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EFFECTS OF BICARBONATE ON GROWTH OF *PASTEURELLA PESTIS*

III. REPLACEMENT OF BICARBONATE BY PYRIMIDINES

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ABSTRACT

BAUGH, C. L. (Fort Detrick, Frederick, Md.), A. W. ANDREWS, AND M. J. SURGALLA. Effects of bicarbonate on growth of *Pasteurella pestis*. III. Replacement of bicarbonate by pyrimidines. J. Bacteriol. 88:1394-1398, 1964.—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, uracil, or citrulline 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.

The nutritional requirements of *Pasteurella pestis* are more exacting at 37 C than at temperatures below 30 C (Hills and Spurr, 1952; Higuchi and Carlin, 1958). Virulent strains of *P. pestis* in broth cultures aerated by agitation were shown to have a temperature-dependent growth requirement in a complex medium (Fukui et al., 1957). 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 (Delwiche et al., 1959; Surgalla, Andrews, and Baugh, 1964) or by calcium, strontium, or zinc ions (Higuchi, Kupferberg, and Smith, 1959).

Several heterotrophic organisms must be supplied with supplemental carbon dioxide for growth, even in a complex medium (Griffin and Racker, 1956; Mueller and Hinton, 1941; New-

ton, Marr, and Wilson, 1954; Steinman, Oyama, and Schulze, 1954). 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 (Ajl and Werkman, 1948); adenylic acid (Pappenheimer and Hottle, 1940); a mixture of guanine, uracil, and cytosine (Tuttle and Scherp, 1952); and a combination of uracil, oxaloacetic acid, and hypoxanthine (Griffin and Racker, 1956).

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, uracil, or citrulline.

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 (BAB) plating media for differential enumeration of avirulent cells were described elsewhere (Surgalla et al., 1964). All test compounds were sterilized by filtration through sintered glass, and were studied at a concentration of 0.01 M, unless the concentration is specifically mentioned.

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 Fig. 1. The total viable count (BAB) and the viable count of the avirulent cells (Mg oxalate agar) are essentially the same at 24 hr and are equal at 48 hr. These results indicate that under these 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.

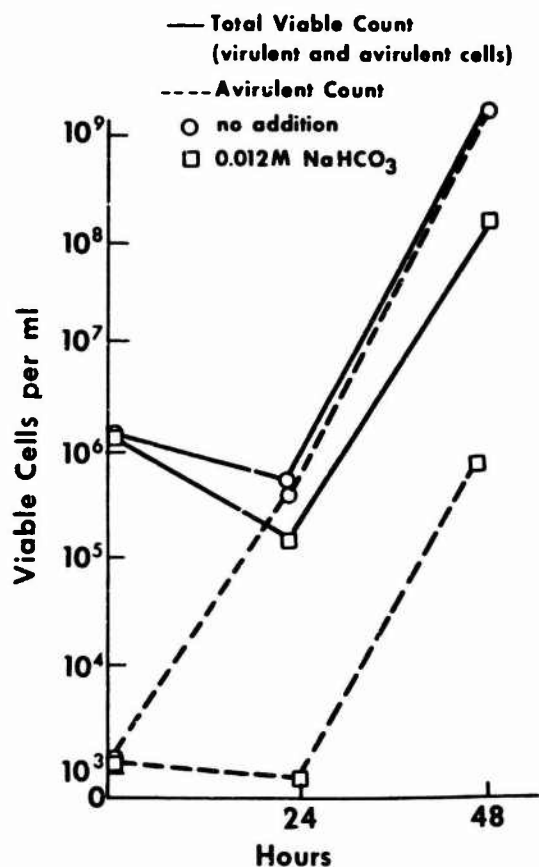


FIG. 1. Effect of NaHCO_3 on the growth of a virulent *Pasteurella pestis* culture.

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 hr 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 (Baugh, Lanham, and Surgalla, 1964). Because carbamyl aspartate was shown to be an intermediate in pyrimidine synthesis (Yates and Pardee, 1956), 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 pyrimidine compounds tested, orotic acid showed the greatest influence on the growth pattern of the virulent cultures (Fig. 2).

At least a two-log differential between the BAB and Mg oxalate counts results when 0.01

M potassium orotate is added. These results demonstrate that, under these conditions, the increased total count is indeed due to the growth of virulent cells. A lower concentration (0.0075 M) of orotate stimulates the growth of the virulent cells, and results in a two-log differential for 24 hr; however, at 48 hr, the two counts are equal. At a higher concentration (0.015 M), both viable counts decrease when measured at 24 hr, but a two-log differential between the two counts is maintained at 24 and 48 hr. The results are quite similar to those obtained with NaHCO_3 (Surgalla et al., 1964).

Cytosine also stimulates the growth of virulent cells under our cultural conditions, but maintains a two-log differential for only 24 hr (Fig. 2). Increasing the cytosine concentration to 0.02 M does not significantly change the growth pattern. The addition of uracil gives essentially the same results as those obtained with cytosine (Table 1).

The possibility that orotate, cytosine, or uracil is simply supplying the virulent cells with carbon dioxide has not been eliminated; however, other compounds known to be decarboxylated by *P. pestis*, such as Krebs cycle intermediates, will not replace NaHCO_3 .

Carbamyl phosphate is involved in the biosynthesis of citrulline as well as the pyrimidines;

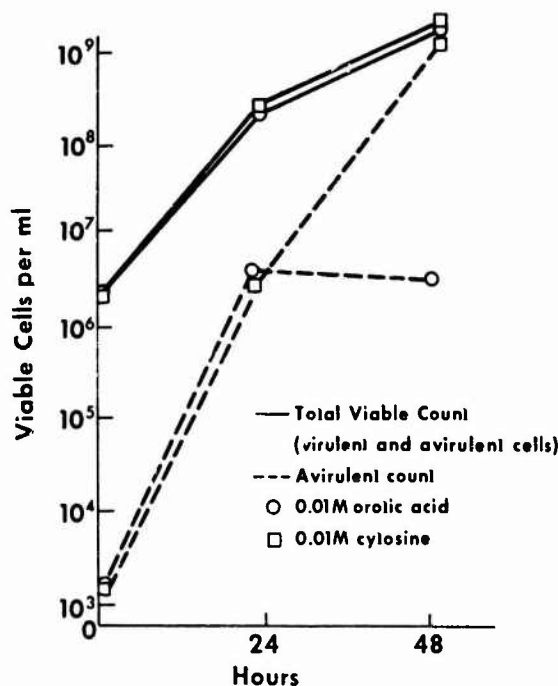


FIG. 2. Effect of K orotate and cytosine on the growth of a virulent *Pasteurella pestis* culture.

TABLE 1. *Effect of various compounds related to carbamyl phosphate on the growth of virulent Pasteurella pestis cultures**

Addition	Concn	Viable count			
		24 hr		48 hr	
		BAB	Mg oxalate	BAB	Mg oxalate
None	M	4.0×10^5	5.9×10^4	1.5×10^5	1.5×10^5
NaHCO ₃	0.012	2.0×10^4	1.0×10^2	6.8×10^7	1.2×10^5
NH ₄ HCO ₃	0.012	9.9×10^6	2.1×10^5	3.9×10^8	9.0×10^7
Na ₂ CO ₃	0.006	1.8×10^5	1.9×10^3	9.0×10^7	8.7×10^6
(NH ₄) ₂ CO ₃	0.006	4.3×10^5	3.2×10^3	1.4×10^8	1.6×10^6
(NH ₄) ₂ CO ₃	0.003	4.2×10^5	5.5×10^3	2.4×10^8	1.4×10^6
Uracil	0.01	4.0×10^6	3.7×10^4	4.4×10^7	1.3×10^7
Arginine	0.06	8.2×10^5	1.3×10^5	1.9×10^8	1.3×10^8
Citrulline	0.054	1.8×10^8	3.4×10^6	1.0×10^9	5.2×10^7

* Inoculum consisted of BAB (blood-agar base), 1.27×10^6 ; and Mg oxalate, 1.02×10^3 .

TABLE 2. *Maintenance of virulent Pasteurella pestis populations by NaHCO₃, K orotate, and cytosine*

Supplement	Concn	Mouse intraperitoneal LD ₅₀ with 95% confidence limits*			
		Age of culture 24 hr		Age of culture 48 hr	
		LD ₅₀	Limits	LD ₅₀	Limits
None	M	15	(8-27)	4,500	(2,500-3,200)
NaHCO ₃	0.012	12	(6-26)	22	(13-41)
Orotate, K salt	0.01	3	(1-5)	5	(3-9)
Cytosine	0.01	4	(2-8)	12	(7-21)

* Initial intraperitoneal mouse LD₅₀ before incubation at 37 C was 135, with 95% confidence limits of 82 to 225.

therefore, arginine and citrulline were also tested for the ability to replace NaHCO₃ (Table 2). The addition of arginine at several concentrations up to 1% did not influence the growth pattern of the virulent cells. Supplemental citrulline stimulates the growth of virulent cells, and the culture usually remains virulent only if the concentration is increased to 0.054 M.

The results with the effective concentration of citrulline were quite erratic. The virulent population of the culture sometimes was increased and was greater than the avirulent population at 24 but not at 48 hr. The variations in the results,

and the requirement for a high concentration of citrulline, probably indicate that the cells are not freely permeable to citrulline.

In investigating further the bicarbonate requirement of virulent cells, it was found that ammonium or sodium carbonate was effective at 0.006 M or one-half the effective concentration of sodium or ammonium bicarbonate (Table 1). Although the addition of ammonium carbonate at 0.003 M resulted in only a twofold differential between the BAB and Mg oxalate counts, the LD₅₀ of the cultures was 78 at 72 hr, whereas the LD₅₀ of the unsupplemented culture was 40,000.

Carbamyl phosphate, which is unstable in solution and decomposes to cyanate (Jones, 1963), did not support the growth of virulent cells, and was slightly inhibitory to the avirulent population. Carbamyl aspartate, carbamyl phosphate plus aspartate, dihydroorotate, cytidine, cytidine monophosphate, thymine, and thymidine will not replace NaHCO₃ 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.

The LD₅₀ values, determined at the same time intervals as the viable counts, demonstrate that orotate and cytosine influence the retention of virulence at 37 C with aeration as well as NaHCO₃ (Table 2). The low LD₅₀ 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 hr, the virulent cells grow well enough to maintain the virulence of the culture.

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 owing to the rapid growth of the avirulent mutants always present in the inoculum.

This loss of virulence by the aerobically grown cultures at 37 C can be prevented by the addition of 0.012 M NaHCO_3 or 0.002 M CaCl_2 . The appearance of a bicarbonate deficiency only in agitated cultures at 37 C indicates that the loss of CO_2 from the culture medium may be involved in the loss of virulence. Because static cultures at 37 C do not rapidly lose virulence, agitation of the culture appears to be the major factor in the loss of the CO_2 . The solubility of CO_2 is less at 37 than at 26 C, and would further influence the loss.

The role of calcium ions in the growth of virulent *P. pestis* is still unknown. One possible function of the supplemental calcium, although it may be minor, could be to prevent the loss of CO_2 from the culture by forming calcium carbonate.

The results obtained by Ogg et al. (1958) support the proposal that the loss of CO_2 from the culture medium, with a resulting bicarbonate and carbonate deficiency, is the primary reason for loss of virulence. These authors found that, if the initial pH of the culture was 7.8, the culture was still virulent at 72 hr. This pH would tend to retain any metabolically produced CO_2 in the form of bicarbonate and carbonate.

The failure of the virulent cells to initiate growth is evidently related to pyrimidine or citrulline biosyntheses, because NaHCO_3 can be replaced with orotic acid and, to a lesser extent, with cytosine, uracil, or citrulline. The major function of the supplemental NaHCO_3 would appear to be the enhancement of carbamyl phosphate production, because it is a common precursor of the pyrimidines and citrulline. Carbamyl phosphate is formed from carbamic

acid, which is in equilibrium with carbonate and bicarbonate ions as well as dissolved CO_2 and NH_3 .

The results obtained with the addition of ammonium carbonate also support the view that a deficiency in carbonate or carbamate needed for synthesis of carbamyl phosphate is the major reason for the failure of the virulent cells to initiate growth. Ammonium carbonate which is in equilibrium with ammonium carbamate is almost four times as effective as is NaHCO_3 .

The specific reason why the virulent cells require a higher carbon dioxide or carbamate concentration than do the avirulent mutants in the inoculum is still unknown. Because supplemental bicarbonate is effective, no thermal or oxidative inactivation of enzymes directly concerned with pyrimidine biosynthesis after carbamyl phosphate formation is indicated. The virulent cells, however, may lack a carbamyl phosphokinase specific for pyrimidine biosynthesis, or the enzyme may be altered at 37 C to require a higher concentration of carbamate. If carbamate is formed enzymatically in *P. pestis*, the virulent cells could be deficient in this enzyme at 37 C. In the latter instance, the supplemental bicarbonate would relieve the enzymatic deficiency in a nonenzymatic manner by supplying additional carbonate and carbamate.

The temperature-dependent biotin requirement of *P. pestis* at 36 C (Brownlow and Wessman, 1960) may also be related to a deficiency in carbamyl phosphate. The nitrogen and the carbamyl carbon of the ureido group of biotin are derived from carbamyl phosphate (Lynen, 1963).

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