

Studies on Immunity in Anthrax

XII. Requirement for Phosphate for Elaboration of Protective Antigen and Its Partial Replacement by Charcoal

GEORGE G. WRIGHT, LORELL H. ANGELETY, AND BEATRICE SWANSON¹

Medical Sciences Laboratory, Fort Detrick, Frederick, Maryland 21701

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Retention of protective antigen on columns of diethylaminoethyl cellulose was inhibited by the 0.01 M phosphate salts in the standard growth medium. Reduction in the concentration of phosphate to 0.001 M allowed satisfactory retention of antigen on diethylaminoethyl cellulose but decreased significantly the elaboration of antigen during growth of the cultures. Growth remained normal at phosphate concentrations as low as 0.0001 M. Inhibition of antigen elaboration in media containing reduced concentrations of phosphate was overcome by addition of 0.006% (w/v) charcoal. It is suggested that antigen elaboration, like encapsulation of virulent strains, is stimulated in the presence of an adsorbent. However, a nonencapsulated strain derived from an F mutant exhibited the same requirement for an adsorbent as nonencapsulated strains derived from the parent strain.

The protective antigen of *Bacillus anthracis* has found practical application in immunization of man against anthrax and in serological measurement of immunity (1, 2, 5, 16). Interest in the antigen also was stimulated by recognition of its role as a component of the anthrax toxin (14). Accordingly, workable procedures for preparation and isolation of protective antigen in good yield are of practical and theoretical significance.

The antigen is elaborated into the culture medium only under particular conditions of growth. Although considerable information has been reported regarding suitable media and cultural conditions, the requirements for elaboration of antigen remain essentially empirical (18). A relatively high concentration of bicarbonate is perhaps the best established requirement. The bicarbonate evidently permits release of antigen which otherwise remains associated with the cells (10). Other requirements for elaboration of antigen in addition to substances needed for growth include a utilizable carbohydrate, guanine or certain other purines, calcium ion, and certain amino acids (11).

Addition of small amounts of charcoal stimulated elaboration of antigen in hydrolyzed casein media and in certain chemically defined media (16). In unpublished experiments in our laboratory with medium 1095 and other defined media, however, no stimulation of antigen elaboration was detected on addition of charcoal. In the present work, difficulties encountered in the concen-

tration of protective antigen by chromatographic methods led to study of the effects of the concentration of phosphate in the medium and to reinvestigation of the addition of charcoal.

MATERIALS AND METHODS

Cultures. The V770-NP1-R and 116-NP1-R2 strains of *B. anthracis* were used for the production of protective antigen (18). F mutants were isolated from strain 116-NP2 by the method of Meynell and Meynell (9). Nonencapsulated mutants were obtained from the F mutants as described by Thorne (15).

Growth media. Medium 1095 (18) was the basal medium for production of protective antigen. Media that contained reduced concentrations of phosphate were adjusted to pH 6.4 to 6.5 before autoclaving. After aseptic addition of all supplements, the complete medium was adjusted to pH 7.8 to 7.9 with 1 N HCl.

Vegetative inoculum. One-liter volumes of medium 1095 were inoculated with 500 spores per ml and incubated for 24 to 25 hr in air at 37 C with moderate stirring.

Preparation of protective antigen. Cultures for routine production of culture filtrate for fractionation studies were grown in 10-liter volumes of medium (18). Cultures were inoculated with 500 spores per ml, adjusted to 37 C, and incubated under anaerobic conditions with constant slow stirring. At the time of complete utilization of glucose, usually 25 to 27 hr, the cultures were adjusted to pH 8 and filtered through Selas filters (Selas Flotronics, Div. of Selas Corp. of America, Spring House, Pa.) of 02 or 03 porosity.

For study of the effect of alterations in the composition of the medium on growth or antigen elaboration,

¹ Present address: Armed Forces Radiobiological Research Institute, Bethesda, Md.

2.5-liter volumes of medium in 4-liter bottles were used. The media were autoclaved for 25 min at 120 C. In some cases the indicated volumes of vegetative inoculum were used rather than spore inoculum. Cultures were incubated in the same manner as the larger cultures, except that at the indicated periods samples were removed, adjusted to pH 8, and clarified by centrifugation for estimates of antigen titer or remaining glucose. In some cases the supernatants were sterilized by passing through membrane filters of 0.45 μm pore size (Millipore Corp., Bedford, Mass.).

Charcoal. Activated charcoal (Norit A) was suspended in double-distilled water at 0.125%, sterilized by autoclaving, and added aseptically to the complete medium as indicated.

Glucose determinations. The glucose oxidase method (12) was employed for glucose determinations, with Glucostat (Worthington Biochemical Corp.) used as reagent.

Phosphate determinations. The method of Fiske and Subbarow (4) was used for phosphate determinations.

Assay of protective antigen. Culture filtrates were titrated for antigen activity by the complement fixation (CF) method (7) by using serum B-48. Most of the filtrates also were examined by gel diffusion to confirm the specificity of the reaction. A circular pattern was used with a central antiserum well containing serum P-8 and six peripheral antigen wells. Protective antigen lines produced by unknown samples were identified by interaction with control lines from wells containing purified protective antigen.

Antisera. Serum B-48, used for CF-titrations, was produced in a burro by four subcutaneous injections of 5×10^9 viable spores of *B. anthracis*, Sterne strain. In immunoelectrophoresis, the serum produced a strong line with protective antigen and two additional weak lines with concentrated culture filtrate. We are indebted to the U.S. Army Medical Research Institute of Infectious Disease for this antiserum. Serum P-8 was obtained from a pony after eight courses of immunization with protective antigen filtrate adsorbed on aluminum hydroxide. The serum produced at least 23 arcs of precipitation with concentrated culture filtrates.

Column chromatography. Diethylaminoethyl (DEAE)-cellulose was suspended as a 7.5% (w/v) slurry in 0.5 N NaOH and shaken at room temperature for 10 min. Then the material was washed thoroughly with deionized water and was washed twice with 0.01 M phosphate buffer, first at pH 6.3 and then at pH 7.2. The weight of DEAE cellulose used for preparation of columns was determined by the volume of culture filtrate to be processed, usually at a ratio of 2 g of DEAE cellulose per liter of undiluted filtrate. The ratio of height to diameter of the columns was approximately 2:1. Columns were equilibrated to pH 7.2 by passage of 0.01 M phosphate buffer.

RESULTS

Initial adsorption of antigen. Exploratory studies revealed the applicability of columns of DEAE cellulose for concentration and fractionation of protective antigen and other antigens in culture filtrates of *B. anthracis*. Dilution of the

culture with 1.25 or 1.5 volumes of water reduced the ionic strength sufficiently so that the antigens were adsorbed on the columns. Passage of phosphate buffers containing appropriate concentrations of sodium chloride eluted the various antigens in acceptable yield.

More detailed study of the recovery of protective antigen during initial adsorption on DEAE cellulose revealed that, after passage of relatively small volumes of diluted culture filtrate, a significant proportion of the antigen remained in the effluent. The attainable loading of the column was much less than in subsequent purification steps. Typical results are presented in Fig. 1, in which the per cent of antigen adsorbed is plotted as a function of volume of filtrate passed.

A similar incomplete adsorption of antigen was observed when suspensions of DEAE cellulose were mixed with diluted culture filtrates, and this more convenient procedure was used for systematic study of factors influencing adsorption of antigen. No significant increase in adsorption was obtained by further dilution of the filtrate, alterations in pH in the range 7.0 to 8.8, use of other brands or types of DEAE cellulose, alkali pretreatment or additional washing of the DEAE cellulose, change in the time of adsorption between 15 and 60 min, or reduction in temperature to 4 C. Prior dialysis of the filtrate against water, however, greatly increased the proportion of protective antigen that combined with DEAE cellulose.

Systematic investigation revealed that the factor that inhibited quantitative recovery of the antigen was the potassium phosphate, which was present in the culture medium at a concentration

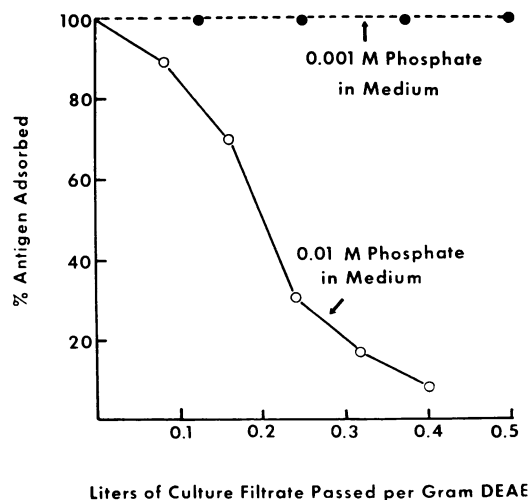


FIG. 1. Effect of concentration of phosphate in growth medium on adsorption of protective antigen from culture filtrate onto DEAE cellulose columns.

TABLE 1. Effect of concentration of phosphate on utilization of glucose and elaboration of protective antigen

Concn of sodium phosphate in medium (M)	Incubation time				
	22 hr	24 hr		27 hr	
	Initial glucose remaining (%)	CF units per ml	Initial glucose remaining (%)	CF units per ml	Initial glucose remaining (%)
0.01	18	120	0	160	0
0.001	21	40	0	40	0
0.0001	25	30	0	30	0
0.00001	82	15	83	15	81

of 0.01 M. Reduction of phosphate in the medium to 0.001 M or dialysis of the filtrate gave greatly increased adsorption of this antigen (Fig. 1).

Effect of phosphate concentration. The concentration of phosphate in the medium had been retained at 0.01 M since the initial studies on antigen elaboration in defined media (17). The phosphate was added as equimolar concentrations of dibasic and monobasic potassium phosphates. Experiments were carried out on the effect of reduction of the concentration of total phosphate on growth as estimated by the time required for complete utilization of the glucose, and on protective antigen elaboration as measured by CF titers. Typical results are presented in Table 1. Reduction in phosphate to 0.001 or 0.0001 M had no significant effect on rate of growth; only when the concentration was reduced to 0.00001 M was growth delayed and decreased. Experiments on uptake of phosphate during growth were consistent with these findings; in cultures with initial phosphate concentrations ranging from 0.01 to 0.0001 M, no change in phosphate concentration could be detected after growth. Optimum elaboration of antigen required a much higher concentration of phosphate than was necessary for growth. Reduction to 0.001 M usually reduced antigen detectably, and further reduction led to progressive loss of antigen elaboration (Table 1).

Various hypotheses were investigated in an effort to determine the basis of the requirement for 0.01 M phosphate. Decrease in phosphate to 0.001 M reduced the buffering power of the medium and lowered the pH during autoclaving from 6.8 to 3.6. However, adjustment of the medium to pH 6.8 with NaOH before autoclaving did not overcome the phosphate requirement. Reduction in phosphate did not alter the pH during growth by as much as 0.05 pH unit; evidently the buffering power of the complete medium results primarily from the sodium bicarbo-

nate added aseptically after autoclaving. Apparently, control of the pH during growth was not the basis of the requirement for phosphate.

The possibility was considered that the requirement for phosphate, in addition to that necessary for growth, involved formation during autoclaving of phosphate complexes with glucose or other medium constituents that might determine the availability of critical metal ions (13). This hypothesis was discarded when it was found that phosphate or glucose could be autoclaved separately from the other constituents of the medium without significant alteration in growth or antigen elaboration.

Synthesis of alkaline phosphatase by *B. anthracis* is controlled by the concentration of phosphate in the medium (6). It seemed possible that 0.01 M phosphate was required to repress production of alkaline phosphatase, which might be competitive with or otherwise inhibit the synthesis of protective antigen. A mutant strain was isolated in which phosphatase was repressed by $\frac{1}{20}$ the concentration of phosphate required by the parent strain. Antigen elaboration by the mutant had the same requirement for phosphate as by the parent strain; evidently, phosphatase synthesis is not involved in the requirement for phosphate.

Reduction in the concentration of phosphates influenced slightly the color of the final medium, perhaps reflecting differences in the state of colloidal phosphate salts. Optimum encapsulation of virulent *B. anthracis* requires not only bicarbonate but also an additional factor present in serum, which is replaceable by serum albumin, charcoal, or certain other adsorbents (8). It seemed possible that optimum elaboration of protective antigen might also require an adsorbent, and that the high concentration of phosphate functioned to form an adsorbent of colloidal phosphates. Accordingly, the effect of addition of charcoal on elaboration of antigen in the presence of reduced concentrations of phosphate was investigated. Preliminary experiments indicated that 0.006% (w/v) charcoal was the highest concentration that did not cause marked delay in growth from spore inocula. Experiments (Table 2) explored the possible interactions of the concentrations of phosphate and charcoal during elaboration of antigen by spore and vegetative inocula of the two strains. Elaboration of antigen was inhibited by reduction of phosphate from 0.01 to 0.001 M, and this inhibition was overcome by addition of charcoal. Charcoal did not increase elaboration of antigen in media containing 0.01 M phosphate. The requirement for 0.01 M phosphate or charcoal appeared to be less marked when vegetative rather than spore inocula were used, especially with

TABLE 2. Effect of concentration of phosphate and addition of charcoal on elaboration of protective antigen

Inoculum	Strain of <i>B. anthracis</i>	Concn of phosphate (M)	Concn of charcoal				
			None	0.002%	0.006%	0.019%	
Spores 500/ml	V770-NP1-R	0.01	80 ^a				
		0.001	40		160		
	V770-NP1-R	0.01	160		160		
		0.001	60		160		
	500/ml	V770-NP1-R	0.001	60	160	160	160
	500/ml	116-NP1-R2	0.001	60	160		160
	500/ml	116-NP1-R2	0.001	30	60		60
500/ml	116-NP1-R2	0.001	15		60		
Vegetative culture	V770-NP1-R	0.01	120				
		0.001	60				
		0.001	20		120		
	V770-NP1-R	0.01	320				
		0.001	160		320		
	V770-NP1-R	0.001	40		160		
	116-NP1-R2	0.001	320	320	320	240	
	116-NP1-R2	0.01	320				
	116-NP1-R2	0.001	240				
	116-NP1-R2	0.01	120				
	116-NP1-R2	0.001	60		120		
	116-NP2-F-R	0.01	120				
		0.001	60		120		

^a Fifty per cent (50%) complement-fixation units per milliliter of culture.

strain 116-NP1-R2. Titers of antigen varied somewhat among experiments, perhaps reflecting variations in sensitivity of the CF titrations, so that comparisons of titer between experiments may not be valid.

Effect of charcoal on rate of growth. Addition of 0.006% charcoal caused no significant effect on growth from vegetative inocula, as judged by the rate of utilization of glucose. With spore inocula, however, addition of 0.006% charcoal increased the time required for complete utilization of glucose by as much as 5 hr. The effect appeared to consist primarily of a lag in the initiation of fermentation. Accordingly, the influence of addition of charcoal at various times after inoculation was investigated. Typical results, showing the delay in glucose utilization when charcoal was added at the time of inoculation, are shown in Fig. 2. The effects of addition of charcoal 4 and 8 hr after inoculation are also shown. Evidently, the growth initiation process that is inhibited by charcoal requires between 4 and 8 hr after inoculation. Delay of up to 8 hr in the addition of charcoal had no consistent effect on elaboration of antigen.

Experiments with F mutants. Mutants that do not require adsorbent for optimal encapsulation

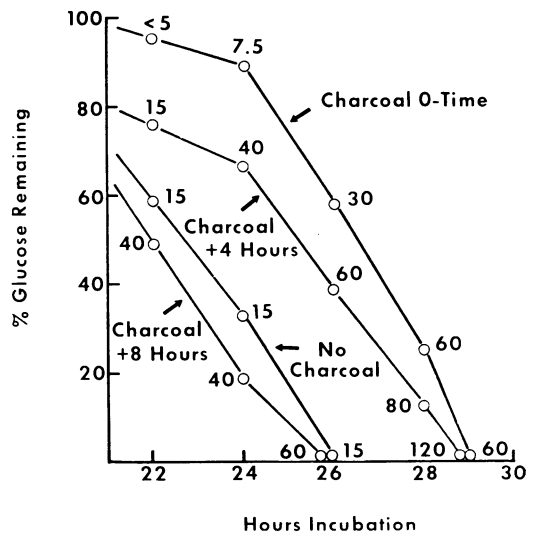


FIG. 2. Effect of time of addition of charcoal on glucose utilization and antigen elaboration in cultures grown from spore inocula. Numbers represent complement-fixing units per milliliter of culture. Curves represent cultures containing no charcoal, or containing 0.006% charcoal added after incubation for 0, 4, or 8 hr.

have been described by Meynell and Meynell (9) and referred to as F mutants. The biochemical changes associated with the mutation have not been elucidated. The nonencapsulated strain 116-NP2-F-R, derived from the F mutant of 116-NP2, was tested for its requirement for an adsorbent for antigen production. Typical results are shown in the last experiment in Table 2. The strain appeared to be no less dependent on adsorbent than the non-F strains tested previously.

DISCUSSION

Presence of an adsorbent represents an additional condition that stimulates significantly the elaboration of protective antigen, and represents another similarity between conditions for antigen elaboration and conditions for synthesis of polypeptide capsule by virulent strains. Evidence has not been obtained regarding the mechanism by which an adsorbent stimulates antigen elaboration.

Evidence was presented that adsorbent stimulates capsule synthesis by removal of a growth product, tentatively identified as fatty acid (8). Changes in the nature and distribution of cellular fatty acids are associated with alterations in the permeability of other microorganisms for amino acids and proteins (3). Control of protective antigen elaboration by bicarbonate also appears to be exerted through changes in cell permeability (10). Furthermore, a relationship between the actions of adsorbent and bicarbonate was indicated by the observation that addition of charcoal reduces the concentration of bicarbonate required for encapsulation (8). It may be proposed that the effect of adsorbent is exerted through alterations in cell permeability.

Because of the numerous parallels between capsule formation and antigen elaboration, the continued requirement for adsorbent of the strain derived from an F mutant was unexpected. However, the biochemical basis of the F mutation is unknown, and its presence can be demonstrated only by its effect on encapsulation. Accordingly, there was no basis for determining whether the F character was maintained in the nonencapsulated mutants used for antigen elaboration. It is also possible that the mechanism by which the F mutation avoids the requirement for adsorbent is not applicable to the process of antigen elaboration.

The clear evidence that charcoal stimulates antigen elaboration only when phosphate is reduced indicates that charcoal replaces the high concentration of phosphate rather than acting by an independent mechanism. No evidence has been obtained of the nature of the adsorbent, the

formation of which is postulated in the presence of 0.01 M phosphate; presumably insoluble phosphates of metal ions are formed. It will be of interest to examine the effect of the addition of charcoal on the requirement for salts of calcium, magnesium, manganese, and iron in the medium. Presence of an optimal concentration of adsorbent may also reduce the requirement for bicarbonate.

The reduction of phosphate concentration, made possible by addition of charcoal, markedly increases the efficiency of the concentration and purification of protective antigen on DEAE cellulose columns. Observations on the isolation and properties of protective antigen will be presented in a subsequent paper.

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