STUDIES ON IMMUNITY IN ANTHRAX

VII. Carbohydrate Metabolism of *Bacillus anthracis* in Relation to Elaboration of Protective Antigen

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The elaboration *in vitro* of protective antigen of *Bacillus anthracis* in chemically defined media has been described in previous reports (Wright *et al.*, 1954*a*; Puziss and Wright, 1954). The alum-precipitated antigen was effective in immunization of laboratory and domestic animals and well tolerated in man (Wright *et al.*, 1954*b*; Schlingman *et al.*, 1956; Jackson *et al.*, 1957). The antigen was elaborated in chemically defined media only under particular cultural conditions, and certain medium constituents were essential for accumulation of antigen in culture filtrates (Puziss and Wright, 1954). Accumulation of protective antigen also occurred under anaerobic conditions in a chemically defined medium (Wright and Puziss, 1957).

Understanding of the mechanisms that determine elaboration of protective antigen of *B. anthracis* may provide a model for investigation of similar factors in other organisms. This paper presents observations on the influence of carbohydrate metabolism on this process.

MATERIALS AND METHODS

Cultures. Several virulent, wild type strains of *B. anthracis* or their mutants were used. The parent strains were Vollum, 107, V770, 116, and 1133 (Auerbach and Wright, 1955). Nonproteolytic mutants are designated NP, and nonencapsulated mutants are designated R. For example, 107-NP2-R1 is a nonproteolytic, nonencapsulated double mutant of strain 107. Strain R1-NP was derived from the Vollum strain. The isolation and characteristics of these avirulent mutants will form the subject of a future paper. Stock suspensions of washed and heated spores of these strains were obtained from cultures on nutrient agar slants and were stored in distilled water in the cold. A standard spore suspension of the virulent Vollum strain of *B. anthracis* was employed for challenge of immunized animals in the assay for protective antigen (Puziss and Wright, 1954).

Media. The basal medium for elaboration of protective antigen was the 968 medium, a modification of the 599 medium of Puziss and Wright (1954). The modifications consisted of omission of cysteine, reduction of ferrous sulfate to 0.00001 M, and addition of 0.0004 M glycine, 0.0001 M L-alanine, and 0.000004 M adenosine. This medium will be discussed more fully in a subsequent paper. The control or reference medium contained 0.1 per cent glucose. Carbohydrates substituted for glucose were tested at the same concentration. Chemicals used in preparation of media were of reagent grade, and water was redistilled in glass. When bicarbonate was omitted, the pH of the medium was adjusted to equal that of the complete medium by addition of sterile N NaOH. For aerobic cultures, 500 ml volumes of media in 4-L Fernbach flasks were inoculated with 500 spores per ml and incubated statically at 37 C for approximately 26 hr, except as noted. For anaerobic cultures, 400-ml volumes of medium in 600-ml bottles were incubated under nitrogen for approximately 48 hr, except as noted (Wright and Puziss, 1957).

Preparation and assay of protective antigen. At harvest the amount of growth in the cultures was estimated visually, since growth of the organism in tenacious chains made ordinary cell counting methods impractical. The cultures were adjusted to pH 8.0 with N NaOH and filtered through ultrafine sintered glass candles. The filtrates were tested for sterility and lyophilized. Protective antigen in the culture filtrates was measured by immunization of guinea pigs with solutions of the lyophilized preparations. Except as otherwise noted, 2 groups of 4 guinea pigs were immunized with 5 subcutaneous injections of antigen on alternate days. One group received a total amount of antigen that represented 7.5 ml

of original filtrate, and the second group received 1.5 ml. Seven days after the last injection the animals were challenged with 1000 spores or about 200 LD_{50} of the Vollum strain (Puziss and Wright, 1954). In some experiments protective antigen in the filtrates was estimated by the complement fixation technique of McGann and Jones (*unpublished data*). Agreement between the two assay procedures was generally satisfactory.

Determination of fermentation products. Samples of culture filtrates were stored at −20 C pending chemical analyses. Determinations were carried out for residual glucose, for lactic, acetic, and formic acids, and for acetoin, glycerol, and 2,3-butylene glycol, according to the methods of Neish (1952). The acetate and formate, contained in steam distillates of the culture filtrates, were separated on a silica gel column and determined by titration with alkali. Lactate and the neutral solvents were determined directly on the culture filtrates. Pyruvate and α-ketoglutarate were identified by paper chromatography of 4-nitro-*o*-phenylenediamine derivatives (Taylor and Smith, 1955), and quantitative analyses were carried out according to the procedures of Koepsell and Sharpe (1952). No attempt was made to obtain a complete fermentation balance.

RESULTS

Effect of carbohydrates and related compounds on antigen production. The three strains were grown in shallow layers of medium in Fernbach flasks. Satisfactory growth and antigen elaboration were obtained in the glucose reference medium. Strain 116-NP1-R2 grew more slowly than the other strains, but the level of protective activity was approximately the same once equal growth was achieved. When carbohydrate was omitted from the medium, the strains grew rather slowly but ultimately reached the same density as in the complete medium; no antigen could be detected in filtrates of these cultures. Other carbohydrates were studied as energy sources for growth and antigen production; average results of several experiments are summarized in table 1. Sucrose, maltose, fructose, and glycerol functioned essentially as well as glucose. Dextrin was less effective, particularly with 2 of the strains. Xylose, ribose, gluconate, glucosamine, mannose, erythritol, and mannitol were inactive in stimulation of growth or antigen formation. Compounds formed during glucose fermentation, such as acetoin and 2,3-butylene glycol, and intermediates such as dihydroxyacetone and glyceraldehyde likewise were inactive. Xylose and ribose were not utilized, as determined by the orcinol-pentose method of Albaum and Umbreit (1947).

Antigen formation and glucose metabolism in aerobic cultures. Elaboration of antigen and utilization of glucose by strain 107-NP2-R1 were studied as functions of incubation time in the presence and in the absence of bicarbonate (figure

TABLE 1

Effect of various carbohydrates on growth and on antigen elaboration by strains of Bacillus anthracis

Carbohydrate	Strain V770-NP1-R		Strain 107-NP2-R1		Strain 116-NP1-R2	
	Growth response*	Protective activity†	Growth response*	Protective activity†	Growth response*	Protective activity†
Glucose	Normal	7/8	Normal	8/8	Normal	7/8
None	Delayed	0/8	Delayed	0/8	Delayed	0/8
Dextrin	Normal	5/8	Normal	1/8	Delayed	0/8
Sucrose	Normal	8/8	Normal	8/8	Normal	7/8
Maltose	Normal	5/8	Normal	6/8	Normal	7/8
Fructose	Normal	3/4	Normal	3/8	Normal	5/8
Glycerol	Normal	4/4	Normal	3/8	Normal	4/4
All others tested‡	Delayed	0/8	Delayed	0/8	Delayed	0/8

* Growth estimated visually as equivalent to the glucose control medium after 24 hr was termed normal. Growth that was less than the glucose control in 24 hr but equivalent to the control medium in 48 hr was termed delayed.

† Ratios refer to the total number of animals surviving divided by the total number challenged.

‡ Xylose, ribose, mannose, mannitol, erythritol, glucosamine, potassium gluconate, acetoin, 2,3-butylene glycol, glyceraldehyde, and dihydroxyacetone.

1). Aerobic cultures exhibited a lag in initiation of growth when bicarbonate was omitted, but after incubation for 38 to 50 hr, growth reached the same level as in the complete medium. Utilization of glucose was complete in both media. The concentration of protective antigen in the complete medium reached a peak after 38 hr of growth and then decreased rapidly. The decrease evidently began after glucose had disappeared from the medium. No antigen was detected in filtrates of cultures from the bicarbonate-free medium.

The concentrations of various products of fermentation were determined at intervals during growth; the results are summarized in figures 2

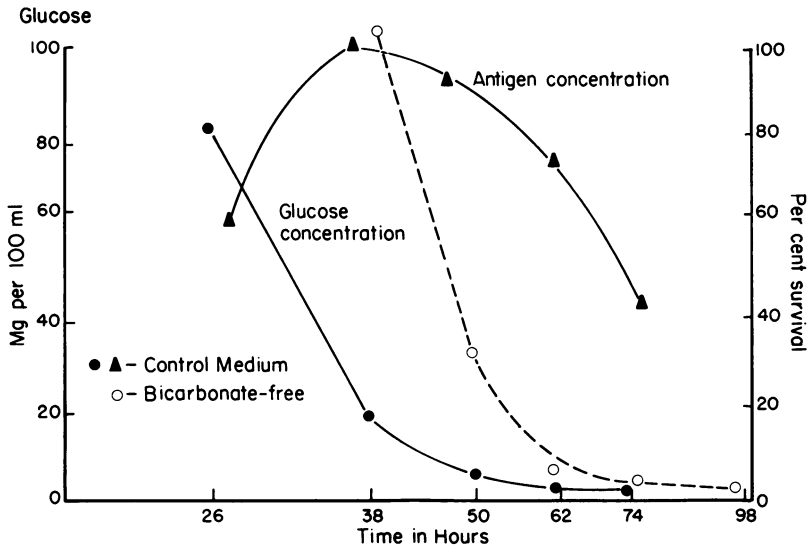

Figure 1. Glucose utilization and antigen accumulation in aerobic culture by *Bacillus anthracis*

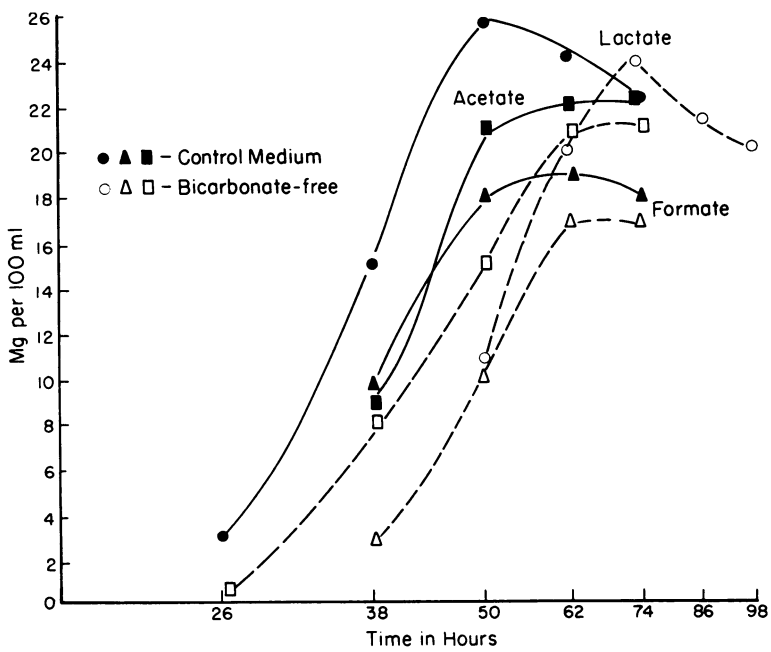

Figure 2. Lactate, acetate, and formate production in aerobic culture by *Bacillus anthracis*

and 3. The concentrations of lactate, acetate, and formate in the control medium were slightly higher than in the bicarbonate-free medium. Glycerol accumulated rapidly in both media and then disappeared gradually; presumably the glycerol is utilized as a substrate by the surviving cells, following disappearance of the glucose. Acetoin and 2,3-butylene glycol were found at low concentrations in filtrates from both media.

Antigen formation and glucose metabolism in anaerobic culture. A similar study was carried out with the same strain in anaerobic culture (figure

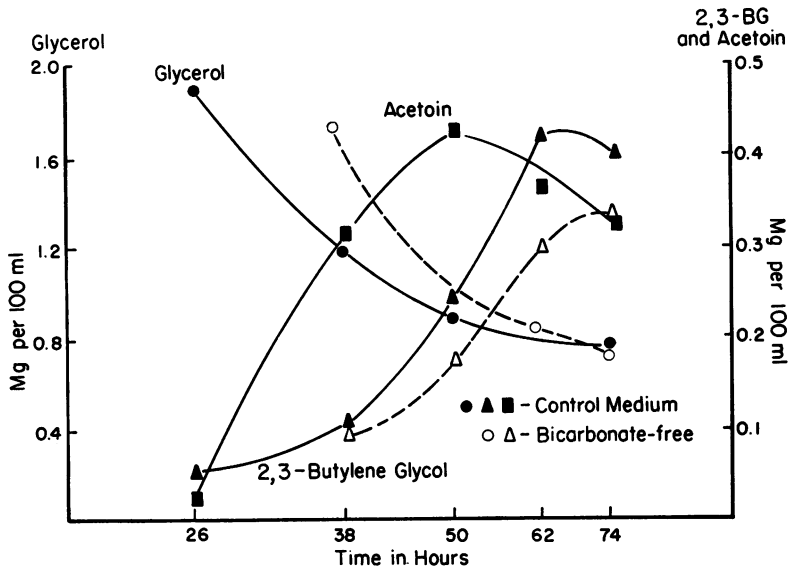

Figure 3. Glycerol, acetoin, and 2,3-butylene glycol production in aerobic culture by *Bacillus anthracis.*

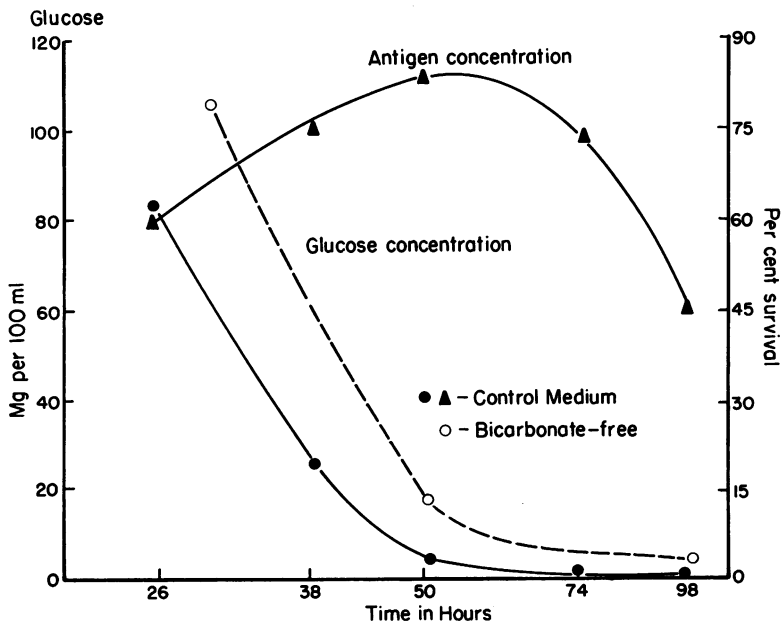

Figure 4. Glucose utilization and antigen accumulation in anaerobic culture by *Bacillus anthracis*

4). The lag in initiation of growth produced by omission of bicarbonate was of shorter duration than with aerobic cultures. Complete utilization of glucose occurred in both complete and bicarbonate-free media. Antigen accumulation in filtrates of the complete medium reached a peak after 50 hr growth, at the point of essentially complete glucose utilization, and then declined rapidly.

Small amounts of antigen were detected in filtrates of cultures in media without added bicarbonate. An occasional animal immunized with these filtrates survived challenge; others showed increased survival times compared with non-immunized control animals. Experiments were carried out with two strains to compare the protective activities of anaerobic cultures grown with and without added bicarbonate. Additional strains were surveyed for ability to elaborate antigen in the absence of bicarbonate. The results are summarized in table 2. It may be noted that under these conditions several strains produced detectable antigen although the amounts were considerably less than in the complete medium. There are tentative indications that the strains vary with respect to antigen production in the absence of bicarbonate.

Cultures grown without added bicarbonate became more acid than cultures in complete medium. Attempts were made to increase the elaboration of antigen in the absence of bicarbonate by periodic adjustment of the pH of the culture during growth. Sterile NaOH was added to the culture at intervals so that the pH remained the same as that of a control culture in complete medium. The results are included in table 3. It may be noted that antigen production in the adjusted culture remained considerably less than that in the complete medium.

TABLE 2

Protective activity of filtrates of anaerobic cultures grown in a complete and a bicarbonate-free medium

Strain of *Bacillus anthracis*	Survival ratios* with immunizing doses as follows:		
	7.5 ml per animal	7.5 ml per animal	1.5 ml per animal
	Bicarbonate omitted	Complete medium	
107-NP2-R1	6/20	16/20	10/20
V770-NP1-R	1/4	13/16	6/12
116-NP1	1/4		
V770-NP1	3/4		
R1-NP	0/4		
1133-NP1	0/4		

* Survival ratios refer to the number of animals surviving divided by the total number challenged.

TABLE 3

Effect of various treatments on elaboration of antigen in a bicarbonate-free medium

Medium	Culture Treatment	Complement Fixation Titers	Survival Ratios* Immunizing doses as follows:	
			7.5 ml per animal	1.5 ml per animal
Without bicarbonate	pH adjustment		1/4	1/4
	None	12.5	2/4	
	Stirred†	0	0/4	
	Sparged with nitrogen†	5	0/4	
Complete	Stirred	60	4/4	3/4

* Ratios refer to the number of animals surviving divided by the total number challenged.

† Cultures were stirred, or sparged with nitrogen as indicated, for 10-min periods after growth for 16, 24, and 30 hr. They were reincubated under nitrogen after each treatment until harvest at 46 hr.

These results suggested that retention of metabolic carbon dioxide was responsible for antigen production in the bicarbonate-free medium. Results of procedures devised to aid escape of metabolic carbon dioxide are presented in table 3. Either stirring or sparging with nitrogen during growth reduced the production of antigen to nondetectable levels.

Analyses of fermentation products in filtrates of anaerobic cultures revealed that the pattern of accumulation of lactate, acetate, and formate was essentially similar in the control and bicarbonate-free media (figure 5). The maximal concentration of lactate in filtrates of anaerobic cultures was nearly twice that found in aerobic cultures; this difference may have been responsible for the greater acidity of the anaerobic cultures. Glycerol in both control and bicarbonate-

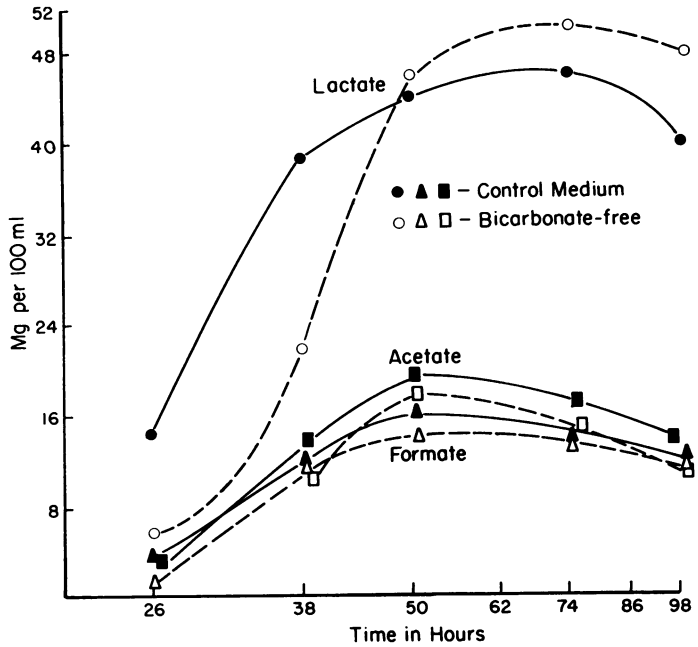

Figure 5. Lactate, formate, and acetate production in anaerobic culture by *Bacillus anthracis*

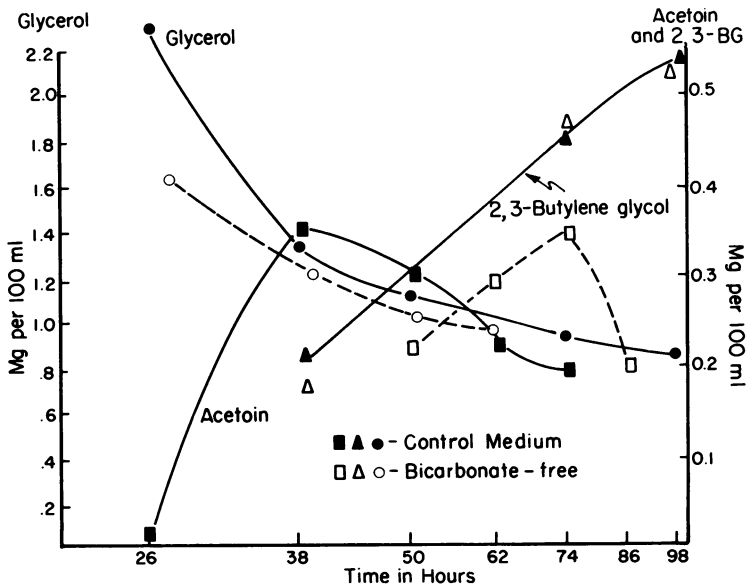

Figure 6. Glycerol, acetoin, and 2,3-butylene glycol production in anaerobic culture by *Bacillus anthracis.*

free cultures accumulated rapidly and then disappeared gradually, as in the aerobic cultures. Acetoin and 2,3-butylene glycol were found in low concentrations, which were nearly identical in the control and the bicarbonate-free cultures (figure 6). The analyses support the conclusion that the organism carries out a mixed lactic fermentation (Puziss and Rittenberg, 1957).

Production of pyruvate in anaerobic and aerobic cultures. Paper chromatograms of filtrates from

anaerobic cultures indicated the presence of pyruvate and α-ketoglutarate. Quantitative determinations of these products were made in filtrates of virulent and avirulent strains grown in complete and in bicarbonate-free medium, under aerobic and anaerobic conditions. Cultures in the bicarbonate-free medium were harvested when growth densities reached those of the complete medium. The results of pyruvate analyses are shown in table 4. Concentrations of α-ketoglutarate in the filtrates were too low for satisfactory quantification. The concentrations of pyruvate were somewhat lower in filtrates from bicarbonate-free medium than in control filtrates; this difference was greater in the anaerobic than in the aerobic cultures. In general, considerably less pyruvate was found when bicarbonate was omitted, although minor variations were observed among the strains. There was no indication that the nonencapsulated mutants differed from their parent strains. Stepwise reduction in the concentration of bicarbonate led to a concomitant decrease in the production of pyruvate, as shown in table 5.

TABLE 4

Effect of omission of bicarbonate on accumulation of pyruvate

Condition of Growth	Strain	Pyruvic Acid, mg/100 ml	
		Complete medium	Bicarbonate omitted
Aerobic	V770	0.62	0.35
	V770-NP1-R	0.80	0.77
	Vollum	—	0.39
Anaerobic	V770	0.98	0.11
	V770-NP1-R	0.87	0.13
	Vollum	0.40	0.11
	R1-NP	0.67	0.18

TABLE 5

Effect of concentration of bicarbonate on accumulation of pyruvate in anaerobic cultures

Strain	Bicarbonate Conc	Pyruvic Acid, mg/100 ml
	%	
V770-NP1-R	0.25	1.19
	0.062	0.45
	0.025	0.78
	0	0.26
V770	0.25	1.15
	0.062	0.53
	0.025	0.34
	0	0.16

The relationship between bicarbonate concentration and the accumulation of pyruvate suggested that pyruvate or α-ketoglutarate might substitute for bicarbonate in stimulation of protective antigen formation. However, addition of 0.004 M sodium pyruvate or 0.0004 M α-ketoglutaric acid did not stimulate antigen formation in a bicarbonate-free medium.

DISCUSSION

Previous experiments had established certain requirements for accumulation of the protective antigen of *B. anthracis* in culture filtrates. These included bicarbonate, calcium ion, and certain amino acids, in addition to the medium constituents necessary for satisfactory growth of the particular strain from a spore inoculum (Puziss and Wright, 1954). The present experiments indicate that a readily utilizable carbohydrate is also essential for significant accumulation of antigen. No protective antigen was detected when carbohydrate was omitted from the medium; growth was somewhat delayed but approached the same final level as in the complete medium. Antigen production was obtained only in the presence of those carbohydrates and related substances that were readily utilized, as shown by their significant stimulation of growth. The observations on carbohydrate utilization are in agreement with the established fermentation reactions of the organism.

An association of carbohydrate metabolism and antigen synthesis is also suggested by the observations on accumulation of protective antigen in growing cultures. The antigen activity reached its peak as the glucose in the medium approached exhaustion; on further incubation of the cultures the activity of antigen decreased. These observations are compatible with the thesis that synthesis of protective antigen requires an active carbohydrate metabolism.

The mechanisms responsible for inactivation of protective antigen on continued incubation of cultures are unknown. Virulent, wild type strains elaborate a protease which was believed to destroy

the antigen (Gladstone, 1946); development of the nonproteolytic mutants that were used in the present work removed this mechanism for destruction of antigen (Wright *et al.*, 1951). It is evident, however, that significant inactivation mechanisms are still operative. Although inactivation of antigen does not become apparent until antigen elaboration has presumably ceased, it is possible that destruction occurs continually during growth and reduces the concentration of antigen even in young cultures. Further study of this inactivation is in progress.

The requirement for bicarbonate in elaboration of the antigen was demonstrated by Gladstone (1946), and has been confirmed repeatedly. The activity of bicarbonate could not be explained by its effect in maintaining a higher pH in the culture, and attempts to replace bicarbonate by functionally related substances were unsuccessful (Puziss and Wright, 1954). In the present work small amounts of antigen were formed in anaerobic cultures in the absence of added bicarbonate. It seems probable, however, that as a result of the closed system used to obtain anaerobiosis, sufficient metabolic carbon dioxide was retained in the medium to permit formation of detectable amounts of antigen. Procedures designed to facilitate removal of metabolic carbon dioxide led to complete suppression of antigen formation. Probably retention of metabolic carbon dioxide was also responsible for elaboration of antigen in aerobic cultures maintained at a high pH by tris(hydroxymethyl)aminomethane buffer (Thorne and Belton, 1957; Strange and Thorne, 1958).

Although the specific effect of bicarbonate in stimulating elaboration of protective antigen is well established, its mode of action remains unknown. After the importance of carbohydrate metabolism in antigen elaboration was demonstrated, it seemed possible that bicarbonate might exert its effect through an influence on the pathways of glycolysis. The present experiments on the products of glucose fermentation were undertaken to test this hypothesis. Omission of bicarbonate from aerobic and anaerobic cultures had no important effect on production of lactic, acetic, and formic acids, glycerol, acetoin, and 2,3-butylene glycol. Only in the accumulation of pyruvic acid was a significant effect of bicarbonate observed. Omission of bicarbonate in anaerobic cultures produced a reduction in pyruvate of fourfold or more; the effect was less marked in aerobic cultures. In this respect no significant differences were seen between virulent strains and nonencapsulated mutants. The inability of pyruvate to replace bicarbonate indicates however, that the effect of bicarbonate on elaboration of antigen is not exerted through accumulation of pyruvate. The crucial influence of bicarbonate on antigen formation is evidently initiated through some mechanism other than an effect on over-all pathways of carbohydrate metabolism.

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SUMMARY

The protective antigen of *Bacillus anthracis* was elaborated only during growth of the organism in the presence of a readily utilizable carbohydrate. Among a variety of carbohydrates and related substances that were tested, only glucose, sucrose, maltose, fructose, dextrin, and glycerol supported significant accumulation of antigen. Utilization of glucose was studied under aerobic and anaerobic conditions. The concentration of antigen reached a peak at approximately the time of exhaustion of glucose, and decreased on further incubation of the culture. Fermentation of glucose was a mixed lactic type, with lactic, acetic, and formic acids the chief products; glycerol, acetoin, 2,3-butylene glycol, pyruvic acid, and traces of α-ketoglutaric acid were also formed. Bicarbonate was required for appreciable elaboration of antigen under both aerobic and anaerobic conditions. Traces of antigen were formed in anaerobic cultures in the absence of added bicarbonate, presumably because of retention of metabolic carbon dioxide. Omission of bicarbonate produced only minor changes in the fermentation products of glucose, except that concentration of pyruvate was significantly reduced, particularly under anaerobic conditions. The meaning of the results is discussed.

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