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STUDIES ON IMMUNITY IN ANTHRAX

XI. CONTROL OF CELLULAR PERMEABILITY BY BICARBONATE ION IN RELATION TO PROTECTIVE ANTIGEN ELABORATION

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ABSTRACT

PUZISS, MILTON (Fort Detrick, Frederick, Md.) AND MARY B. HOWARD. Studies on immunity in anthrax. XI. Control of cellular permeability by bicarbonate ion in relation to protective antigen elaboration. J. Bacteriol. **85**:237–243. 1963.—No elaboration of *Bacillus anthracis* protective antigen was demonstrated after addition of bicarbonate to cultures incubated 42 hr in a bicarbonate-free medium. Antigen accumulation in culture filtrates was greater if the bicarbonate was added to these cultures early in the growth period. Cell-free extracts of washed cell suspensions, prepared by hand grinding or lysozyme treatment, had no demonstrable intracellular protective antigen. Sonic treatment released antigen in measurable amounts, but tended to destroy or degrade the labile antigen. Freezing and thawing disrupted the cells and liberated the antigen; protective antigen was shown to be of internal rather than of extracellular origin. Maximal intracellular antigen concentration occurred before the peak concentration was reached in the culture filtrates. Antigen also was found in cell extracts from bicarbonate-free culture media when none was present in the corresponding culture filtrates. A high level of internal protective antigen was present in cells grown in a bicarbonate-free medium maintained at a constantly alkaline pH; only a trace of antigen was present in the corresponding pH-adjusted culture filtrate. The hypothesis is presented that bicarbonate functions to control cellular permeability and release of antigen from the cells into the ambient medium.

Previous reports from this laboratory have shown that a protective antigen was produced by nonencapsulated, nonproteolytic mutants of *Bacillus anthracis* grown in a chemically defined medium under certain cultural conditions (Puziss and Wright, 1954; Wright and Puziss, 1957). Sodium bicarbonate was one of the specific requirements for elaboration of antigen into the medium; this requirement was demonstrated by Gladstone (1946), and has been confirmed repeatedly in both aerobic and anaerobic cultures. Small amounts of antigen were demonstrated in filtrates from anaerobically grown cultures in a bicarbonate-free medium, probably as a result of metabolic carbon dioxide retained in the closed system used to obtain anaerobiosis (Puziss and Wright, 1959). Thorne and Belton (1957) also considered retention of metabolic carbon dioxide in an alkaline medium a factor in antigen accumulation in a bicarbonate-free culture. The bicarbonate could not be replaced by functionally related compounds (Puziss and Wright, 1954); its mode of action in antigen elaboration has not been defined. Bicarbonate had no influence on carbohydrate metabolism of the organism during growth and elaboration of protective antigen in culture filtrates (Puziss and Wright, 1959). The metabolism of amino acids similarly was not significantly affected by bicarbonate in aerobic or anaerobic culture (Wright, Puziss, and Neely, 1962).

This report describes the results of studies of the influence of bicarbonate on accumulation of extracellular and intracellular protective antigen, and presents a plausible hypothesis for the mode of action of bicarbonate.

MATERIALS AND METHODS

Cultures. The strains of *B. anthracis* were nonencapsulated, nonproteolytic, avirulent mutants of the virulent strains 107 and V770 (Wright et al., 1962). Spore suspensions for inocula were adjusted to give a final concentration of 500 spores per ml of medium. Immunized animals were challenged by a standard spore suspension of the virulent Vollum strain of *B. anthracis*.

Medium and methods of culture. Preparation of the chemically defined medium 1095 for anaerobic cultures has been described (Wright et al., 1962). The pH of the complete medium was approximately 7.8. When sodium bicarbonate was omitted, the pH of the medium was adjusted to the same level as the control by addition of sterile 1 N NaOH. Media for anaerobic growth were prepared in volumes of 4 to 10 liters in glass aspirator bottles, and were incubated under nitrogen as already described.

Harvest and preparation of cells. After the required incubation at 37 C, the culture was dispensed via the delivery tube of the aspirator bottle into sterile 600-ml centrifuge bottles. Cells were readily collected by centrifugation at 1,500 × *g* for 20 min.

When cells from larger volumes of anaerobic culture (10 liters) were harvested, the culture vessel was placed in a cold bath (4 C); the cells sedimented satisfactorily in 3 to 4 hr at this temperature. Most of the supernatant was discarded, and the concentrated cells in suspension were collected by centrifugation.

In the initial studies, the packed cells were washed five times by suspension in 500-ml portions of cold sterile distilled water. It was later found that one washing for the removal of soluble surface antigen was as adequate as five washings; this was the method used in the latter part of this study. Washed cells were resuspended in sterile buffer and adjusted to give an optical density reading (with a blue filter) of 500 ± 50 Klett-Summerson units. Buffers were either 0.05 M phosphate (pH 7.9) or tris(hydroxymethyl)-aminomethane (tris), 0.04 M (pH 8.0).

Processing of cell extracts. After the completion of the cell extraction procedures (see Results), the extracellular fluid and the cell debris were separated by centrifugation 4,000 × *g* for 30 min) at 4 C. The supernatants were drawn off and were sterilized by filtration, tested for sterility, and stored either in the frozen or the lyophilized state.

Assay for protective antigen in culture filtrates and cell extracts. The in vivo assay method for estimation of protective antigen by immunization and challenge of guinea pigs has been described by Puziss and Wright (1959). Cell-free extracts in the normal fluid state, or the lyophilized product, were stored at −20 C for use during the period required for animal immunization. The crude dried cell-free extracts were reconstituted at 25 mg/ml concentration. Guinea pigs (250 to 350 g) were immunized, each animal receiving a total of 62.5 mg of the cell-free extract, given subcutaneously in five doses of 0.5 ml each on alternate days. Culture filtrates obtained from the complete medium were used as controls; these were reconstituted at the routine 15 mg/ml concentration. Cell extracts were assayed, in most cases, in parallel with their corresponding culture filtrates. The extracts and filtrates were also titrated in vitro for antigenic activity by the complement-fixation method of McGann, Stearman, and Wright (1961). The Ouchterlony agar diffusion method of Thorne and Belton (1957) was used to identify and to titrate the intracellular antigen.

RESULTS

Effect of bicarbonate additions to incubated cultures. Experiments were performed to determine the effect on antigen elaboration of addition of bicarbonate to a mature cell culture previously grown in a bicarbonate-free medium. Bicarbonate was added aseptically, at double the normal concentration, to bicarbonate-free cultures after 42 hr of incubation (the normal harvest period for antigen-containing cultures). Part of the culture was filtered at once, along with a complete medium control. Another part of the culture was reincubated anaerobically for an additional 3 hr before filtration. A surface-active agent (Tween 80) was also added to 42-hr bicarbonate-free cultures, in an attempt to remove any protective antigen that might have accumulated on the bacterial cell surface. This addition was without effect on the accumulation of antigen in the culture filtrate. Normal antigen accumulation occurred in the control culture filtrates, whereas only traces of antigen were detected in the filtrates of cultures in which the bicarbonate was added to the 42-hr-old cultures. Other cultures in which the bicarbonate was added after 42 hr of incubation were reincubated with supplemental glucose as an additional energy source, since the initial glucose was exhausted after 42 hr. The presence of this extra source of energy, and the addition of bicarbonate, did not influence the accumulation of protective antigen; only a trace of antigen was found in the culture filtrates, if the bicarbonate was added after the 42-hr period. Evidently, bicarbonate had no effect on antigen

elaboration when it was added to an already mature culture.

To determine at what point during growth the bicarbonate effect on antigen elaboration could be demonstrated, bicarbonate was added to bicarbonate-free cultures grown for 20, 28, or 35 hr. These cultures were then reincubated anaerobically, until harvest at 42 hr. Assay results showed that the earlier the bicarbonate was added to the cultures, the more antigen accumulated in the filtrate. No antigen, or at most only trace amounts, was detected if bicarbonate was added after 35 hr of incubation (Table 1). General agreement between animal assay and complement-fixation titers was obtained.

TABLE 1. *Protective antigen elaboration in culture filtrates after addition of bicarbonate to an incomplete medium*

Culture medium	Post-inoculation bicarbonate addition	Antigen assay: Survival ratio*	Antigen assay: Avg day of death	Antigen assay: Complement fixation†
	hr			
Complete	0	7/8	5.0	120
HCO_3-free	No addition	0/8	3.7	5
HCO_3-free	20	3/8	5.3	30
HCO_3-free	28	2/8	3.5	30
HCO_3-free	35	0/8	3.9	5
Normal animals		0/4	3.9	

* Ratio of the number of animals surviving over the total number challenged.

† Expressed as 50% units per ml.

Preliminary studies on extraction procedures. In an effort to understand the factors controlling the accumulation of extracellular protective antigen, an attempt was made to detect the presence of intracellular antigen. Washed cells were ground in a chilled mortar and extracted with 0.05 M cold phosphate buffer, pH 8.0 (McIlwain, 1948). After centrifugation, the supernatant was assayed for antigen activity by complement-fixation and agar diffusion methods. For a control in the agar diffusion test, a highly immunogenic dried culture filtrate was used at a concentration of 5 mg/ml. This control material formed a single precipitation line with horse antispore serum that merged with the line formed by the purified protective antigen of Strange and Thorne (1958). Cell disruption by hand grinding did not release protective antigen.

Treatment with lysozyme was investigated as a means of liberating intracellular antigen. Cells from a 23-hr culture were incubated at 37 C with lysozyme, at a final concentration of 0.5 mg/ml, and sampled after 0.5, 1, 2, 3, and 5 hr. Optical density measurements and stained smears were made; the suspensions were then filtered and the supernatants frozen. Increasing the concentration of lysozyme and incubating the treated cell suspensions at different temperatures did not lead to liberation of antigen detectable by in vitro assay methods. A slight increase in optical density of the lysozyme-containing suspension, and a decrease in that of the control, were noted. This was similar to the findings of Gladstone and Johnston (1955).

Ultrasonic disintegration of cell suspensions. Washed cells suspended in buffer were subjected to ultrasonic treatment in a 10-kc oscillator (Raytheon Co., Waltham, Mass.) for 40 min. An alcohol-Dry Ice mixture was circulated through the jacket of the treatment cup in an attempt to minimize destruction of labile material liberated during sonic treatment. Good cell disruption was achieved, as evidenced by loss of turbidity and an increase in viscosity of the clarified supernatant from the disrupted cells. Cell breakage was confirmed by microscopy. The cell-free extracts released by sonic treatment were too highly anticomplementary for use in complement-fixation reactions. The sonically treated extracts, filtered through a membrane filter, and lyophilized and concentrated threefold, showed the presence of protective antigen; a line was evident on the agar diffusion plates that joined the line produced by a lyophilized protective antigen control.

Sonic liberation of antigen was investigated further. A 15-min sonic treatment was sufficient to liberate the maximal amount of protective antigen. Cultures incubated less than 44 hr were then investigated, to determine whether protective antigen within the cells was present in amounts large enough to be detected without concentration, and at what period during growth it appeared. Volumes (10-liter) of culture media were used to obtain cells at different periods of time from a single culture source. Results of the serological assays on the sonic extracts of these cells are presented in Table 2. The data indicate that protective antigen was present in the cell ex-

TABLE 2. *Protective antigen in cell extracts and culture filtrates after ultrasonic treatment of cell suspensions*

Incubation time	Antigen source	Antigen titers	
		[illegible] fixation	Agar plate diffusion
hr		*50% units/ml*	
16	Filtrate	5	Neg
	Extract	AC*	1:1
23	Filtrate	30	Neg
	Extract	AC	1:4
28	Filtrate	70	1:2
	Extract	AC	1:2
34	Filtrate	80	1:8
	Extract	AC	Neg
47	Filtrate	120	1:8
	Extract	AC	Neg
64	Filtrate	80	1:4
	Extract	AC	Neg

* Anticomplementary; no complement-fixation titer could be obtained.

tracts in measurable amounts before a comparable concentration was detectable in the culture filtrate.

There were indications that sonic treatment of culture filtrate degraded the protective antigen. Protection of guinea pigs was lower with sonically treated than with untreated filtrate antigen. The protective effect of addition of serum during sonic treatment of cell suspensions was investigated, since Strange and Belton (1954) found that normal horse serum stabilized their filtrate antigen. Normal horse serum (5%, v/v) added to a 23-hr cell suspension, or to culture filtrate antigen prior to sonic treatment, had no stabilizing effect. Accordingly, less drastic methods of cell disruption were investigated.

Effect of freezing and thawing on release of intracellular antigen. Cells from 4 liters of 23-hr culture were subjected to three cycles of freezing and slow thawing. After centrifugation to remove cellular debris, the extracts were sterilized by filtration through either membrane or ultrafine sintered-glass filters. The extracts showed no evidence of anticomplementary activity; they had complement-fixation titers of approximately 120. Agar plate diffusion titers were the same as those of the sonically treated cell extracts. Increasing the number of freeze-thaw cycles did not release more protective antigen. This method of extracting intracellular material was used throughout the remainder of the study, since the frozen-thawed extracts possessed somewhat greater protective activity than the sonic extracts and had the additional advantage of not being anticomplementary.

Protective antigen in extracts from a complete medium. Prior to cellular disruption and release of intracellular contents, it was necessary to determine whether the soluble protective antigen could be removed from the cell surface by washing. All the washings from the cell suspensions were pooled, lyophilized, and reconstituted at a 40-fold concentration. No protective antigen was demonstrated in the concentrated washings by either in vitro or in vivo assay. The negative results of these assays indicated that no soluble protective antigen was present on the cell surface.

The extracts of cells incubated for 42 hr in a complete medium contained only small amounts of antigen; antigen in the corresponding culture filtrate, however, was at the usual level. This observation indicated that the optimal time for antigen elaboration into the medium was not the optimum for accumulation of intracellular antigen. To clarify this point, 10-liter batches of culture grown in complete medium were sampled at intervals during incubation, to obtain equal volumes of cells for preparation of cell extracts and of culture filtrates from a single source. Assay results of the cell extracts are presented in Table 3; it can be seen that maximal intracellular antigen was present at about 23 hr of incubation. This was similar to the results first obtained with the sonically treated cell extracts (Table 2). Direct comparison of antigen in the

TABLE 3. *Protective antigen in extracts of cells from a complete medium*

Growth time	Antigen assay			
	Survival ratio*	Avg day of death	Complement fixation†	Diffusion titer
hr				
20	2/4	7.0	100	1:2
23	2/4	5.0	120	1:6
26	1/4	5.0	100	1:4
29	1/8	4.8	75	1:2
Control filtrate	7/8	5.0	160	1:8

* Ratio of the number of animals surviving over the total number challenged.

† Expressed as 50% units per ml.

extracts and in the culture filtrates revealed that intracellular antigen reached a maximum, and then began to disappear from the internal environment concurrently with the accumulation of antigen in the culture filtrate (Fig. 1).

Protective antigen in extracts from a bicarbonate-free medium. Preliminary studies on extracts of cells obtained from a bicarbonate-free medium indicated the presence of protective antigen in these extracts. To examine this more fully, large volumes of cells were grown in the bicarbonate-free medium, and were sampled at intervals during growth to obtain both cells and culture filtrates. Extracts of these cells showed that protective antigen was present in a measurable amount within the cells, and that a peak of intracellular antigen was reached after about 34 hr of incubation. No protective antigen could be demonstrated in the corresponding culture filtrates from the bicarbonate-free medium (Table 4). The difference in time of occurrence of maximal internal antigen in bicarbonate-free cell extracts and in control cell extracts corresponded to the lag in initiation of growth between these two types of cultures, as described in a previous report (Puziss and Wright, 1959). Cells that were grown anaerobically in a bicarbonate-free medium in the presence of 0.04 M tris buffer (pH 8.4) showed normal growth. Only a trace of protective antigen, however, was detected in this culture filtrate, as shown by a complement-fixation titer of 5 and a guinea pig survival ratio of 1/8.

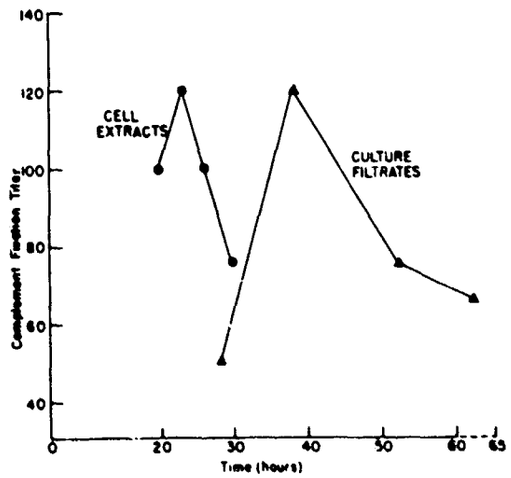

FIG. 1. *Intracellular and filtrate antigen accumulation in a complete medium.*

TABLE 4. *Protective antigen in extracts and culture filtrates from bicarbonate-free cultures*

Antigen type	Growth time	Antigen assay: Survival ratio*	Avg day of death	Complement fixation†	Gel diffusion titer
	hr				
Filtrate	23	0/8	3.5	0	Neg
Extract		0/7	3.5	6	Trace
Filtrate	34	0/7	3.9	0	Neg
Extract		3/7	6.2	80	1:4
Filtrate	41	0/16	3.5	0	Neg
Extract		1/15	4.5	24	1:2

* Ratio of the number of animals surviving over the total number challenged.

† Expressed as 50% units per ml.

TABLE 5. *Protective antigen in extracts and culture filtrates from pH-adjusted bicarbonate-free cultures*

Antigen type	Growth time	Antigen assay: Survival ratio*	Complement fixation†	Gel diffusion titer
	hr			
Filtrate	40	—	17.5	Neg
Extract		1/8	80	1:4
Filtrate	50	1/8	10	Neg
Extract		2/8	40	1:2

* Ratio of the number of animals surviving over the total number challenged; no animal data for the 40-hr filtrate available.

† Expressed as 50% units per ml.

It was observed that anaerobic bicarbonate-free cultures, in the absence of any other buffer, became more acid after the 40-hr incubation period than did control cultures, despite the initially high pH in the medium. This may have resulted from the absence of the buffering effect of bicarbonate. The greater acidity in the medium was one possible reason for the low level of protective antigen in extracts of cells grown longer than 34 hr in the bicarbonate-free medium. To determine the effect of the low pH on the internal protective antigen, bicarbonate-free cultures containing a pH indicator (*m*-cresol purple) were adjusted at 3- to 4-hr intervals during incubation by periodic addition of sterile 1 N NaOH to maintain the pH at the initial level of pH 7.6 to 7.8. Extracts prepared from cells grown in the

pH-adjusted bicarbonate-free medium for 40 hr had considerable protective antigen activity. Protective antigen was still evident in the cell extracts even after 50 hr of incubation of culture. Despite the alkaline pH level maintained in the culture vessel, however, only a trace of antigen was detected in the corresponding bicarbonate-free culture filtrates (Table 5).

DISCUSSION

It is evident from these studies that bicarbonate had no effect on antigen elaboration, if it was added to a suspension of matured cells grown in the absence of bicarbonate ion. High bicarbonate concentrations, as well as addition of a surface-active agent, and the presence of additional glucose, did not act upon these cells to remove or "strip" any protective antigen from the cell surface. Bicarbonate was necessary, apparently, during the early growth of the cell for any appreciable antigen accumulation in the culture medium. The earlier the cells were exposed to the bicarbonate, the more antigen accumulated.

Several methods of disrupting the cell structure and extracting the internal cell contents were explored. The results with lysozyme treatment showed the ineffectiveness of this method. As Gladstone and Johnston (1955) reported, not all *B. anthracis* strains are susceptible to lysozyme action; it is also possible, as they indicated, that the bicarbonate and CO_2 concentrations present during growth were too low for synthesis of the lysozyme substrate in the cell walls. Freezing and thawing of cells suspended in buffer was the method chosen for obtaining intracellular antigen with minimal destruction. The cell-free extracts could be lyophilized, in a manner similar to culture filtrates, without appreciable loss in antigen activity. It is difficult to understand why the frozen-thawed extracts of Smith, Keppie, and Stanley (1953) should not have contained at least some protective antigen, since this is the least rigorous extraction method. Cell disruption by shaking with "ballotini" (Smith et al., 1953; Gladstone, 1946), in the absence of a suitable buffering system, might readily result in destruction of the labile antigen liberated, particularly at pH levels below 8.0. In the study of Smith et al. (1953), as well as in our study, no antigen was detected in the washings from the cell suspensions. In addition to these authors, others have reported that cell-free extracts of the organism are free from any toxic activity (Gladstone, 1946; King and Stein, 1950).

The extracts described in this study contained significant concentrations of the protective antigen; this is in contrast to the findings of Keppie, Smith, and Harris-Smith (1953) that no antigen was present in the internal contents of cells, and that antigen was, therefore, essentially extracellular. Results of the studies reported here show that protective antigen of *B. anthracis* is probably of internal origin and is not a surface type of cellular antigen. As demonstrated in this study, antigen is apparently formed fairly early within the cells, when grown anaerobically in the complete medium. Peak protective antigen concentration within the cells occurs before peak accumulation in the culture medium is found. As the cells approach maturity and exhaustion of nutrients, antigen apparently passes out of the cells and into the ambient medium, as though it were a metabolic by-product; it is not, however, a product dependent on cellular autolysis. In cells grown in a bicarbonate-free medium, internal antigen reaches a maximum later than in cells from a complete medium; this corresponds closely to the lag in growth initiation of cells grown anaerobically in the absence of bicarbonate (Puziss and Wright, 1959).

The salient observation reported in this paper is that of significant antigen formation within cells grown in the absence of bicarbonate, but with no significant antigen accumulation in their corresponding culture filtrate. Culture of cells in a bicarbonate-free medium, maintained at a constantly alkaline pH, supported the observation of high antigen levels within the cell and very low levels in the culture medium outside the cell. Strange and Thorne (1958) reported that some antigen elaboration occurred in a bicarbonate-free medium buffered at an alkaline pH with tris buffer. This was confirmed in our anaerobic cultures grown in the presence of tris buffer, where the concentration of antigen in the culture filtrate was very low in comparison with control culture filtrates. The small accumulation of protective antigen can be attributed to the efficient retention of metabolic CO_2 by the tris buffer in a bicarbonate-free medium. In this study, an alkaline pH was maintained by intermittent addition of small amounts of NaOH to the bicarbonate-free cultures. The data obtained from study of the

cell extracts strongly suggest that bicarbonate in the medium affects cellular permeability, allowing passage of the internally formed antigen across the cell membrane. In the absence of bicarbonate, antigen apparently is unable to pass across the cell membrane and accumulates within the cell. Bicarbonate-free anaerobic cultures incubated for 3 weeks retain normal turbidity and very little autolysis. Cultures grown in a complete medium, however, usually show the beginnings of autolysis after about 72 hr of incubation; nearly complete clearing of the medium is seen by 3 weeks.

The mechanism by which bicarbonate could affect cellular permeability is not understood; perhaps some alteration of the integrity of the cellular membrane occurs. Similar effects on cellular permeability by other inorganic ions have been observed with mammalian cells. For example, Dubes and Klingler (1961) increased monkey kidney cell permeability to polio virus by calcium ion depletion. Adams and Burgess (1960) decreased catalase migration from mouse-liver granules by addition of calcium or magnesium ions. Bicarbonate ion concentration, therefore, may indeed affect permeability of some species of bacterial cells.

Acknowledgments

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