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Biochemistry of *Vibrio cholerae* Virulence

II. Skin Permeability Factor/Cholera Enterotoxin Production in a Chemically Defined Medium

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A chemically defined medium capable of eliciting high titers of skin permeability factor (PF)/enterotoxin from three different strains of *Vibrio cholerae* has been developed. Toxin/antigen elaboration in synthetic and in complex media was monitored by a specific passive hemagglutination-inhibition test. A distinct temporal difference in the pattern of toxin/antigen elaboration was noted when the two types of media were compared. In complex media, PF activity and corresponding antigen release were coincident, whereas in the defined medium a biphasic pattern resulting in elaboration of nontoxic antigen during the second phase was seen. Possible reasons for the latter observation are discussed, and several experiments illustrating the unique utility of the defined medium are presented.

In what now seems to be a prophetic statement concluding a paper describing partial purification of cholera toxin, Finkelstein (4) remarked: "research in this area appears to be entering the logarithmic phase of growth." As if to corroborate this prediction, the ensuing 4 years saw the conditions necessary for in vitro toxin production worked out and cholera enterotoxin extensively purified by three separate groups of investigators using three completely different purification procedures (1, 5, 15). As with most such developments, the questions most raised by these accomplishments are more numerous than those which were answered. Some of these questions are as follows. What is the biochemical and biophysical nature of the toxin molecule? What are the exact kinetics of its production in the growth cycle of the vibrio population? What are the immediate precursors of the toxin molecule in the cell? Is the toxin molecule derived from cell material (e.g., cell envelope) or is it synthesized de novo? What initiates or controls, or initiates and controls, toxin biosynthesis in the cell?

Meaningful answers to these questions can only be achieved by working with an exactly defined system in which all of the parameters of the growth environment are strictly controlled. The keys to such a system are (i) a synthetic growth medium which contains as few components as are compatible with good growth and toxin production and (ii) a sensitive yet quantitative method for measuring toxin or antigen, or both.

We describe here for the first time a completely synthetic culture medium which supports growth and toxin production on a par with the more complex media reported earlier (13). Also presented are details of a sensitive, reproducible, passive hemagglutination-inhibition (PHI) test for the quantitation of toxin/antigen in a wide variety of biological samples. The utility of these two investigative tools will be shown in this communication.

To remain consistent with our previous publications (13-15) we use the terms toxin, enterotoxin, toxic antigen, and skin permeability factor (PF) to connote that antigenically reactive material which has biological (i.e., PF) activity. Because the PHI test does not distinguish between toxic and nontoxic antigen, we use the term toxin/antigen to refer to antigen quantitated by the PHI test alone.

MATERIALS AND METHODS

Microorganism. Inaba strain 569B of *Vibrio cholerae* was used in all of these experiments except as noted. Culture maintenance, inoculum preparation, and conditions of growth were as previously described (13).

Biological assays. PF activity was estimated essentially as described by Craig (2). Twofold dilutions of samples (0.1 ml each) were injected intradermally into the shaved backs of rabbits. After 18 to 24 hr, positive reactions became grossly edematous and indurated as result of a localized increase in vascular permeability. A 0.5-ml amount of 5% Evans blue dye was injected intravenously 1 hr before the time of reading. End points (a blue area at least 7 by 7 mm) were expressed as blueing doses (BD) per milliliter or as the $\log_{10}$ of

this value. Each dilution was tested in duplicate or usually in quadruplicate. Under these assay conditions, the observed end points of replicate titrations of the same sample varied between two- and fourfold.

Preparation of antisera. Purified toxin prepared by the method of Richardson and Noftle (15) was mixed with an equal volume of Freund's complete adjuvant and administered to a pair of rabbits. Samples (0.5 ml) of the mixture were injected into the region of the axillary, inguinal, and cervical lymph nodes on day 0, followed by single injections into the cervical site on days 7, 14, 21, and 28. The animals were rested for 1 additional week and exsanguinated. The resultant sera were pooled, absorbed twice with washed whole 569B cells to rid them of possible trace amounts of contaminating antisomatic antibodies, and stored in small portions at −70 C. At a later time, the serum was pooled again and absorbed with fresh sheep erythrocytes to eliminate cross-reactions of the Forssman type which might interfere with the specificity of the PHI test.

PHI test. Formalinized sheep erythrocytes were sensitized with purified toxin by the method of Hochstein et al. (9). The doubly absorbed antiserum described above was titrated by the technique outlined in reference 9, except that the volumes were reduced to adapt the method to the Microtiter apparatus. The inhibition test was carried out essentially as described by Finkelstein and Peterson (7) with two minimum hemagglutinating (HA) units of the standard antiserum. Serial twofold dilutions (final volume, 25 μliters) of the samples to be assayed were made in duplicate by using 1.0% normal rabbit serum as a diluent. Twenty-five microliters containing 2 HA units of freshly diluted antiserum was added to each cup, and the mixture was incubated for 1 hr in a moist chamber at 38 C. Fifty microliters of a 0.5% suspension of the standard sensitized sheep erythrocyte antigen was added, and the plates were reincubated at 38 C. Visible agglutination occurred in positive cups after 1 to 2 hr, but the readings were usually made after overnight incubation at room temperature. The results are expressed as the reciprocal of the highest dilution showing clear-cut inhibition of hemagglutination. This value in turn was converted to micrograms of antigen per milliliter by comparison with a standard curve prepared with known quantities of pure toxin and identical assay conditions. The method is reproducible (95% confidence limits) in the range of 4 to 15 μg of Lowry protein per ml, and samples were adjusted by dilution to bring them to appropriate antigen concentrations.

Biochemical assays. Protein was determined by the method of Lowry et al. (11) with crystalline bovine serum albumin as a standard. Proteinase activity in culture supernatant fluids was assayed by the technique of McDonald and Chen (12) with 2% USP casein as substrate. Activity is expressed as the amount of acid-soluble Lowry-positive material (measured at 700 nm) released from the substrate per hour per milliliter of sample. The ninhydrin assay was described previously (8).

RESULTS

In the course of our studies on in vitro toxin production, we have developed several semisynthetic media which support good growth of vibrios and promote toxin production (13). The basic composition of all of these media is a mineral salt base supplemented with casein hydrolysate. [Basal salts contain, in grams per liter of 5 mM tris-(hydroxymethyl)aminomethane(Tris)-maleate buffer (*p*H 7.5): NaCl, 2.5; KCl, 2.5; Na_2HPO_4, 0.2; glycerol, 0.5 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.] Through a largely empirical process, it was found that the casein hydrolysate could be replaced by a synthetic mixture of amino acids similar in composition to the hydrolysate. However, growth and toxin production in this medium fell far short of growth and toxin production achieved in the presence of the casein hydrolysate. This difficulty was partially overcome when it was discovered that addition of potassium and phosphate to the basal medium stimulated growth and toxin production to levels one-third to one-half of those reached with the complex medium. With this somewhat simplified medium as a starting point, an attempt was made to determine which of the 16 amino acids present were required for maximum growth and toxin production.

Specific activity of amino acid mixtures. A series of nine pools of the amino acids contained in the synthetic mixture was compounded. The pools (four amino acids per pool) were arranged so that there was an overlap of the constituent amino acids. Through this device, it was possible to determine whether any single amino acid inhibited, had no effect on, or stimulated growth, toxigenicity, or both. The results of several such experiments are summarized in Table 1. By comparing the total amount of growth and PF titer resulting from each pool, it was possible to eliminate those amino acids which had no marked effect on PF production. The results show that appreciable (10^4 BD/ml or greater) PF titers were obtained when pools 1 and 8 were used in the growth medium. Pools 7 and 9 exhibited excellent growth, but relatively little toxin was produced All of the other mixtures (except 5 and 6 in which growth was very poor) evoked at least a moderate level of toxigenicity.

Based on these results, experiments (Table 2) were conducted to determine the minimum number of amino acids necessary to support growth and toxigenicity levels equivalent to those in the complex media. During the course of these studies, it was found that the normally employed trace amounts of yeast extract had no effect on either growth or toxin production so this component was subsequently eliminated from the base medium. Inclusion of glutamate and aspartate in combination decreased growth,

TABLE 1. *Effect of amino acid mixtures on growth and PF production*[a]

Pool	1 (5×10^4; 2.9)	2 (10^4; 2.4)	3 (0; 1.1)	4 (10^4; 2.7)
5 (0; 0.4)	Histidine	Glycine	Cystine	Methionine
6 (0; 1.0)	Phenylalanine	Leucine	Isoleucine	Valine
7 (0; 3.1)	Glutamic acid	Tyrosine	Tryptophan	Threonine
8 (5×10^4; 3.4)	Arginine	Serine	Alanine	Aspartic acid
9 (10^3; 3.2)	Proline	Lysine	Alanine	Isoleucine

[a] Each amino acid was present in a final concentration of 0.25%. The first number in each bracket is the PF titer (blueing doses per milliliter) obtained after 16 hr of growth at 30 C. The second number is the final turbidity of the culture at 640 nm. Pool 9 was separate and nonoverlapping.

TABLE 2. *Effect of different amino acid supplements on production of PF in TA medium*

Amino acid supplement[a]	BD/ml[b]	Specific activity[c]
Glu, asp, ser, arg[d]	400	1.15
Glu, asp, ser	200	2.58
Glu, arg, ser	200	3.06
Asn	200	5.24
Asp, ser, arg	100	3.10
Glu, asp, arg	100	3.85
Glu	10	1.41
Glu, ser	10	1.42
Glu, arg	10	1.61
Glu, asp	10	1.64
Ser, arg	10	3.28
Ser, asp	10	3.64
Ser	1	1.14
Arg	1	1.15
Asp	1	1.16
Asp, arg	1	2.50

[a] Total amino acid concentration for each supplement was 1.0 g/100 ml. Equal weights of each amino acid were used when added as mixtures. Each medium was inoculated with strain 569B and incubated at 25 C and at 250 rev/min for 16 hr.

[b] Blueing doses per milliliter. Values are expressed $\times 10^{-2}$.

[c] Log_{10} toxin titer per 16-hr optical density at 640 nm.

[d] Abbreviations: arginine, arg; asparagine, asn; serine, ser; aspartic acid, asp; glutamic acid, glu.

presumably because of their competition for a common transport locus in the cell membrane. Similarly, it was found that the addition of asparagine to the medium had a marked stimulatory effect on toxigenicity. Attempts to reduce the number of requisite amino acids below four were unsuccessful.

The medium (TA) as finally compounded contained, in addition to the mineral salts base, 0.25% each of arginine, asparagine, glutamate, and serine. Although the final cell yield of this medium [optical density (OD), 3.6 to 4.0 at 16 hr] was equivalent to that of TRY (consisting of, in grams per liter of Tris-maleate buffer: NaCl, 2.5; KCl, 2.5; Na_2HPO_4, 0.2; yeast extract, 0.05; and glycerol, 0.5), the toxin titers (maximum 80,000 BD/ml) and the growth rate were still not equal to those obtainable with the latter medium. Since it has been shown (3, 13) that the growth rate plays an essential but as yet unknown role in determining the final toxin level, attempts were made to increase the growth rate in the artificial medium. It was found that the main cause of the growth lag in the synthetic medium was the formation of insoluble complexes composed of the amino acids and the bivalent cations in the base medium. Resolution of this problem was achieved by the addition of minute amounts of a metal chelator (nitrilotriacetic acid) to the stock solution of trace salts from which the base medium is compounded. The results of this addition are shown in Fig. 1, in which growth curves of TRY and TA are compared. The growth rates (generation times of 112 and 120 min, respectively) and the final cell yields are nearly identical. The maximum PF titers (160,000 for the former and 120,000 for the latter) are within the range of error for the bioassay.

Kinetics of toxin/antigen production in TRY and TA. To ascertain how closely toxin and antigen production in TA medium paralleled that in TRY, several experiments were carried out in which antigen production was measured by the PHI test and toxin was measured by the usual bioassay. [Toxic antigen (toxin) is measured as PF activity in BD per milliliter. Since the PHI test measures total antigen, both toxic and nontoxic, we refer to the moiety it measures as toxin/antigen.] Each medium was tested in duplicate flasks from which samples were removed at the times designated in Fig. 1. Samples were removed from alternate flasks of each pair to diminish the effects of volume reduction on the outcome of the experiments. Portions of each

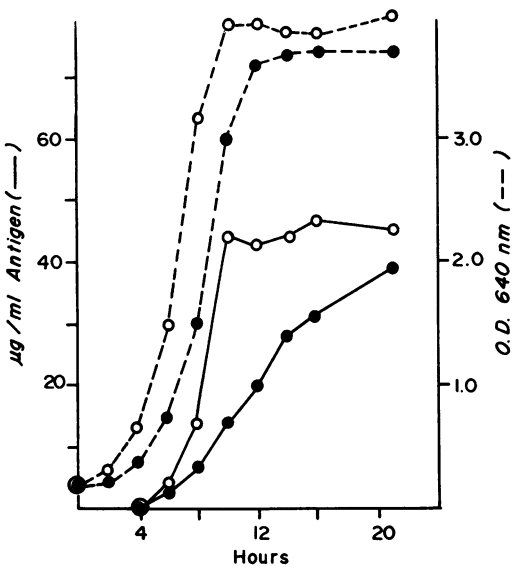

FIG. 1. *Relationship between growth (OD at 640 nm) and toxin antigen production in TA medium and in TRY medium. Toxin antigen was measured by PHI. Symbols: ●, TA; ○, TRY.*

sample were assayed for antigen content by the PHI test and for toxicity by the PF test. Antigen was first detected in both media at 6 hr by the PHI test; toxicity at this time was below 1,000 BD/ml. In TA, the antigen level increased at a more or less constant rate up to the last reading which was taken at 21 hr. In TRY, antigen levels increased to a maximum at 10 hr and remained constant throughout the rest of the experiment. PF titers in both media rose in parallel with the increase in antigen and peaked at 12 hr. The titer in TRY appeared to be twice that in TA.

Comparison of rates of antigen synthesis in TRY and TA. To define the phase of growth correlated with the highest rate of antigen accumulation, the data from Fig. 1 were plotted with increments in antigen concentration as a function of time (Fig. 2). In TRY, the major burst of antigen increase occurred between 8 and 10 hr, fell to 0 by 12 hr, and remained there for the duration of the experiment. In TA, the maximum rates of appearance fluctuated and spanned the extended time period from 10 to 14 hr. The second increase in rate at 14 hr may represent either regrowth of the culture and elevated biosynthesis or intracellular antigen released as a result of autolysis. There was a pronounced drop in rate at 16 hr, but, in contrast to the pattern seen in TRY, a significant rate of accumulation was maintained through 21 hr, the point at which the experiment was terminated.

These data show that, although the antigen concentrations reached in 21 hr in both media were nearly equivalent, the rate and temporal relationship of antigen synthesis or release in TRY, or both, were quite different from those observed in TA. As is the case with PF activity (13), the maximum rate of antigen accumulation coincided with the period of transition from exponential to linear growth in both media.

Growth and antigen production by other vibrios in TA medium. To test the capacity of TA to evoke in vitro antigen synthesis from toxigenic vibrios other than 569B, two previously well studied (3, 15) strains, VC12 and B1307, were incubated under conditions (25 C, initial medium *p*H 6.5) known to elicit maximum PF production. Growth of both strains was comparable to that attained in TRY; thus samples were removed at periodic intervals and tested for antigen by the PHI technique. When the change in antigen content per unit of time was plotted as in Fig. 3, each organism presented a different accumulation pattern. VC12 reached a maximum rate from 8 to 12 hr, released an additional small burst at 16 hr, and then leveled off with no additional net synthesis between 18 and 36 hr. This pattern is similar to that exhibited by 569B in TRY. In contrast to VC12, B1307 exhibited a significant elevation in antigen level between 18 and 36 hr. This observation is consistant with the

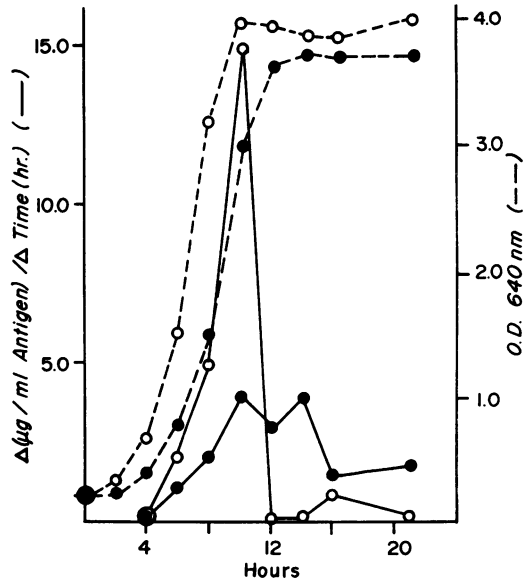

FIG. 2. *Relationship between growth (OD at 640 nm) and the change in toxin antigen concentration with time in TA medium and in TRY medium. Toxin antigen was measured by PHI. Symbols: ●, TA; ○, TRY.*

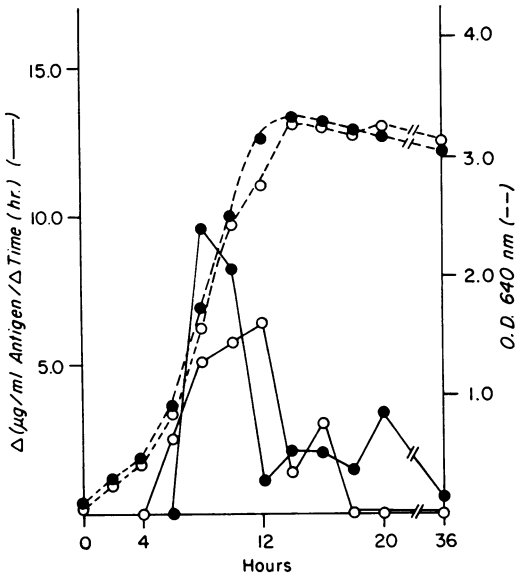

FIG. 3. *Relationship between growth (OD at 640 nm) and the change in toxin antigen concentration with time in TA. Symbols: ○, VC12; ●, B1307; dotted line, growth; solid line, antigen.*

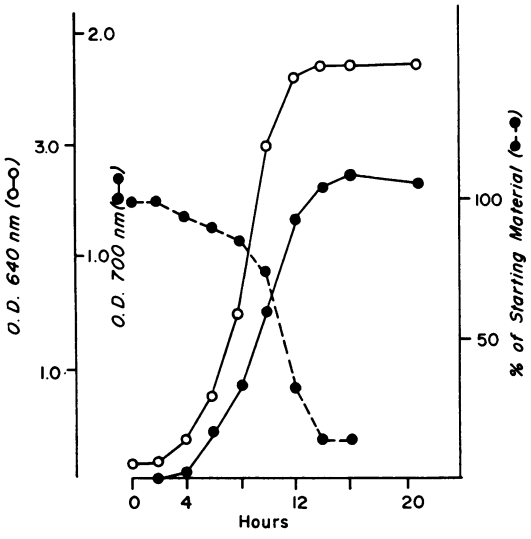

FIG. 4. *Relationship between growth, proteinase activity, and ninhydrin-positive material in TA medium. Symbols: ○, growth; ●–●, proteinase activity; ●---●, ninhydrin-positive material.*

findings of Craig (2) and Kusama and Craig (10) that PF titers in B1307 rose with cessation of active growth and coincident with autolysis. When the same strains were grown at 38 C in TA with an initial *p*H of 8, no antigen was detectable even after 36 hr. This result corroborates our earlier demonstration (3) that little or no PF is synthesized by strains other than 569B when both the *p*H and temperature are elevated.

Biochemical changes in TA medium during antigen accumulation. It has been reported (10, 13) that a nonspecific extracellular proteinase is released into TRY culture fluids concomitant with PF accumulation. Because of possible adverse effects of the proteinase on the toxin/antigen, it was of interest to relate the proteinase activity of TA supernatant fluids to antigen stability. TA cultures of 569B were grown under standard conditions, and samples were removed at prescribed intervals and assayed for antigen content by the PHI test and for proteinase activity with casein as a substrate. Not only was proteinase activity released but it parallelled growth, leveling off at the same time the culture entered the maximum stationary phase (Fig. 4). In contrast, the antigen content of the medium (Fig. 1) rose at a slower pace and continued to increase at a steady rate until the end of the experiment. These results indicate that the antigen concentration, at least as measured by the PHI assay, is unaffected by the proteinase under these conditions of growth. Identical experiments with TRY medium revealed that proteinase release followed the same general pattern but that its specific activity was 25% less than was observed with TA.

Another advantage of using TA is also seen in Fig. 4: the assimilation of ninhydrin-positive material (amino acids) by the growing cells was correlated with growth and the release of proteinase into the culture fluid. This direct comparison is possible because there are no aromatic amino acids in the medium to interfere with the proteinase (and total extracellular protein) determinations (Lowry), and no ninhydrin-positive substances other than the amino acids are initially present.

As expected, during exponential growth there was an inverse relationship between amino acids uptake on the one hand and growth and extracellular protein accumulation on the other. The cessation of active growth coincided almost exactly with the termination of amino acid assimilation, whereas protein accumulation (including antigen) in the medium continued at a reduced rate. These results indicate that when one or more of the amino acids reach limiting concentrations, growth switches from logarithmic to linear and antigen accumulation is occurring at its maximum rate (Fig. 2).

Accordingly, an attempt was made to determine which of the constituent amino acids was involved in triggering antigen synthesis or release, or both. Amino acid standards and portions

of samples from late log and early exponential phases of growth were spotted on silica gel thin-layer chromatograms and processed as described earlier. A representative chromatogram is reproduced in Fig. 5. The initiation of the stationary phase (maximum rate of antigen accumulation) coincided with the disappearance of serine and asparagine from the medium. By 14 hr, all detectable amounts of these amino acids were absent. These data (although only qualitative) also suggest that very little arginine is assimilated by the cells during growth and that glutamic acid was only sparingly used towards the end of the experiment after depletion of the other amino acids.

Relationships between total protein, total antigen, and PF activity. By combining the data from various experiments, it was possible to relate total extracellular protein and antigen with toxicity (Table 3). In TA medium the proportion of antigen in the total extracellular protein increased dramatically between 6 and 12 hr as did the PF activity. At 12 hr, the PF titer levelled off, but the antigen continued to increase through the duration of the experiment. By contrast, in TRY, antigen and PF levels roughly parallelled each other throughout the experimental period. Because of the complexity of TRY, it was not possible to determine the total protein; thus the percentage of antigenic protein present cannot be compared with TA. It appears that antigen synthesis in the two growth media is quite different even though PF synthesis seems to be very similar. In TRY only one burst of antigen release occurred (Fig. 2), and toxin and antigen were synthesized concomitantly. This

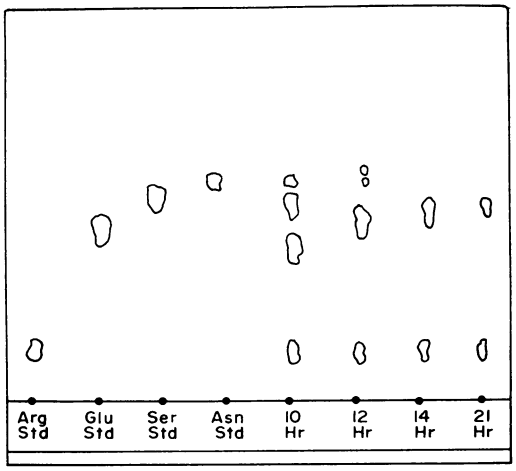

FIG. 5. *Thin-layer chromatogram of TA culture samples taken at 10, 12, 14, and 21 hr. Solvent: chloroform-methanol-ammonium hydroxide (2:2:1, v/v). Support medium: Silica Gel 1B.*

TABLE 3. *Relationships between total extracellular protein, toxin/antigen, and PF activity*

Time of growth (hr)	TA antigen (μg/ml)	Extracellular protein as antigen (%)	TA PF activity (BD/ml)[a]	TRY antigen (μg/ml)	TRY PF (BD/ml)[a]
4	0		0	0	0
6	2	9	0	4	0
8	6	24	5	14	40
10	14	32	10	44	80
12	20	36	80	43	160
14	28	42	80	44	160
16	31		80	47	160
21	39		80	45	160

[a] Values are expressed $\times 10^{-3}$.

was also true of the initial rate of increase in TA, but the second and extended period of synthesis seemed to be directed towards nontoxic antigen. Such an interpretation might be reinforced by noting that at 12 hr the PF titer and antigen value of TA were both approximately one-half of TRY, whereas at 21 hr the antigen concentration had increased 50% whereas the PF titer remained the same.

DISCUSSION

These studies represent the first successful attempt to produce high titers of cholera toxin in a chemically defined medium. Craig's (2) original studies on PF production in vitro were carried out in a basal salts medium supplemented with sucrose as the sole carbon source. However, the maximum PF titers obtained were only on the order of 10,000 BD/ml, or less than 20% of that routinely attained in TA. In a more complete study, Finkelstein and LoSpalluto (6) systematically investigated syncase plus various protein hydrolysates and amino acid mixtures as possible stimulators of toxigenicity. They monitored antigen production (by radial immunodiffusion) and concluded that none of their defined media could approach Casamino Acids in terms of supporting antigen synthesis. Interestingly, all of the simple trial media which elicited significant antigen production contained arginine and glutamic acid in addition to other amino acids. They did not report a requirement for serine per se, but several of the amino acids employed are metabolic equivalents of serine.

We previously reported (15) that a simple medium much like TA (aspartate replaced asparagine, no chelator was present, and yeast extract was included) would yield PF titers of about 10,000 BD/ml. As pointed out here, the major problem with this semisynthetic medium, aside from the ill-defined components of the yeast extract, was not the cell yield but the

limited rate of growth due to the unavailability of complexed iron and magnesium at *p*H 7.5. Addition of the chelator seems to have overcome this adverse effect, and for all practical purposes cell yields and growth rates in TRY and TA are identical. The fact that VC12 and B1307 also produce good toxin titers in TA suggests that this medium will serve as an excellent basis for comparative toxigenicity studies of a wide variety of vibrio strains.

In corroboration of our earlier studies (3, 13), it is clear from the data presented here that the maximum rate of toxin/antigen synthesis or release occurs during the phase of declining growth. This is true not only for 569B (Fig. 2) but for VC12 and B1307 as well. From the limited observations made until now, it would seem that toxigenic vibrios can be separated into two distinct groups: those which produce a burst of toxin antigen in the declining growth phase with little or no evidence of lysis and those which, concomitant with lysis, continue to release toxin/antigen subsequent to the initial burst. This phenomenon may be related to the observations of Kusama and Craig (10), who showed that approximately one-half of their test strains exhibited marked autolysis and prolonged incubation, presumably as a result of an as yet uncharacterized "lytic factor" (proteinase?). Thus, the possibility that the biphasic release of toxin (Fig. 2 and 3) in some instances is due to "compartmentalization" of the molecules into extracellular and intracellular (or loosely and tightly bound) components seems plausible and amenable to additional experimental scrutiny with the PHI-TA system.

The accumulation of nontoxic antigen in TA culture supernatant fluids invites a number of interesting experimental approaches to the study of toxin/antigen biosynthesis and release. The most tenable explanation for our observations is that, for presently unknown reasons, organisms beyond the phase of declining growth begin to synthesize or release a modified molecule which is no longer toxic but which is antigenically closely enough related to the toxin to cross-react in the PHI test. This is apparently not a conversion of toxin (choleragen) to toxoid (choleragenoid) as described by Finkelstein and LoSpalluto (6) because, in TA, PF titers are maintained at their maximum level and, in TRY cultures under identical conditions of incubation, accumulation of nontoxic antigen cannot be detected. It is possible that during later stages of growth and "regrowth," factors in the medium, such as increased *p*H, lowered oxygen tension, accumulation of metabolites, depletion of trace elements, etc., selectively bring about inactivation or cause disaggregation or aggregation of newly synthesized released toxin. However, this is hard to reconcile with the observed stability of PF synthesized before the onset of the stationary phase.

The most enticing observation related to nontoxic antigen synthesis is the change in the pattern of amino acid assimilation occurring when the medium is depleted of serine and asparagine. Appearance of nontoxic antigen and cessation of PF synthesis are coincident with the onset of glutamate and arginine uptake, tempting one to speculate that these alterations in amino acid metabolism may result in formation of nontoxic antigen. If this proves to be the case, it may be possible to affect total biosynthesis of nontoxic antigen through slight changes in amino acid concentration ratios or by the addition of amino acid analogues to the culture medium.

The release of extracellular proteinase in parallel with growth of cholera vibrios has been noted before in studies from our laboratory (13) and in studies by Kusama and Craig (10). The data presented here confirm two important aspects of proteinase activity in toxin-containing supernatant fluids: (i) the proteinase and toxin are not the same molecule; (ii) toxin/antigen as formed in TA or TRY under our growth conditions is apparently unaffected by the proteinase. The first conclusion is based on the observed kinetics of accumulation of the two activities, which are quite different, and on heat-inactivation studies (Richardson, *unpublished data*) which showed that the proteinase maintains 90% of its original activity after being subjected to a time and temperature (56 C, 60 min) which destroyed 100% of the PF activity. The second conclusion is supported by the data of Table 3 which show that in both TA and TRY the peak PF titer is unchanged from the 12th through the 21st hr, in spite of a marked increase in proteinase activity. In TA, the level of accumulated antigen doubled during this same time period and was apparently unaltered to the extent that it still could react with specific antibody in the PHI assay. The principal reason that the proteinase has little effect on the toxin/antigen is that the enzyme is relatively inactive at 25 C (growth temperature), its optimum activity being manifested at 40 C (13). A more serious problem posed by the proteinase is that it is very close to the toxin in molecular size (Richardson, *unpublished data*), making separation of the two molecules during toxin purification a difficult task. Table 3 highlights another advantage of using TA for kinetic studies. By comparing the concentration of the total extracellular protein to that of toxin/antigen, the percentage contributed by the latter can be

determined. This is possible because there are no aromatic amino acids initially present in the medium to interfere with the protein assay. With this technique, the exact time when toxin/antigen reaches its maximum percentage of the total protein in the supernatant fluid can be ascertained, thereby facilitating subsequent purification procedures. The relatively high ratio of toxin/antigen to total protein produced in TA is consistent with our earlier observations (15) that the increase in specific activity (BD per microgram of protein) during purification of toxin produced in semidefined media is fivefold lower than the corresponding value from peptone, implying that there is much less initial contamination with nonrelated protein in the simpler medium.

From the data presented, it seems clear that some or all of the questions posed at the beginning of this paper can be answered by employing TA medium and various combinations of toxin/antigen assay systems. For example, with single-pulse, pulse-chase, and isotope dilution techniques with radio-labeled precursors or analogues, or both, it should be feasible to reconstruct the exact pathway of toxin biosynthesis in a hypertoxigenic strain like 569B and in more conventional recently isolated strains. The employment of isotopes will offer a sensitive method for distinguishing between the biosynthetic and release phases of toxin/antigen accumulation. Regulation of toxigenicity at the biochemical and genetic levels can also be more easily studied in a medium such as TA in which each substituent can be precisely varied in quantity from complete absence to excess. With TA (and perhaps even less complex modifications of it) as a basic tool, many of the molecular mechanisms attendant to cholera enterotoxin formation may soon be elucidated.

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