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TECHNICAL MANUSCRIPT 119

EFFECTS OF BICARBONATE  
ON GROWTH  
OF PASTEURELLA PESTIS.

III. REPLACEMENT OF BICARBONATE  
BY PYRIMIDINES

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EFFECTS OF BICARBONATE ON GROWTH OF PASTEURELLA PESTIS .

III. REPLACEMENT OF BICARBONATE BY PYRIMIDINES

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ABSTRACT

The effect of carbon dioxide on the growth of virulent Pasteurella pestis cultures at 37°C with aeration was studied by substituting known products of carbon dioxide fixation for bicarbonate in the test system. The growth of the virulent cells in the inoculum is stimulated and the culture remains virulent if bicarbonate is replaced by orotic acid. The addition of cytosine or uracil also results in the retention of virulence but the effect on the growth of the virulent cells is not as pronounced as with bicarbonate or orotic acid. It is proposed that an impaired pyrimidine synthesis due to a deficiency in carbamyl phosphate is responsible for the loss of virulence by P. pestis in aerated broth cultures at 37°C. The carbamyl phosphate deficiency may be enhanced by the loss of metabolically produced carbon dioxide at 37°C.

## I. INTRODUCTION

The nutritional requirements of Pasteurella pestis are more exacting at 37°C than at temperatures below 30°C.<sup>1,2/</sup> Virulent strains of P. pestis in broth cultures aerated by agitation have been shown to have a temperature-dependent growth requirement in a complex medium.<sup>3/</sup> This growth requirement, which apparently does not exist at 26°C or in static or anaerobic cultures at 37°C, can be satisfied with supplemental sodium bicarbonate<sup>4,5/</sup> or by calcium, strontium, or zinc ions.<sup>6/</sup>

Several heterotrophic organisms must be supplied with supplemental carbon dioxide for growth even in a complex medium.<sup>7-10/</sup> With some organisms, the carbon dioxide requirement can be replaced with products of carbon dioxide fixation reactions or closely related compounds, such as dicarboxylic acids,<sup>11/</sup> adenylic acid,<sup>12/</sup> a mixture of guanine, uracil, and cytosine,<sup>13/</sup> or a combination of uracil, oxalacetic acid, and hypoxanthine.<sup>1/</sup>

This study demonstrates that the temperature-dependent requirement for supplemental carbon dioxide or metal ions of a virulent P. pestis strain can be satisfied with orotic acid and to a lesser degree with cytosine or uracil.

## II. MATERIALS AND METHODS

The growth conditions, the method of determining virulence by intraperitoneal injection of mice\*, and the use of magnesium oxalate agar and blood agar base plating media for differential enumeration of avirulent cells have been described elsewhere.<sup>5/</sup> All test compounds were sterilized by filtration through sintered glass and studied at a concentration of 0.01 M unless the concentration is specifically mentioned.

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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 aerobic growth pattern obtained with an inoculum from the virulent Alexander strain of *P. pestis* in modified brain heart infusion broth at 37°C is shown in Figure 1. The total viable count (BAB) and the viable count of the avirulent cells (Mg oxalate agar) are essentially the same at 24 hours and are equal at 48 hours. These results indicate that under these experimental conditions the virulent cells do not start to grow or, if they do grow, they have a very long lag phase as compared with the avirulent mutants present in the inoculum.

Figure 1 also shows the response obtained under the same cultural conditions when the growth medium is supplemented with 0.012 M sodium bicarbonate. The two-log differential between the BAB and Mg oxalate agar counts at 24 and 48 hours indicates that the virulent cells are able to grow under these conditions and that the culture is still virulent.

Carbamyl aspartate is one of the radioactive compounds formed when washed cells of avirulent *P. pestis* strain A-4 are incubated with  $C^{14}O_2$ .<sup>14</sup> Since carbamyl aspartate has been shown to be an intermediate in pyrimidine synthesis,<sup>15</sup> it and other intermediates of pyrimidine metabolism were tested for the ability to support the growth of virulent cells under our growth conditions. Of the compounds tested, only orotic acid, cytosine, and uracil definitely influenced the growth pattern of the virulent cultures.

Figure 2 shows the results obtained when the growth medium is supplemented with potassium orotate or cytosine. At least a two-log differential between the BAB and Mg oxalate counts results when orotate is added. These results demonstrate that under these conditions the increased total count is indeed due to the growth of virulent cells. Cytosine also stimulates the growth of virulent cells under our cultural conditions but maintains a two-log differential for only 24 hours. The addition of uracil gives results similar to those obtained with cytosine. The retention of virulence by cytosine and uracil has been somewhat erratic and may indicate an indirect mode of action for these compounds.

A lower concentration of orotate (0.0075 M) stimulates the growth of the virulent cells and results in a two-log differential for 24 hours; however, at 48 hours the two counts are equal. At a higher concentration (0.015 M), both viable counts decrease when measured at 24 hours, but a two-log differential between the two counts is maintained at both 24 and 48 hours. These results are similar to those obtained with various concentrations of  $NaHCO_3$ .<sup>2</sup> Increasing the cytosine concentration to 0.02 M does not significantly change the growth pattern. The possibility that orotate, cytosine, or uracil is simply supplying the organisms with carbon dioxide has not been eliminated; however, other compounds known to be decarboxylated by *P. pestis* will not replace  $NaHCO_3$  in our system.

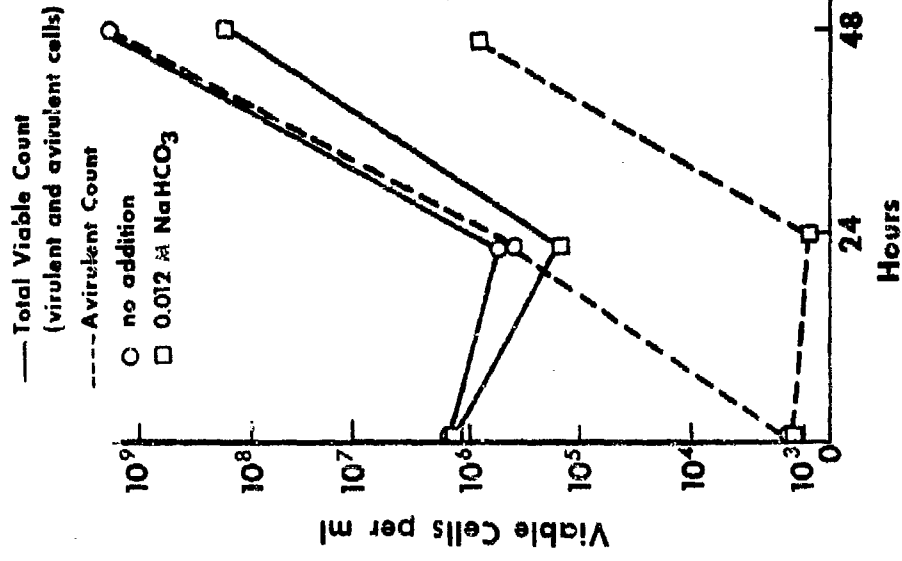


Figure 1. Effect of  $\text{NaHCO}_3$  on the Growth of a Virulent *Pasteurella pestis* Culture.

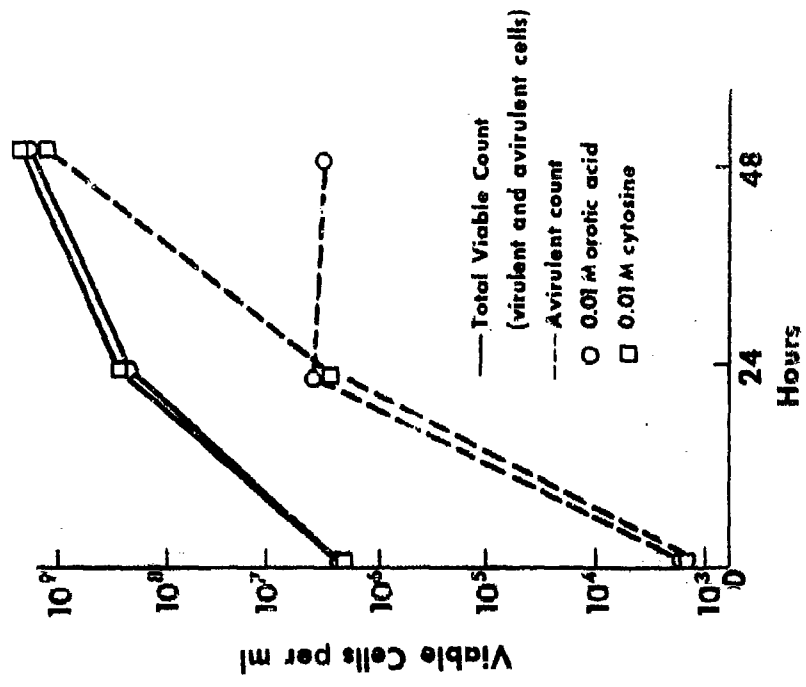


Figure 2. Effect of Potassium Orotate and Cystine on the Growth of a Virulent *Pasteurella pestis* Culture.

Carbamyl phosphate (Lithium salt), carbamyl phosphate plus aspartate, dihydroorotate, cytidine, cytidine monophosphate, uridine, uridine monophosphate, thymine, and thymidine will not replace  $\text{NaHCO}_3$  at the concentrations tested. None of the intermediates of the citric acid cycle, purines, or purine derivatives tested influenced the retention of virulence in our system.

$\text{LD}_{50}$  values, determined at the same time intervals as the viable counts, demonstrate that orotate and cytosine influence the retention of virulence at  $37^\circ\text{C}$  with aeration as well as  $\text{NaHCO}_3$  (Table I). The low  $\text{LD}_{50}$  value obtained when cytosine is added confirms the initial growth stimulation of virulent cells by this compound as determined with the BAB and Mg oxalate counts. Even though the avirulent count is almost the same as the total count at 48 hours, the virulent cells grow well enough to maintain the virulence of the culture.

TABLE I. MAINTENANCE OF VIRULENT *P. PESTIS* POPULATIONS BY  $\text{NaHCO}_3$ , POTASSIUM OROTATE, AND CYTOSINE

Supplement	House Intraperitoneal $\text{LD}_{50}$ and 95 per cent Confidence Limits <sup>a/</sup>			
	<u>24-hr Culture</u>		<u>48-hr Culture</u>	
	$\text{LD}_{50}$	Limits	$\text{LD}_{50}$	Limits
None	15	(8-27)	4500	(2500-8200)
$\text{NaHCO}_3$ (0.012 M)	12	(6-26)	22	(13-41)
Orotate, K salt (0.01 M)	3	(1-5)	5	(3-9)
Cytosine (0.01 M)	4	(2-8)	12	(7-21)

a. The initial intraperitoneal mouse  $\text{LD}_{50}$  before incubation at  $37^\circ\text{C}$  was 135 with 95 per cent confidence limits of 82 to 225.

IV. DISCUSSION

The virulent cells in an inoculum of P. pestis will grow in certain broth media at 26°C with or without aeration and at 37°C if the culture is incubated statically or anaerobically. However, when the culture is agitated at 37°C, the virulent cells either have a very long lag phase or do not grow and the culture loses virulence because of the rapid growth of the avirulent mutants always present in the inoculum.

The failure of the virulent cells to initiate growth is evidently related to pyrimidine synthesis, as the addition of orotic acid, cytosine, or uracil will stimulate the growth (or shorten the lag phase) of virulent cells. Since supplemental bicarbonate also allows the virulent cells to grow, no thermal or oxidative inactivation of enzymes directly concerned with pyrimidine biosynthesis is indicated unless carbamate is formed enzymatically from  $(NH_4)_2CO_3$  by P. pestis.

In view of the retention of virulence by pyrimidines, the major function of the supplemental bicarbonate appears to be the enhancement of carbamyl phosphate production, an intermediate in pyrimidine synthesis. A deficiency in carbamyl phosphate is therefore proposed as the major reason for the failure of virulent cells to initiate growth. The occurrence of a bicarbonate requirement for virulent cells in agitated cultures at 37°C but not at 26°C supports this view, as carbon dioxide is less soluble at the former temperature. Agitation of the culture would also be a contributing factor in the loss of carbon dioxide.

Under our growth conditions, both virulent and avirulent cells produce a capsular antigen (Fraction I). In addition to many common antigens, the virulent cells produce two additional antigens, V and W.<sup>16/</sup> These three antigens are not produced at 26°C or in the absence of oxygen.<sup>16,17/</sup> This additional protein synthesis may result in an unfavorable competition for carbamyl phosphate, as it is an intermediate in arginine synthesis as well as for pyrimidines. This competition would be more detrimental to the virulent cell because of V and W formation. Aeration by agitation at 37°C could further accentuate any carbamyl phosphate deficiency by imposing a greater carbamyl phosphate demand due to increased protein synthesis as well as by promoting a greater loss of carbon dioxide.

It may be fortuitous but, when either V and W antigen or Fraction I production is inhibited or depressed at 37°C with aeration by certain cultural conditions, the culture remains virulent. The addition of calcium stimulates the growth of virulent cells<sup>2/</sup> and inhibits V and W antigen production.<sup>17/</sup> An initial pH of 7.8 was found to retain virulence<sup>18/</sup> and was also found to depress Fraction I production approximately fifty per cent.<sup>19/</sup>

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