ENVIRONMENTAL CONDITIONS AFFECTING THE POPULATION DYNAMICS AND THE RETENTION OF VIRULENCE OF *PASTEURELLA PESTIS*: THE ROLE OF CARBON DIOXIDE

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Fukui *et al.* (1957) reported that virulent strains of *Pasteurella pestis* consistently lost virulence when grown at 37 C under conditions of aeration in media generally suitable for the proliferation of the species. This loss of virulence was attributed to the selection of avirulent mutants which multiplied much more rapidly than virulent cells. Ogg *et al.* (1958) observed that the loss of virulence could be diminished by the adjustment of the initial pH of the medium to pH 7.7 to 7.8, by the reduction of oxygen tension, or by the addition of filtered, spent medium.

In recent studies we have been able to define certain cultural conditions which contribute markedly toward limiting or inhibiting the loss of virulence. The following modifications of the usual cultural conditions independently greatly enhanced the retention of virulence as determined by the mouse titrations: (a) the inclusion in the medium of 0.10 per cent sodium bicarbonate; (b) cultivation under an atmosphere of nitrogen; (c) cultivation under an atmosphere of nitrogen containing 1.0 per cent CO_2; (d) the inclusion in the medium of 0.25 per cent glutamic or aspartic acids. An additive effect was observed in the retention of virulence when certain of these environmental factors existed in combination.

MATERIALS AND METHODS

The virulent and avirulent strains of *P. pestis* designated ASC 1 and A1224, respectively, were obtained in our laboratory as single colony isolates from the virulent "Alexander" strain originally isolated in 1949 from a case of bubonic plague in New Mexico.

The cultures were grown in brain heart infusion broth (Difco) supplemented with 0.06 per cent $MgSO_4 \cdot 7H_2O$. Fifteen ml of the medium were dispensed into 250-ml Erlenmeyer flasks, inoculated to give an initial viable count of approximately 10^4 to 10^5 cells per ml, and incubated at 37 C with shaking, or under the desired gas atmosphere in desiccator jars placed on the reciprocating shaking apparatus. When it was necessary to grow the cultures in the complete absence of oxygen, the desiccator jar was evacuated to approximately 500 mm of mercury negative pressure; and nitrogen was permitted to flow into the desiccator until the pressure was reduced to approximately 25 mm. This procedure of evacuating and filling with nitrogen was repeated 5 times before the desiccator was finally sealed. One per cent CO_2 gas was added when desired by admitting the correct amount of CO_2 in the final dilution step, as measured by the proportionate pressure release. Fresh sodium bicarbonate solution was prepared as a 10 per cent solution in 0.06 M phosphate buffer (pH 7.2 to 7.4), sterilized by filtration, and added aseptically to give a final concentration of 0.1 per cent (0.012 M). Growth was measured turbidimetrically with a Coleman model 9 Nepho-Colorimeter using the 655 mμ filter. To facilitate the determination of optical densities, 250-ml Erlenmeyer flasks with a convenient cuvette fused to the flask were constructed and used in the studies on growth rates. Virulence was determined by intraperitoneal challenge of Swiss-Webster mice (18 to 22 g) with 0.2 ml of appropriate serial dilution of the culture in brain heart infusion broth. This diluent permitted better survival of the microorganisms than did the 0.06 M phosphate buffer previously utilized (Fukui *et al.*, 1957). A minimum of 5 dilutions with 10 mice per dilution was used for each virulence titration. The LD_{50} after 10 days was calculated by the method of Goldberg *et al.* (1954). Viable cell counts were determined by the pour plating methods using blood agar base (Difco) medium supplemented with 0.1 per cent glucose and 0.04 per cent sodium sulfite. The plates were incubated at 26 C for 48 to 72 hr before they were counted.

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RESULTS AND DISCUSSION

When the virulent strain ASC 1 of *P. pestis* was grown in the complete absence of oxygen (under nitrogen) at 37 C in brain heart infusion broth with shaking, a minimum of 12 to 15 generations was obtained within 40 to 44 hr. The cultures were relatively virulent with LD_{50}'s in the order of magnitude of 10^2 to 10^3 cells. They were approximately 10^4 to 10^6 times more virulent than the cells from the same strain grown in the presence of air (table 1). This correlation of reduced oxygen tension to the selective production of virulent cells substantiated unpublished experiments performed earlier by one of us (G. M. F.) and may be considered as an extension of similar observations published by Ogg *et al.* (1958).

The addition of 0.1 per cent sodium bicarbonate, 0.25 per cent glutamic acid or 0.25 per cent aspartic acid to the brain heart infusion broth greatly reduced the rate of loss of virulence of strain ASC 1 grown with aeration at 37 C. After 40 to 44 hr of incubation the viable cell count of these cultures was usually found to be in the range of 5×10^8 to 5×10^9 cells per ml (12 to 15 generations) and the LD_{50}'s elicited were in the order of 10^2 to 10^3. These cultures were also found to be 10^4 to 10^6 times more virulent than similar cultures grown under air in brain heart infusion broth without the additions (table 1).

TABLE 1

Virulence of cultures of Pasteurella pestis (ASC 1) when grown under differing environmental conditions

Conditions	Mouse Intraperitoneal LD_{50} (Range)
Heart infusion broth (Difco) incubated at 37 C for 40–44 hr with shaking after an inoculation of 10^4–10^5 cells/ml, and with *air* as the gas phase	10^7–10^8
except N_2 as the gas phase	10^2–10^3
plus added 0.1% $NaHCO_3$	10^2–10^3
plus added 0.25% glutamic acid	10^2–10^3
plus 0.25% aspartic acid	10^2–10^3
except N_2 + 1% CO_2 as the gas phase	10 –10^2
except N_2 as the gas phase and medium containing 0.1% $NaHCO_3$	1 –10
except N_2 as the gas phase and medium containing 0.25% aspartic acid	1 –10
except N_2 as the gas phase and medium containing 0.25% glutamic acid	10 –10^2

With regard to the bicarbonate and CO_2 effects (table 1), it is reasonable to assume that they are identical since other experiments have established that sodium concentration is not a significant factor in retention of virulence. The observation of Ogg *et al.* (1958) on the preservation of virulence by the addition of various culture filtrates and by media that had been adjusted to pH 7.7 to 7.8 prior to inoculation can be explained in terms of the CO_2 or bicarbonate effects described in this report. Culture filtrates from most microorganisms (including *P. pestis*) contain large amounts of metabolically produced CO_2; and the neutralization of media and culture filtrates with even analytical grades of alkali introduces appreciable amounts of chemically bound CO_2 unless very special precautions are taken to prepare carbonate-free solutions of the alkali. In view of the effect of high hydroxyl ion concentration on the retention of virulence (pH 7.7 to 7.8) as reported by Ogg *et al.* (1958), it becomes important, therefore, to distinguish, if possible, the "bicarbonate effect" from the "pH effect," since sodium bicarbonate, when placed in aqueous solution, slowly loses CO_2 and becomes alkaline. Our observations show, however, that the effect of this alkalizing process is minor in the concentration of sodium bicarbonate employed in the culture medium the initial pH being changed only to the extent of approximately 0.2 of a pH unit at neutrality. With cultures buffered at pH 6.8 to 7.0 the virulence preserving properties of sodium bicarbonate remain unchanged. The relatively high hydroxyl ion concentration concerned in the "pH effect" is unnecessary, and might be regarded as but a cause of CO_2 retention in spent media, which are usually alkaline, or as a secondary factor involved in the introduction of carbonate into the medium as an impurity present in the alkali. Under nitrogen where the cultures become acid (with no interim pH rise) because of the production of fermentation acids, the "bicarbonate effect" is still apparent. Perhaps the most cogent argument that can be advanced, however, involves the observation that gaseous CO_2 in nitrogen exerts a favorable effect in retention of virulence. In this case the pH change, although minor in extent, is toward the acid range of the scale. It can be concluded, therefore that the

sodium bicarbonate exerts its effect in the retention of virulence through a mechanism not directly related to pH alteration.

Table 1 summarizes the effects of the various factors which influence the retention of virulence as tested individually and in certain combinations. The combinations of sodium bicarbonate or aspartic acid under nitrogen seemed to be particularly efficacious. In each case, however, the mouse intraperitoneal LD_{50} is drastically lower than that of the control culture (LD_{50} 10^7 to 10^8).

Whether aspartic and glutamic acids function as a source of CO_2, indirectly to suppress the tricarboxylic acid cycle in the avirulent mutant, or in some other, unknown manner is a matter of conjecture and is reminiscent of the dilemma with regard to the abilities of CO_2, tricarboxylic acid cycle intermediates, or glutamic and aspartic acids to initiate growth of *Escherichia coli* in synthetic media, as described a number of years ago (see, for example, Ajl and Werkman, 1948; Lwoff and Monod, 1949; and more recently Wiame *et al.*, 1956). It could be argued that CO_2 (and hence bicarbonate) functions in the synthesis of aspartic and glutamic acids and that these amino acids in some unknown manner suppress the cyclic oxidative mechanisms and thereby selectively inhibit the avirulent forms, which have a more active oxidative metabolism. In support of this hypothesis is the proved value of anaerobiosis in the preservation of virulence. It could also be hypothesized that glutamic and aspartic acids function by producing CO_2 and that the CO_2 exerts a selective effect, either by inhibiting oxidations, or via some unknown mechanism. There also exists the possibility that CO_2 and the amino acids accomplish the same end by means of totally different devices. A cause and effect relationship needs yet to be established in this particular case between CO_2 and these amino acids.

With regard to CO_2 (and bicarbonate) alone, the mechanism seems more easily explained. It was approached through a series of growth studies related to the population dynamics of the virulent and avirulent cells. In this aspect of the work the growth rates of the virulent strain (ASC 1) and the avirulent mutant (A1224) cultured under various conditions were compared. When these cultures were grown in air with shaking, the avirulent strain invariably initiated growth 10 to 15 hr earlier than did the virulent strain and reached the stationary phase of growth 15 to 20 hr earlier. As illustrated in figure 1, it can be seen that the addition of 0.1 per cent sodium bicarbonate to the medium consistently delayed to approximately the same extent the initiation of growth in air of both the virulent and avirulent cultures. Once the growth process was well established, however, the avirulent strain still usually proliferated at a greater rate and attained a greater maximum population than did the virulent strain (ASC 1). On first examination of the curves, it appeared that the bicarbonate exerted the same inhibitory effect on the growth of both types of organism. An analysis of the population of the virulent strain, by the method described previously (Fukui *et al.*, 1957) however, revealed that avirulent cells are present (table 2) in normal virulent cultures even when the stock cultures are carried in such a manner (i. e. in the presence of reducing agents, under nitrogen, and in the presence of bicarbonate) as to display con tinuously low LD_{50}'s. This fact becomes quite plausible when one realizes that a culture which becomes 90 per cent avirulent *in the sense that only 10 per cent of the cells are virulent* merely increases its LD_{50} approximately by a factor of 10. The curve (figure 1) for strain ASC 1 (virulent) grown in air without sodium bicarbonate represents the selective outgrowth of the avirulent mutants present in the inoculum. This fact is obvious since the LD_{50} as measured at 72 hr was 1×10^6 cells. The curve for strain ASC 1 grown in air in the presence of bicarbonate represents growth of virulent cells as manifested by the mouse virulence titration at 72 hr, which established an LD_{50} of 2 cells (see also table 2). This latter curve represents, therefore, most realistically *the growth of the virulent members* of the inoculum and the manintenance of strain ASC 1 in the virulent form. The application of the virulence titration has, in a sense, given these growth experiments another dimension. It becomes obvious that the presence of dissolved CO_2 suppresses the proliferation of the avirulent organisms. The simplest interpretation is that this inhibition is accomplished through interference with oxidative metabolism, a type of process most characteristic of the avirulent mutant. We are reminded here that strain A1224 and other strains of avirulent *P. pestis* which we have studied will invariably initiate growth under aerobic conditions much more rapidly than their virulent cousins; and, when the virulent cultures do initiate growth under aerobic conditions, the culture invariably

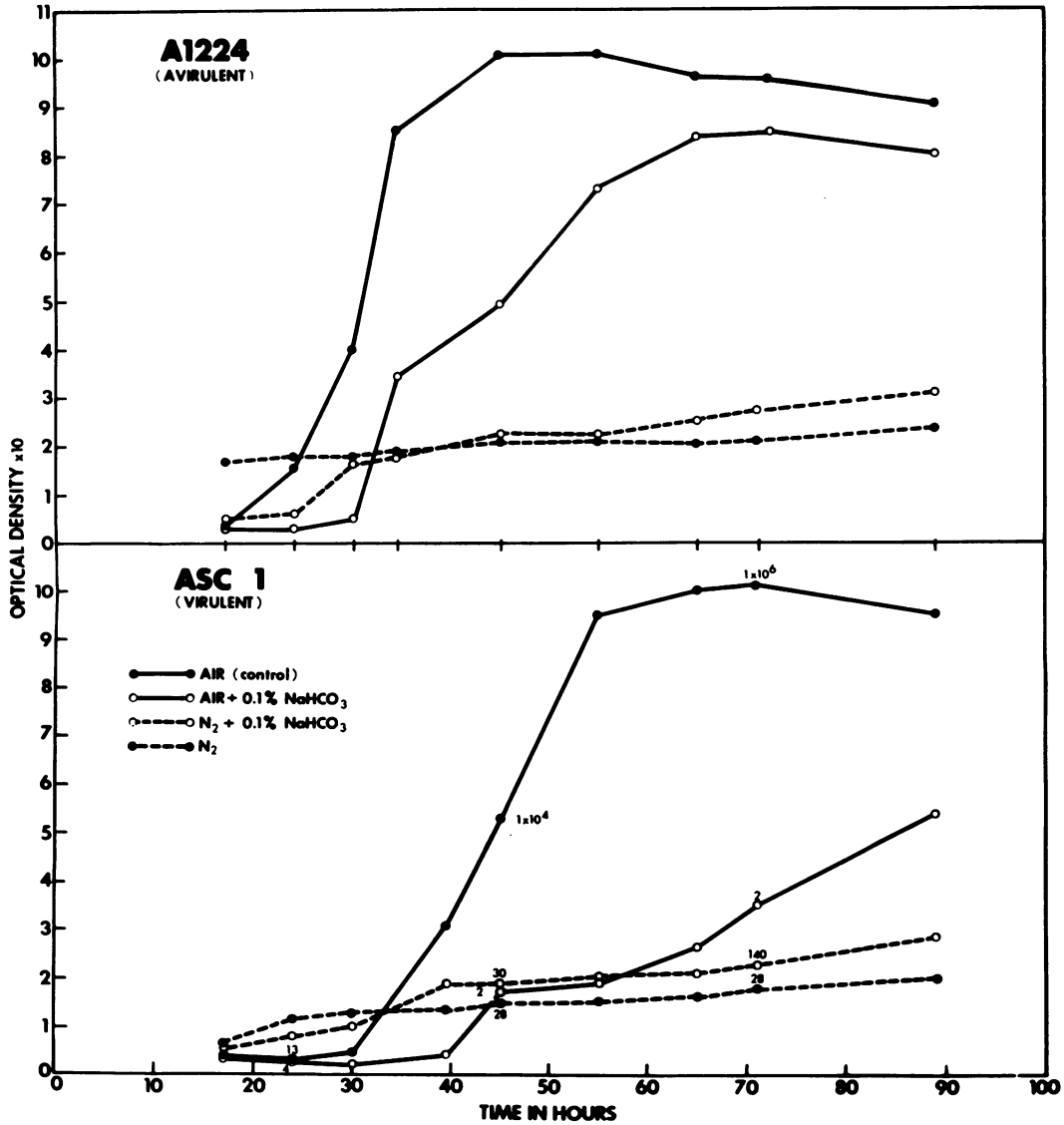

Figure 1. Effect of $NaHCO_3$ on the growth and virulence of *Pasteurella pestis* in air and under N_2

becomes avirulent. The interpretation also receives support from the studies regarding growth and virulence in the absence of oxygen. As recorded in figure 1, it has been established that the virulent and avirulent strains grow at approximately the same rate when cultivated under an atmosphere of nitrogen, and the virulent culture (ASC 1) retains virulence. Insofar as anaerobic metabolism is concerned the virulent members of the inoculum can compete in the growth process.

It was observed that the addition of sodium bicarbonate (0.1 per cent) or the inclusion of 1 per cent CO_2 in the gas atmosphere, when the cultures were grown under nitrogen, resulted in more consistent growth, fewer growth failures when inocula were very small, and a somewhat greater yield of cells. Growth was initiated more rapidly under the nitrogen-CO_2 atmosphere than under air although the cell yield was less than that attained under aerobic conditions.

When cultures grown in air were permitted to incubate further or serial transfers made so that a greater number of generations (30 to 40) was obtained, a progressive loss of virulence was

TABLE 2

Effect of sodium bicarbonate on the population and virulence of Pasteurella pestis grown at 37 C with aeration

Additive	Age (hr)	Viable Count/ml	Ratio: Virulent/ Avirulent	LD_{50}	95% Confidence Limits
None	0	1.7×10^5	9/1	43.0	28.0–75.0
	24	6.3×10^4	9/0	13.0	7.5–21.0
	48	8.6×10^6	0/10	1.4×10^4	7.7×10^3–2.5×10^4
	72	1.1×10^9	0/10	1.25×10^6	7.3×10^5–2.1×10^6
	96	7.0×10^8	0/10	8.2×10^5	3.9×10^5–1.7×10^6
0.1% $NaHCO_3$	0	1.7×10^5	9/1	29.0	17.0–48.0
	24	6.3×10^6	10/0	3.9	2.3–6.7
	48	2.4×10^8	7/1	2.3	1.4–3.9
	72	5.7×10^8	9/0	1.45	0.8–2.7
	96	6.4×10^8	10/0	52.0	31.0–89.0

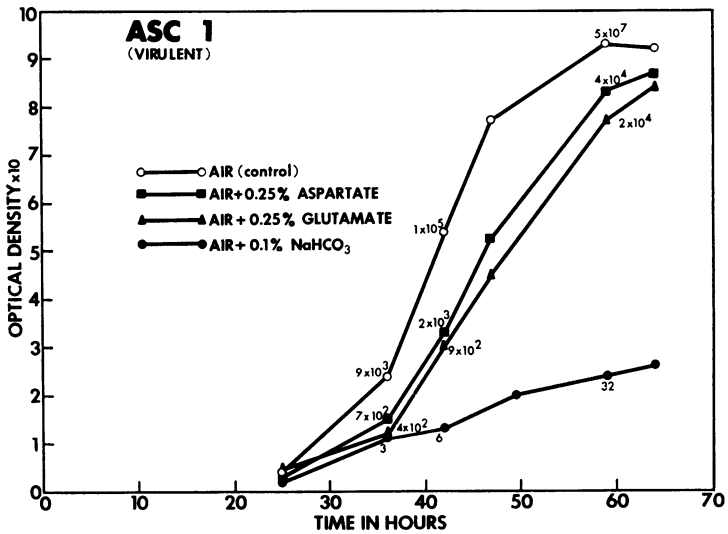

Figure 2. Effect of glutamic and aspartic acids and bicarbonate on the growth and virulence of *Pasteurella pestis.*

noted even in the presence of sodium bicarbonate, aspartic or glutamic acids. Further incubation of cultures grown in the absence of oxygen (under N_2) did not manifest any loss of virulence.

Further evidence to support our findings that CO_2 potentiates the preservation of virulence was obtained by correlating the quantity of bound CO_2 in complex media with the virulence of the resulting cultures. Of six different lots of complex medium examined, the one lot which displayed the ability to grow cultures with a positive degree of virulence retention also contained appreciable quantities of bound CO_2.

Our studies have not progressed to the extent that any definitive interpretation can be made with respect to the amino acid effects on virulence retention. Preliminary observations (figure 2) display results which are at least quantitatively quite unlike those observed in the case of sodium bicarbonate. A much shorter growth lag is apparent, but nevertheless, the lag such as it is might suggest a similar differential inhibitory mechanism. Studies involving population analyses and relative rates of loss of virulence over continued subculture are in progress and may contribute toward the elucidation of the mode of retention of virulence in these cases.

In conclusion the following interpretations can

be given in gross physiological terms. The avirulent cells have a more effective oxidative metabolism (figure 1) than have the virulent cells, hence under aerobic conditions the avirulent mutants in a culture will outgrow the virulent cells and the culture will become avirulent. In the absence of oxygen, the virulent cells can compete successfully on the basis of their anaerobic metabolism and hence the cultures retain virulence. Carbon dioxide (sodium bicarbonate) inhibits oxidative metabolism, and since the avirulent cells are more active metabolically, insofar as their oxidative metabolism is concerned, the net result is the suppression of the avirulent mutants and the retention of virulence of the culture. This CO_2 effect can be interpreted as an inhibition of CO_2 release in the terminal oxidative mechanisms.

SUMMARY

Studies on the growth characteristics of virulent and avirulent strains of *Pasterurella pestis* at 37 C in air and under nitrogen and in the presence of CO_2 (bicarbonate), aspartic acid or glutamic acid, have furnished the following results: (a) under aerobic conditions the avirulent cells in a population are capable of outgrowing the virulent members; (b) in the absence of oxygen (under N_2) the virulent cells in the inoculum can grow at least as rapidly as the avirulent cells, and the culture retains virulence; (c) carbon dioxide suppresses the initial rate of growth in air of both virulent and avirulent cultures, but in such a manner that the virulent cells of the "virulent" inoculum continue to proliferate, with the end result that the initial ratio of virulent to avirulent cells is held approximately constant, and the culture retains virulence for the test animal; (d) aspartic or glutamic acids contribute toward the retention of virulence in a manner which may or may not be related to the CO_2 effect.

An interpretation of the favorable effect of CO_2 (bicarbonate) on the retention of virulence is given in terms of CO_2 inhibition of terminal oxidative mechanisms with the consequent selective inhibition of the avirulent forms and the competitive outgrowth of the virulent members of the population within the intitial inoculum.

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