INFECTION AND IMMUNITY, Apr. 1973, p. 567-572

Vol. 7, No. 4
Printed in U.S.A.

Biochemistry of *Vibrio cholerae* Virulence

III. Nutritional Requirements for Toxin Production and the Effects of pH on Toxin Elaboration in Chemically Defined Media

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Received for publication 27 November 1972

The investigations described in this report concern the metabolic and physiologic parameters governing cholera enterotoxin production in chemically defined growth media. The results indicate that the minimal nutritional requirements for growth of pathogenic *Vibrio cholerae* are the same as those for toxin production, and that toxin production parallels growth of the organisms. Studies of the relationship between toxin accumulation and pH reveal that toxin biosynthesis can be separated from toxin release. Toxin is synthesized below pH 7.0, but release and accumulation of extracellular toxin occur only at neutral or alkaline pH values.

In spite of extensive literature on bacterial exotoxins, there is relatively little information on the regulation of their synthesis or the mechanism(s) of their release. This is especially true for exotoxins produced by gram-negative bacteria, and in particular *Vibrio cholerae*, for which the environmental and nutritional requirements for in vitro toxin production have only recently been studied (1, 2, 8). The investigations described in this report concern the metabolic and physiologic events which control cholera toxin biosynthesis or release, or both.

We describe here the development of a synthetic medium which, at present, represents the minimal known nutritional requirements for in vitro growth and toxin production. The medium is designated AG medium. This report also presents experimental evidence which indicates that toxin release can be separated, on a temporal basis, from toxin biosynthesis and growth, and that toxin release is largely controlled by a single physical parameter: pH.

In the present study, a passive hemagglutination inhibition (PHI) test was used to measure toxin. Since the PHI assay is not capable of distinguishing between toxic and nontoxic antigen (i.e., toxoid), the results are expressed here in terms of micrograms of "antigen" per milliliter. In keeping with our previous reports (1, 9), we consider enterotoxin and skin permeability factor as being the same antigenically reactive substance. (These experiments represented a portion of a dissertation submitted by the senior author to Wake Forest University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

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MATERIALS AND METHODS

Microorganism. Inaba strain 569B of *V. cholerae* was used throughout these investigations. Inoculum size and culture maintenance were as previously described (8). All incubations were at 25 C.

Culture media. Three types of synthetic media were used in this study: TA medium (1), AG medium, and PA medium. TA medium contained in grams per liter of 5 mM tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 7.5): NaCl, 2.5; KCl, 2.5; Na_2HPO_4, 0.2; glycerol, 0.5; asparagine, serine, glutamic acid, arginine, 2.5 each plus 1 ml/liter of a mixture of 5% $MgSO_4$, 0.5% $MnCl_2 \cdot 4H_2O$ and, 0.5% $FeCl_3$ in 0.4% nitrilotriacetic acid.

AG medium differed from TA medium in that it contained: 0.25% NaCl, 0.05 M potassium phosphate buffer (pH 7.5), 0.1% (vol/vol) trace metals (same as in TA medium), and 0.25% each asparagine and glucose. AG medium was sterilized by filtration.

PA medium represented a composite of some of the components found in both TA and AG medium. It contained: 0.25% NaCl, 0.05 M potassium phosphate buffer (pH 6.3), 0.1% (vol/vol) trace metals, and 0.25% each asparagine, serine, glutamic acid, and arginine. PA medium was used to compare and contrast antigen elaboration at low (pH 6.5) and high pH (i.e., pH >7.0) in the absence of glucose.

Antigen assay. A PHI test for the presence of antigen was performed by the method described by Callahan et al. (1). The assay was standardized by using known concentrations of purified toxin and was found to be reproducible with a variation of ± 2 μg of antigen per ml in the range of 2 to 20 μg. Samples containing more than 21 μg of antigen

per ml were diluted to fall within the above range. Test samples were routinely run in triplicate. During these investigations, the PHI assay was not affected by pH as low as 6.0.

Biochemical assays. Protein was determined by the method of Lowry et al. (7) by using crystalline bovine serum albumin as a standard. Glucose concentrations were determined colorimetrically by the method of Hoffman (5). The ninhydrin assay (4) and the method of thin-layer chromatography (1) were described previously.

RESULTS

Antigen elaboration in media supplemented with glucose. Previous experiments in this laboratory (unpublished work) and elsewhere (3) suggested that toxin biosynthesis in chemically complex media was sensitive to catabolite repression. Since antigen elaboration has been shown (1) to occur throughout the growth cycle in TA medium which lacks glucose, an effort was made to determine if the addition of glucose repressed toxin synthesis.

A preliminary experiment was performed to evaluate the effect of glucose utilization on the growth of strain 569B in TA medium. Two culture flasks were used. One contained TA medium and the other contained TA medium supplemented with 0.5% glucose. Both flasks were inoculated and incubated for 24 h. Samples were withdrawn from each flask at 2-h intervals and used to measure the turbidity and pH of the culture medium. The most obvious effect of glucose utilization was the accompanying drop in pH to 5.8 which appeared to limit growth. In contrast, growth in TA medium not containing glucose resulted in a steady rise in pH to 8.2. Based on these results, an attempt was made to control the pH by neutralizing the acid produced during glucose utilization by periodic additions of NaOH (1.0 N). A control flask containing TA medium and an experimental flask containing TA medium supplemented with 0.5% glucose were inoculated and incubated for 24 h. The pH of the control medium was determined at hourly intervals for 16 h, and the pH of the experimental medium was adjusted to that of the control. Samples obtained from the experimental flask were used to determine the glucose, antigen concentrations, and turbidity of the medium. As shown in Fig. 1, antigen elaboration paralleled growth and occurred concomitantly with glucose utilization when the pH of the medium was controlled. Growth and antigen production in the control flask occurred as previously described (1).

Growth and antigen production in phosphate-buffered TA media supplemented with glucose. Acid production accompanying glucose metabolism was controlled by substituting 0.05 M potassium phosphate buffer (pH 7.5) for the 0.001 M Tris-maleate buffer in TA medium. The addition of potassium phosphate ions also permitted the elimination of KCl and Na_2HPO_4 from the medium, and glucose replaced the glycerol. Thus, the modified TA medium contained, in addition to trace metals, 0.25% NaCl, 0.05 M phosphate buffer, 0.25% of each amino acid, and 0.5% glucose.

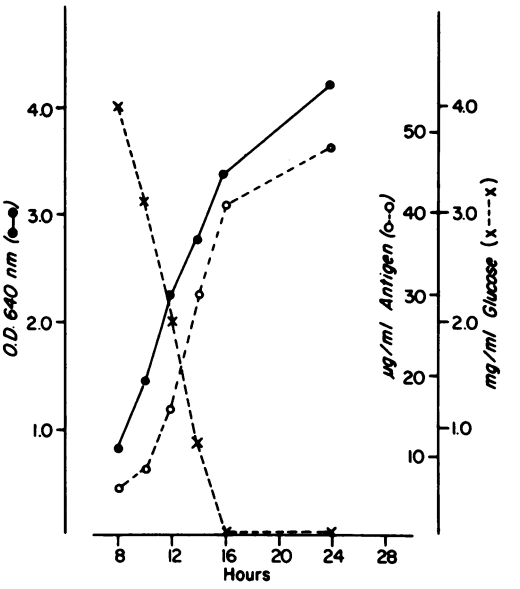

FIG. 1. *Relationship between growth (OD at 640 nm), antigen production, and glucose utilization in media titrated with NaOH.*

A culture flask containing modified TA medium was inoculated and incubated for 24 h. Samples were withdrawn periodically from the culture flask to measure the turbidity, pH, and antigen concentration of the medium. As illustrated in Fig. 2, during the first 12 h of growth the pH decreased to 6.7. After this initial drop there was a steady rise in pH to 7.8. The concentration of antigen accumulated in the first 12 h of growth was negligible in comparison to the amount accumulated during the rise in pH accompanying the final 12 h of growth.

Analysis of the amino acid requirements for growth and antigen production in modified TA medium. In previous experiments (1), thin-layer chromatographic analysis of TA cultures showed that serine and asparagine were exhausted from the medium by the end of the exponential growth phase. By comparison, only small amounts of arginine and glutamic acid were utilized. Because of the apparent preferential

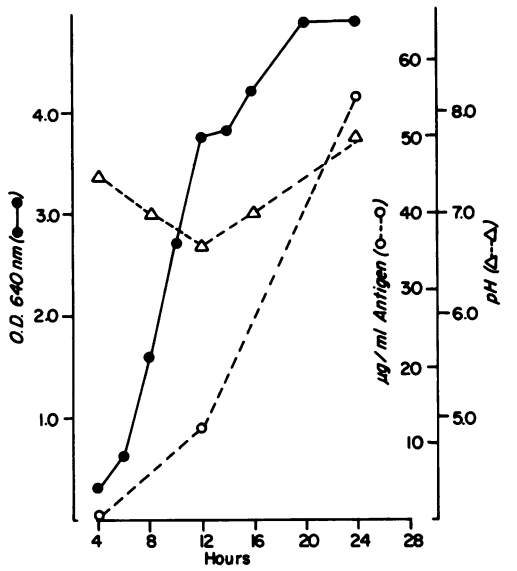

FIG. 2. *Relationship between growth (OD at 640 nm), antigen production, and pH in TA medium buffered with potassium phosphate (pH 7.5) and containing glucose.*

utilization of serine and asparagine, an experiment was designed to evaluate the individual capacity of these two amino acids to support growth and toxin biosynthesis in media containing glucose. Four different media were used in the experiment. One contained 0.25% each asparagine and serine, a second contained 0.5% serine, a third contained 0.5% asparagine, and the fourth was supplemented with 0.2% NH_4Cl. In addition, each contained trace metals, 0.25% NaCl, 0.5% glucose, and 0.05 M potassium phosphate buffer (pH 7.5). The turbidity and antigen concentration of each medium was determined after the cultures reached the stationary phase. The results, presented in Table 1, indicated that NH_4Cl was a poor substitute for the amino acids. Asparagine, added singly, supported growth and antigen production equivalent to that observed when asparagine and serine were added together.

Growth and antigen production in AG medium. A combination of glucose and asparagine used to supplement a defined basal medium supported high cell yields of strain 569B and satisfied the nutritional requirements for the production of high titers of antigen. The complete medium, buffered with 0.05 M potassium phosphate (pH 7.5), was designated AG medium.

To study the relationship between growth and antigen production in AG medium, duplicate culture flasks were inoculated and incubated for 24 h. The turbidity, pH, and antigen concentration were determined at 2-h intervals. These same samples also were used to measure the disappearance of glucose and asparagine (i.e., ninhydrin-positive material) from the medium.

TABLE 1. *Capacity of different nitrogen supplements to support growth and antigen production in media containing glucose*

Nitrogen source[a]	Maximum OD at 640 nm[b]	Antigen produced[c] (μg/ml)
Asparagine and serine, 0.25% each	4.2	48
Asparagine, 0.50%	4.0	45
Serine, 0.50%	3.6	25
NH_4Cl, 0.20%	3.0	10

[a] Nitrogen source used to supplement media containing 0.25% NaCl, 0.50% glucose, 0.05 M potassium phosphate buffer (pH 7.5), and 0.1% (vol/vol) trace metals (see Materials and Methods).

[b] Optical densities of stationary-phase cultures of strain 569B incubated at 25 C and 250 rpm.

[c] Antigen was measured by the PHI assay.

As shown in Fig. 3, antigen elaboration in AG medium did not parallel growth of the organisms, and antigen production appeared to be affected by the pH of the medium since extensive accumulation did not occur when the pH of the medium was below 7.0. During the first 16 h of growth, all of the glucose and about 50% of the asparagine (i.e., ninhydrin-positive material) were utilized and the initial pH of the medium fell from 7.5 to 6.7 (Fig. 3, 4). Asparagine (ninhydrin-positive material) continued to be metabolized after the complete utilization of glucose and the pH of the medium rose to 8.2 by 24 h.

Effects of pH on antigen elaboration in AG medium. As indicated in Fig. 3, antigen elaboration in AG medium appeared to be inhibited below pH 7.0. To evaluate this finding further, two flasks of AG medium were inoculated with strain 569B, and one culture was maintained at pH 7.5 by continuous titration with NaOH. The other culture was not titrated and thus the pH was allowed to drop below 7.0 during acid accumulation. Samples were taken periodically from each culture to measure the turbidity, pH, and antigen concentration of the medium. Although there was no detectable difference in the amount of growth present in each culture, the amount of antigen accumulated in the culture maintained at pH 7.5 was approximately twice that found in the other culture below pH 7.0 (Table 2). A dramatic increase in the accumulation of antigen

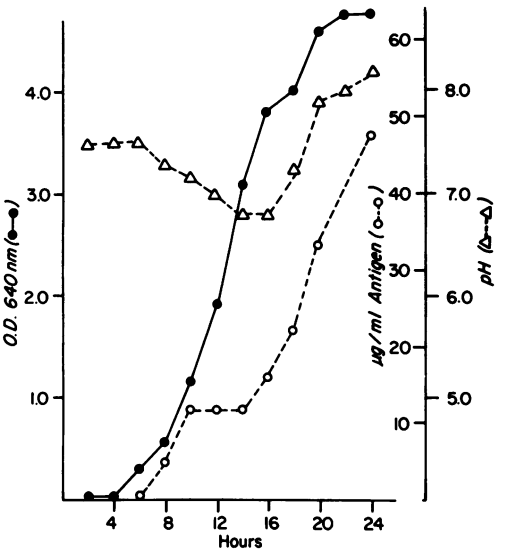

FIG. 3. *Relationship between growth (OD at 640 nm), antigen production, and pH in AG medium.*

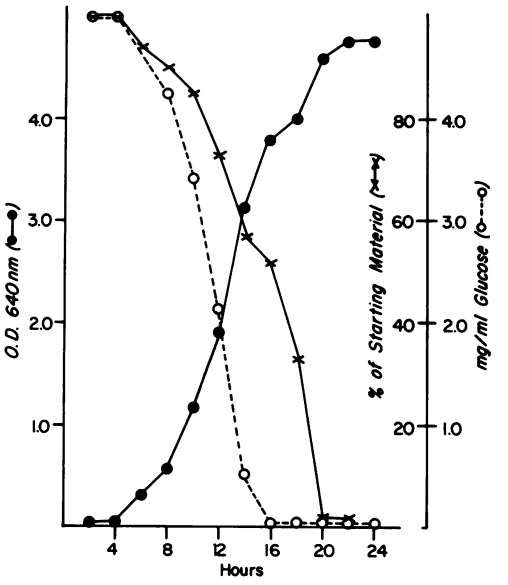

FIG. 4. *Relationship between growth (OD at 640 nm) and utilization of glucose and ninhydrin-positive material (×—×) in AG medium.*

occurred in 2 h when the pH of the control culture rose to 7.1.

Effects of pH on antigen elaboration in PA medium. An experiment was performed to determine the effects of pH on antigen elaboration in TA medium buffered with potassium phosphate (0.05 M, pH 6.3) and lacking glucose. This medium was designated PA medium. Two cultures of PA medium were incubated for a total of 21 h. After the pH of one culture had risen to 6.5 (about 12 h of growth), it was maintained at pH 6.5 by continuous titration with HCl. The pH of the other culture was allowed to rise above 7.0. At 15, 17, 19, and 21 h of growth, samples from each culture were removed and used to measure the pH, turbidity, and antigen concentration of the medium (Table 3). The two cultures exhibited the same maximal growth (i.e., OD 2.5), but the one maintained at pH 6.5 contained less than one-third of the amount of antigen.

Effect of pH on antigen accumulation by non-proliferating cells. An experiment was performed to determine if the effect of pH on antigen elaboration (shown in Tables 2 and 3) could be demonstrated with non-proliferating cells. In addition, it was of interest to determine if the pH effect involved inhibition of toxin synthesis or inhibition of toxin release. To do this, replicate flasks of PA medium (initial pH 6.3) were used. After 13 h of incubation, the mid-logarithmic (OD 1.4) cultures (pH 6.5) were centrifuged to sediment the cells, and the supernatant fluids were decanted. The cells were suspended to their original volume in a nitrogen-free basal medium containing trace metals, 0.25% NaCl, 0.05% (wt/vol) glycerol, and 0.05 M potassium phosphate buffer. One of the suspended cultures was buffered at pH 6.5, and the other two were buffered at pH 7.5. One of the pH 7.5-buffered cultures received 10 μg of chloramphenicol per ml to inhibit protein synthesis. All three suspensions were incubated with shaking at

TABLE 2. *Effects of pH on antigen production by strain 569B in AG medium*

Growth[a] (h)	AG medium 1[b]		AG medium 2[c]	
	pH	Antigen (μg/ml)	pH	Antigen (μg/ml)
8	7.5	3	7.4	3
10	7.5	10	7.1	9
12	7.5	21	6.8	12
14	7.5	28	6.7	14
16	7.5	42	7.1	32

[a] Growth was measured turbidimetrically at 640 nm. Turbidities of each culture were the same during the experiment.

[b] AG medium 1 was maintained at pH 7.5 by continuous titration with NaOH.

[c] AG medium 2 was not maintained at pH 7.5, allowing acid accumulation in the medium to lower the pH.

TABLE 3. *Effects of pH on antigen production by strain 569B in PA medium*

Growth (h)	PA medium 1[a]			PA medium 2[b]		
	pH	OD[c]	Antigen (μg/ml)	pH	OD	Antigen (μg/ml)
15	6.5	2.0	8	7.1	2.0	28
17	6.5	2.3	10	7.5	2.5	35
19	6.5	2.5	10	7.8	2.5	35
21	6.5	2.5	10	8.0	2.5	35

[a] PA medium 1 was maintained at pH 6.5 by continuous titration with HCl.

[b] PA medium 2 was not maintained at pH 6.5, allowing base accumulation in the medium to raise the pH above 7.0.

[c] Optical densities were determined at 640 nm.

TABLE 4. *Effects of pH on antigen release from nonreplicating cultures*[a]

Time after resuspension of culture (min)	Medium 1[b] (μg/ml of antigen)	Medium 2[c] (μg/ml of antigen)	Medium 3[d] (μg/ml of antigen)
0	0	8	8
10	0	8	8
20	0	10	8
30	0	12	8
40	0	12	8
50	0	12	8
60	0	12	8

[a] Mid-logarithmic growth cultures of strain 569B, grown in PA medium, were centrifuged to sediment the cells and the supernatant fluids were decanted. The cells were resuspended to their original volume in a nitrogen-free basal salts medium to prevent further multiplication. Each culture was reincubated for a total of 60 min.

[b] Medium 1 was a basal salts medium buffered at pH 6.5.

[c] Medium 2 was a basal salts medium buffered at pH 7.5.

[d] Medium 3 was a basal salts medium buffered at pH 7.5 and contained 10 μg of chloramphenicol per ml.

25 C. Samples were removed from each culture immediately after resuspension (i.e., time zero) and at 10-min intervals for 1 h. These samples were used to determine the turbidity and antigen concentration of the medium. The results of this experiment are presented in Table 4. Cells suspended at pH 6.5 released no detectable amounts of antigen during the 60-min incubation period. Cells suspended at pH 7.5, in the presence of chloramphenicol, released 8 μg of antigen per ml at time zero and none thereafter. Cells suspended at pH 7.5 without chloramphenicol released 8 μg of antigen per ml at time zero, and an additional 4 μg/ml during the first 30 min of incubation.

The turbidity of each suspension was 1.4 OD units at time zero and at 60 min of incubation, indicating that the cells were non-proliferating in the presence or absence of chloramphenicol. The major portion of antigen was synthesized during growth, when the pH was still below 7.0, and released from the cells by the simple expedient of suspending the cells in buffer at pH 7.5.

DISCUSSION

A combination of asparagine and glucose (i.e., AG medium) apparently represents the minimal nutritional requirements for maximal growth and antigen biosynthesis. The maximal cell yield usually obtained in AG medium (OD of 4.7 at 24 h) (Fig. 3) is slightly greater than that obtained in chemically complex media (OD of 4.0 at 16 h) (1). The amount of antigen produced in AG medium is 47 μg/ml at 24 h (Fig. 3) and is equivalent to that produced in complex media (i.e., 45 μg/ml) (1).

This is a somewhat unusual finding because, as is often the case, completely synthetic media which support the production of reasonable amounts of toxin usually are not suitable for maximal growth. But with AG medium, maximal growth and antigen production appear to coexist.

During the development of AG medium, it was found that a major disadvantage in having glucose in the medium was that glucose utilization resulted in a drastic decrease in pH. To offset this undesirable effect, NaOH was added to neutralize the acid produced during glucose utilization. When the pH was controlled, glucose stimulated growth and toxin production paralleled cellular multiplication (Fig. 1), suggesting that in a chemically defined medium catabolite repression of antigen production does not occur.

In contrast, when 0.05 M potassium phosphate buffer is used to control the pH, the kinetics of antigen production do not parallel growth (Fig. 2, 3). Instead there is a transient pause in antigen elaboration accompanying a drop in pH during the first 12 h of growth. After glucose is depleted from the medium, the pH rises and antigen accumulation is greatly increased. This would suggest that the rise in pH of the medium after glucose is utilized triggers increased synthesis or release of antigen.

This hypothesis may clarify the findings of Kusama and Craig (6), who demonstrated that growth of strain 569B in the presence of glucose

results in a drop in pH from 7.0 to 6.2 during the first 4 h of growth, after which there is sharp rise in pH to 7.3 at about 10 h followed by a gradual increase to pH 8.5 at 96 h. According to their findings, only small amounts of toxin were produced during the first 4 h of growth when the organisms were in the mid-logarithmic growth phase, but between 4 and 12 h there was a 44-fold increase in toxin. The sharp rise in toxin was coincident with the rise in pH.

The effect of pH on antigen accumulation appears to involve inhibition of antigen release rather than antigen biosynthesis. This is clearly demonstrated by the findings that non-proliferating cells suspended in a nitrogen-free medium release antigen immediately after suspension in the presence or absence of chloramphenicol at pH 7.5 but not at pH 6.5 (Table 4).

The observation that cholera toxin is released from cells without delay by the simple expedient of washing the cells at pH 7.5 but not at 6.5 indicates that the antigen is bound, probably by ionic interactions, to the cell's surface at the low pH.

From a practical standpoint, these findings suggest a way of purifying and concentrating cholera toxin in bulk. By growing strain 569B to high cell yields at pH 6.5, it should be possible to obtain reasonably pure preparations of toxin by harvesting the cells and suspending them in small volumes of an alkaline salts solution. The cells could then be removed by centrifugation, leaving the toxin material in the supernatant fluid. Studies are presently underway to determine the feasibility of this approach as a large-scale purification procedure.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI 07772 from the National Institute of Allergy and Infectious Diseases, by Public Health Service research career development award AI 09018 to S.H.R., and by the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases.

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