from the BAB viable assay plates at 24 hours, most would be genetically completely avirulent). On the other hand, the total viable counts do reflect accurately the growth of the virulent inoculum upon addition of either bicarbonate or calcium.

Addition of both calcium and bicarbonate apparently combined the favorable effects of both for maintenance of the virulent population; growth was stimulated as with calcium alone, and viability was usually maintained beyond 72 hours. The combined effect of magnesium and bicarbonate in five experiments resulted in a relief of the toxicity of magnesium for virulent cells and growth curves that might be expected if smaller amounts of bicarbonate alone had been added.

IV. DISCUSSION

It may appear to the reader that the growth curves in Figures 1 and 2 would more clearly present the facts if, instead of plotting total viable counts, avirulent counts were deducted from the total and "virulent" cell viable counts were plotted. This can be done and is useful when virulent cells, which have a "calcium requirement" and will not grow on the MGOX agar, are clearly predominant. However, whenever the avirulent population makes up more than about 50 per cent of the culture, or when it becomes predominant, any attempt to calculate a "virulent" count would be unreliable because of increasing significance of normal plate counting errors and would only help to conceal the facts. It is safer to look at the total and "avirulent" counts together; then, the places where virulent counts may be estimated with any degree of accuracy become more obvious.

Magnesium stimulates production of virulence antigens, whereas calcium suppresses the VW antigen production but stimulates growth,[8] and the intriguing possibility is presented that growth and VW antigen production are incompatible.[9] What is the case when growth of virulent cells is stimulated by bicarbonate in the absence of calcium, which is known to suppress VW antigen production? Cultures that are kept virulent by addition of 0.1 per cent bicarbonate produce VW antigens, but this does not answer the question whether a cell can grow while producing these antigens. Since the growth rate under bicarbonate stimulation is not optimum, it is possible that some virulent cells grow while others remain static and synthesize VW antigens. Quantitative measurement of VW antigen per cell at less than optimum growth rates as done by Lawton[8] would not be sufficient to answer the question.

In their synthetic medium, which was satisfactory for aerated growth of P. pestis at 26°C, Higuchi et al[3] found that raising the temperature to 37°C made the medium completely inadequate for most avirulent strains, whereas the virulent strains still appeared to grow reasonably well. A tenfold increase in the magnesium concentration in their medium permitted avirulent growth but suppressed virulent growth. Addition of calcium then made the medium satisfactory for growth of both virulent and avirulent cells and virulent P. pestis cells have subsequently been said to have a "calcium requirement." The differential growth response of virulent and avirulent P. pestis in one direction to magnesium, in the opposite direction to bicarbonate, and the observed effects of combined magnesium and bicarbonate are of special interest. The reason for the toxicity of magnesium for virulent cells is still unknown, as is the mechanism of the counteracting effects of calcium or bicarbonate. Perhaps some light will be shed on these mechanisms by continuing investigations in this laboratory of carbon dioxide fixation in pathways of RNA synthesis and related magnesium effects.

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