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Factors Influencing In Vitro Skin Permeability Factor Production by *Vibrio cholerae*

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The development of a new semisynthetic medium which stimulates in vitro production of the skin permeability factor (PF) by *Vibrio cholerae* is described. The effects of *p*H, aeration, temperature, and length of incubation on PF formation or release in strain 569B and several other strains, or both, are compared. Data are presented which show that maximal PF accumulation occurs during a transitional period of growth joining the exponential and stationary phases of the growth cycle. PF elaboration is completed well ahead of any visible signs of lysis in the culture and the PF activity appears to be proportional to the length of the linear growth phase. Possible mechanisms of toxigenicity and the nature of PF are discussed.

Since man is the only host for *Vibrio cholerae* in the natural disease state of cholera, opportunities to evaluate toxin(s) produced under experimental conditions in vitro have been limited. Consequently, the relationships of the physiology and nutrition of *V. cholerae* to the biosynthesis and secretion of toxin(s) or virulence factors are poorly understood at the present time.

Data will be presented in this paper which define optimal conditions for in vitro toxin production by Inaba strain 569B of *V. cholerae*. These conditions will also be compared with those shown to affect maximal toxin production by other cholera vibrio strains (7). Throughout this report, cholera toxin will be referred to as vascular permeability factor (PF) and defined as an exocellular, heat-labile, nondialysable moiety produced by cholera vibrios in response to certain specific environmental alterations. Intradermal injection of this material into guinea pigs or rabbits causes a localized increase in capillary permeability resulting in an indurative response of the delayed (16 to 24 hr), prolonged (24 to 36 hr) type (4). PF is closely associated with or identical to the cholera enterotoxin which elicits fluid accumulation in the intestines of humans (1), dogs (18), infant rabbits (8), and ligated ileal loops of adult rabbits (12).

MATERIALS AND METHODS

Organisms. Stock cultures of rabbit-passed *V. cholerae* strain 569B (Inaba) were obtained in the lyophilized state from John Feeley of the National Institutes of Health, Bethesda, Md. After reconstitution, transfer to Columbia agar (BBL) slants, and overnight incubation at 38 C, the resultant heavy growth was suspended in Heart Infusion Broth (Difco) containing 15% glycerol and frozen in small samples at −50 C. For each experiment, a fresh vial was rapidly thawed and the suspension was used to inoculate an appropriate number of Columbia agar slants. After overnight incubation at 38 C, the growth was washed off in saline and the combined suspensions [about 10^{10} colony forming units (CFU)] per ml, were used as inocula for the experiments to be described. All of the experiments were carried out using a single batch of frozen samples derived from the original lyophilized culture.

Growth curves. Growth was followed turbidimetrically at 640 nm (A640) in 1-cm cuvettes in a Beckman model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Incubation was carried out at various temperatures in a reciprocal water bath shaker operated at 100 to 160 4-inch cycles per min, or on a rotory incubator shaker operating at 250 rev/min. Viable counts were obtained by spreading samples of appropriate saline dilutions of growing cultures over the surface of prepoured and dried Columbia agar plates.

Toxin assay. The basic assay employed in these studies was the permeability factor skin test of Craig (4). Samples (0.1 ml each) of full strength and serially diluted test materials were injected intradermally into the shaved flanks of guinea pigs. Dilutions were made in sterile, buffered saline containing 0.1% gelatin. After 18 to 24 hr, positive reactions became grossly edematous and indurated at the site of injection due to a localized increase in vascular permeability. In most experiments, 1.0 ml of 2% Evans blue dye in buffered saline was injected intraveneously 1 hr prior to the time of reading. Endpoints (a blue area at least 8 by 8 mm) were expressed as blueing doses (BD) per ml or as the $\log_{10}$ of this value.

The titrations were usually done in duplicate on the same or separate animals. The observed endpoints did not vary more than twofold when the titrations were done concurrently, or more than threefold when the same samples were retitrated after storage at 4 C for varying lengths of time. At low dilutions (1:5 to 1:20) the diameter of the blue areas was found to be roughly proportional to the titer of the sample injected.

RESULTS

To assess the influence of nutritional and physical variations in the experimental environment on the response of *V. cholerae* in terms of PF titers, a series of studies concerned with the composition of the growth medium was carried out. Preliminary studies showed that PF formation was directly related to growth above optical densities (OD) of 1.0 to 1.5, so the initial experiments were designed to obtain maximal growth in the most simple medium.

PF production in semisynthetic media. Since casein hydrolysate stimulates PF production (10), our starting point was a tris(hydroxymethyl)-aminomethane (Tris)-buffered 1% solution of Casamino Acids (Difco). Tris was employed as the buffer system because phosphate interferes with many assays useful in characterizing lysis during the growth period. Initially, the *p*H (7.5 to 8.2) and the NaCl concentration (0.5%) resulting in the least lag period were determined. Because of variations in the vitamin content of different lots of Casamino Acids, a trace of yeast extract (50 mg/liter) was also added to the base medium. Low levels of growth could be obtained using the basal medium alone (Fig. 1). Addition of 0.05% sucrose did not alter the growth rate, although it appeared to eliminate the lag period and make the rate linear throughout the growth period, thereby doubling the cell yield at 24 hr. The addition of 0.005% $MgSO_4$ and 0.0005% $FeCl_3$ to the sucrose-supplemented basal medium increased the rate of cell growth by a factor of nine and the final turbidity of the culture increased fourfold. Manganese chloride (0.0005%) was found to partially satisfy the Mg^{++} requirement and was therefore routinely included in the trace metals mixture. Substitution of Na_2SO_4 for $MgSO_4$ caused a reduction in growth response back to basal levels, showing that Mg^{++} was stimulatory.

Reducing the Casamino Acids concentration by one-half (to 0.5%) decreased the growth rate by a factor of three and resulted in a twofold drop in maximum turbidity (Fig. 2). Further investigation revealed that growth rate and final cell yield are directly proportional to the Casamino Acids concentration of the medium over a range of 0.005% to 1.0%. The elimination of sucrose (or an alternate carbon and energy source) from the medium (Fig. 2) resulted in a pronounced growth lag and a significant (25%) drop in cell yield. Substitution of lactate for sucrose seemed to increase the growth rate and final density;

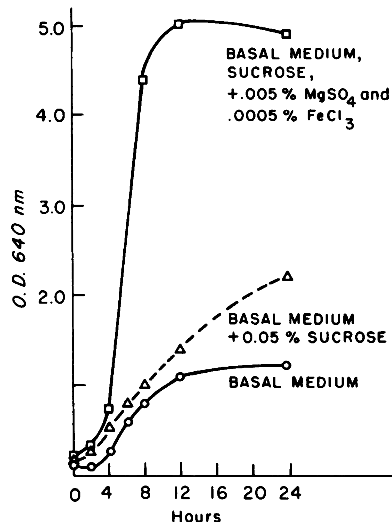

FIG. 1. *Growth of V. cholerae as a function of the growth medium composition. The basal medium contained 1% Casamino Acids, 0.005% yeast extract, and 0.5% NaCl in 0.05* M *Tris-hydrochloride, pH 8.0. A 50-ml amount per 500-ml flask was placed on a rotary shaker at 130 4-inch oscillations/min at 30 C.*

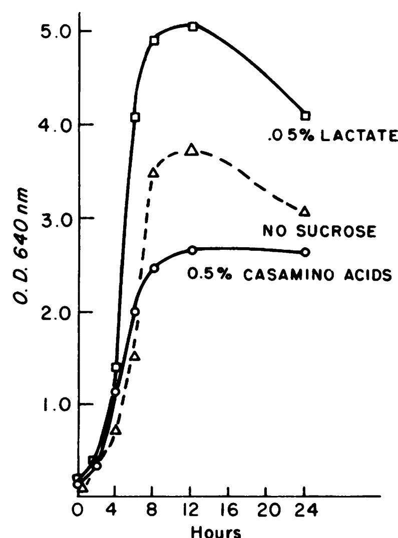

FIG. 2. *Growth of V. cholerae as a function of the concentration and type of substrate added. Basal medium as in Fig. 1, plus 0.1% trace metals and indicated changes. Growth conditions as in Fig. 1.*

at the same time, the accumulation of acidic end products and the attendant *p*H drop characteristic of fermentable substrates were eliminated.

Effect of aeration on PF titer and cellular localization. In the experiments described so far, an arbitrary volume of medium (one-tenth that of the culture flask employed) was used. The selection of this surface to volume ratio was based on observations by previous workers (6, 8) that maximal toxin production was attained only when cultures were vigorously aerated. To investigate the effects of aeration on PF titer, four 500-ml flasks containing 50, 100, 250, and 450 ml of medium were inoculated to an equal cell density and placed on a shaker at 130 4-inch oscillations per min at 30 C. In these experiments, sucrose was used as the energy source since lactate is not utilized under anaerobic conditions. To offset the *p*H drop caused by sucrose fermentation, the Tris concentration was raised to 0.1 M. Samples were removed at predetermined intervals and the growth rate was estimated. At the end of the experiment, the cells were removed from the growth liquor by centrifugation; equivalent quantities of cells from each flask were suspended in 10 ml of 0.05 M Tris buffer, *p*H 8.2. Each suspension was treated by sonic oscillation for a total of 3 min at 5 C, using the full power setting on a Biosonik 20-kc sonic generator (Will Scientific, Inc., Rochester, N.Y.) Whole cells and debris were removed by centrifugation at 15,000 × *g* for 30 min in the cold. The results of these studies (Table 1) indicate that both growth rate and total cell yield are directly proportional to the surface to volume ratio of the culture. No marked difference was observed between the 50-ml and the 100-ml cultures in terms of total cell yield, in spite of the fact that the exponential growth rate in the 100-ml culture was only about two-thirds that of the 50-ml culture.

The PF titers of the supernatant solutions and of the sonicates were identical in the 50-ml and 100-ml cultures. The maximal titer (50-ml flask) in this experiment was about 8% of that ordinarily obtained with standard conditions (*see* Table 2). When the medium volume was increased to 250 ml, the final turbidity and the exponential growth rate decreased by factors of 2.6 and 6.5 in comparison to the rate and yield from the 50-ml flask. This decline in rate and yield was accompanied by a significant decrease in the PF titer of the supernatant fluid (16-fold) and the sonic-treated material (eightfold). In the flask containing 450 ml of medium, no PF activity was detected and the low level of growth which did occur was attributable to fermentation of the sucrose present in the medium (*see* Fig. 1). In the 50-ml and 100-ml cultures, the normal initial drop in *p*H due to the rapid utilization of the sucrose was followed by a slow rise to *p*H values above the starting level. In the deeper cultures (250 and 450 ml), the initial drop was not reversed during further growth. Failure to detect PF in the deep culture suggested that anaerobic conditions or poor growth, or both, inhibit PF production.

PF titer as a function of Tris concentration. From initial experiments, it was found that 0.05 M Tris was adequate to maintain the *p*H of the growth medium within 0.2 to 0.3 units of the starting value. However, in the volume experiment (Table 1) and in certain other studies where the medium was supplemented with abnormally high levels of sucrose (up to 0.5%), it was necessary to increase the molarity of the buffer system to as much as 0.2 M to avoid large *p*H fluctuations. In each experiment where the Tris concentration was elevated above normal, the

TABLE 1. *Toxin production and cell localization as a function of culture volume*[a]

Medium vol.	A640 (24 hr)	*p*H			Growth rate[b]	Titer[c]	
		0 hr	6 hr	24 hr		Supernatant fluid	Sonic-treated material
ml							
50	3.60	8.2	7.9	8.4	1.30	6,400	800
100	3.42	8.2	7.8	8.3	0.90	6,400	800
250	1.38	8.2	7.8	7.9	0.20	400	100
450	0.50	8.2	7.8	7.9	0.15	0	0

[a] Except for the volume and a buffer concentration of 0.1 M, growth conditions are as in Fig. 2.

[b] Expressed as Δ A640/hr, log phase.

[c] Expressed as BD per ml of test material. One BD produces a skin reaction at least 8 × 8 mm in diam.

TABLE 2. *PF titer as a function of Tris concentration in the growth medium*[a]

Hour	0.10 M		0.05 M		0.02 M		0.01 M	
	A640	*p*H	A640	*p*H	A640	*p*H	A640	*p*H
0	0.125	7.8	0.130	7.9	0.130	7.9	0.150	7.8
4	0.970	7.8	1.380	7.9	1.520	7.9	1.650	7.9
6	2.100	7.8	3.150	8.0	3.600	8.1	3.600	8.2
12	4.600	7.8	4.800	8.0	4.600	8.1	4.600	8.2
12[b]	10,000		40,000		80,000		240,000	

[a] Growth conditions as in Fig. 2. The medium contained 0.05% sodium lactate as the energy source.

[b] Blueing doses per ml of supernatant fluid.

PF titers were 4- to 15-fold lower than normal. To determine if this phenomenon could be reversed and PF titers could be elevated by lowering the buffering capacity of the medium, a series of 500-ml flasks containing 50 ml of medium differing only in buffer concentration was inoculated and grown as usual. Turbidity determinations were made hourly and larger samples were removed at prescribed intervals for PF titrations. The data presented in Table 2 reveal that the Tris concentration does indeed influence the PF titer. At 0.1 M, the titer was only 10,000 BD/ml (about eightfold less than normal for these conditions), whereas the 0.01 M Tris-buffered medium yielded a titer of 240,000 BD/ml, about three times the normal value. The *p*H in all of the flasks, with the exception of the one buffered at 0.1 M, followed the same pattern (i.e., a slow rise from the starting level of *p*H 7.8 or 7.9 to a final value of *p*H 8.0 to 8.2). The most obvious effect of increasing the Tris concentration was a lag in growth (compare the 6-hr turbidities of the flasks with concentrations of 0.01 and 0.1 M) which resulted in a 2 hr differential in the time required to complete the exponential phase of growth.

PF titer as a function of temperature. It has been suggested by Craig (5) that PF titers are inversely proportional to the temperature of growth. Accordingly, temperature to PF relationships in our system were investigated employing a series of culture flasks (50 ml of medium/500-ml flask) incubated at different temperatures over a 25 C to 40 C range on a rotary shaker at 250 rev/min. Samples were removed at the end of the exponential phase of growth and at 24 hr. The PF titer and turbidity achieved at the end of the exponential phase (Table 3) were inversely proportional to the growth temperature, as is the time required to reach the end of logarithmic growth (12 hr at 25 C versus 7 hr at 40 C). Both turbidity and PF titers decreased in direct proportion to the temperature at 24 hr of incubation. At 40 C there was a twofold decline in turbidity and a fourfold decrease in PF titer between 7 and 24 hr, whereas at 25 C there was a slight increase in turbidity and PF titer during the second 12 hr of the growth cycle. The optimal temperature range for PF production in this system appears to be 25 to 30 C.

Wet mounts of each culture were examined by phase contrast microscopy at the intervals studied. The marked decrease in PF titer and turbidity was accompanied by a conversion of the typical curved rod-shaped vibrios to small, dense, spherical forms. The rates of this transformation and the concomitant decrease in PF titer were found to be directly related to the growth temperature. At 37 and 40 C, the conversion to spheres was complete in 24 hr or less, whereas at 25 C total transformation to the spherical forms requires 48 hr or longer. This morphological transition and its relevance to in vitro PF formation will be the subject of a separate communication (Kennedy and Richardson, *in press*).

PF titer as a function of pH and inoculum size. Two other factors involved in in vitro PF elaboration, initial *p*H and inoculum size, were also examined. When the starting *p*H was varied from 6.5 to 8.5 the maximal difference in PF titer observed was 10-fold. In this range the only noticeable effect of *p*H on growth was a slight alteration in the length of the lag period (i.e., from essentially no lag at *p*H 7.5 to a lag of 1 hr at *p*H 6.5). In general, PF titers were highest in the 7.0 to 7.8 range and were reduced when the starting *p*H was 8.0 or above. The peak PF values coupled with the shortest incubation intervals occurred in the *p*H 6.5 to 7.5 range.

The length of the lag phase was found to be proportional to inoculum size over a range of 10^3 to 10^8 CFU/ml. There was no observable effect either on the logarithmic growth rate or the final cell yield within this range. The PF titer was identical in all supernatant fluids over the range studied regardless of the length of the lag period. The inoculum resulting in the shortest total time of growth (10^8 CFU/ml) was therefore used in all subsequent experiments.

Kinetics of in vitro PF formation. The results of the temperature studies suggested that PF begins to accumulate in the culture medium in the early phases of the growth cycle and that its appearance is independent of cell autolysis.

TABLE 3. *PF titer as a function of growth temperature*[a]

Temp	Time	A640	Log_{10} BD/ml	Log_{10} BD/ml[b]
C	*hr*			
25	12	4.2	4.24	
25	24	5.0	4.78	+0.54
30	10	4.6	4.90	
30	24	3.2	4.81	−0.09
35	7	4.6	4.18	
35	24	2.7	3.93	−0.25
37	8	4.3	3.90	
37	24	2.6	3.60	−0.30
40	7	4.6	2.81	
40	24	2.3	1.93	−0.88

[a] Growth conditions as in Fig. 2. Supplemental carbon source was 0.05% sodium lactate.

[b] Represents the difference in BD/ml between the peak titer and the titer at 24 hr.

Accordingly, a series of experiments was conducted to attempt to correlate PF titer with viable cell numbers and turbidity.

Replicate 500-ml flasks each containing 50 ml of medium were inoculated at 12-hr intervals and incubated with shaking (130 4-inch cycles/min) at 30 C. Samples were removed hourly and dilutions were made for viable counts. PF titrations were carried out on appropriate dilutions of selected samples. The data from several such experiments are summarized graphically in Fig. 3.

The usual rapid growth rate was observed with the exponential phase extending from 2 to 7 hr. In this period of time, the viable count rose from 1.3×10^8 to 1.7×10^{10} CFU/ml. This increase represents a total of approximately eight cell doublings, with an average generation time of 76 min. During most of the exponential phase (2 to 6 hr), the generation time dropped to 56 min and 4.3 cell divisions took place. The turbidity and the viable count correlated well throughout the experimental period. At the end of the log phase, the cell count leveled off at approximately 1.5×10^{10} CFU/ml; it remained at this level for 16 hr. At this point which is coincidental with a marked decrease in turbidity, the cell count began to fall rapidly, until at 36 hr it was only slightly greater than the starting value. Microscopic observation, showed that transformation to the previously described spherical forms was essentially complete by 36 hr.

The PF titers of the samples were found to reach maximal values concurrent with the late logarithmic phase of growth of the culture well ahead of any significant autolysis of the cells. In agreement with the data of the temperature experiments, the titers started to drop slowly at about 24 hr, and by 36 hr a fourfold reduction from the maximal titer was recorded.

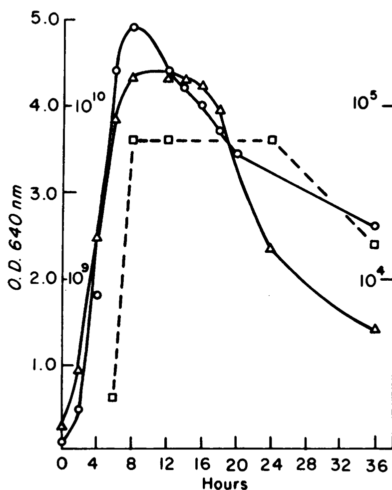

FIG. 3. *PF titer as a function of viable cell numbers and turbidity. Growth conditions were the same as in Fig. 2. Symbols:* log_{10} *CFU/ml* (△), *left ordinant;* log_{10} *BD/ml* (□), *right ordinant; turbidity* (○).

Standard conditions for PF production. As a result of the studies reported above, we have developed a standard method for producing PF in 1.0 to 2.0 liter batches (the routine procedure employs 25 to 30 individual flasks). The Tris-Casamino-Acids-yeast (TCY) medium employed is compounded as indicated in Table 4. Using initial cell densities of 10^8 CFU/ml and 50 ml of TCY per 500-ml baffle flask, PF titers of 250,000 to 350,000 BD/ml have been obtained after overnight (14- to 16-hr) incubation at 25 C on a rotary shaker at 250 rev/min. These cultures show little decrease in turbidity from their peak at about 14 hr; when viewed by phase microscopy, the cells appear quite normal with no evidence of lysis or spheroplast formation. Data from typical experiments relating growth, PF titer, and the area of blueing produced by the injection of sequential samples (1:10 dilutions) from the supernatant fluids are presented in Fig. 4. There is a good correlation between growth, PF titer, and the area of blueing. The maximal rate of PF increase occurs during the transition from the logarithmic to the stationary phase, whereas the peak titer is reached at about 18 hr. The low titers shown in Fig. 4 are the result of overnight storage of the diluted samples; overnight storage is necessary to bioassay all the samples in the experiment at the same time. In the usual large-scale experiment which is uninterrupted by sampling, the titer peaks at 15 to 16 hr and was found to be 2.5 to 3.5×10^5 BD/ml. The maximal

TABLE 4. *Composition of TCY medium*[a]

Substance	Amount
NaCl	5[a]
Casamino Acids	10[a]
Sodium lactate	0.5[b]
Yeast extract	0.05[b]
5% $MgSO_4$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $FeCl_3 \cdot H_2O$ in 0.001 N H_2SO_4	1.0[c]
Tris-hydrochloride (0.01 M, *p*H 7.8)	1,000[c]

[a] Lowering the NaCl concentration to 0.25% and adding 0.25% KCl and 0.02% Na_2HPO_4 allows synthetic mixtures of pure amino acids and certain single amino acids to be used in place of Casamino Acids with no appreciable decrease in PF titers. Substitution of maleic acid for HCl in the buffer system allows poising of the medium's *p*H at any point between 6.0 and 8.0.

[b] Value expressed in grams.

[c] Value expressed in milliliters.

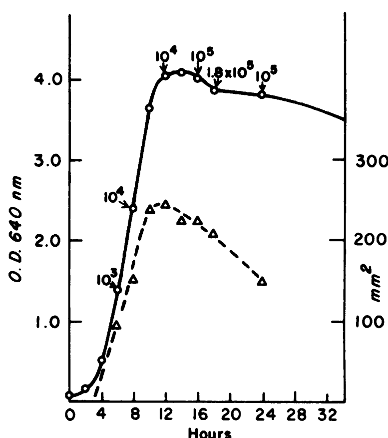

FIG. 4. *PF production under optimal in vitro conditions. A 50-ml amount of TCY (as in Table 4) per 500-ml baffle flask. Symbols: (○), incubation, 250 rev/min at 25 C, OD at 640 nm; (△) blue area produced by 1:10 dilutions of the indicated samples. Numbers (expressed as BD/ml) along the top of the curve are titers obtained at the times noted.*

cell density obtained under these conditions is 5×10^9 CFU/ml, with the final *p*H of 7.8 to 8.0.

Comparison of PF production in 569B and in other choleragenic vibrios. In light of the experience that has been gained in this study and in correlative studies which have been carried out on other strains of vibrios (7; S. H. Richardson, Bacteriol. Proc., p. 89, 1969), optimal conditions for in vitro PF production by several different strains can be summarized as shown in Table 5. All the strains reach peak titers in 10 to 14 hr, which is coincidental with the end of the exponential phase of growth. In every strain the PF peak was reached before any visible signs of lysis were seen in the culture. In addition, maximal rates of PF titer increase always occurred during the period of unbalanced growth which links the exponential and stationary growth phases.

Further incubation (4 to 5 hr beyond peak titer) caused a rapid decline in PF activity of culture fluids from organisms other than 569B. In the case of 569B, the PF titer was stable for at least 8 hr after the peak had been reached. These results and the repeated observation that PF titers from 569B are consistently higher than those from other strains help to explain why in vitro PF production had been described only in 569B until recently (9).

The other major differences seen when 569B and other strains are compared are the following. (i) 569B has no sharp optima for PF production when factors, such as initial *p*H, growth temperature, or the medium's buffer system, are varied. (ii) PF is produced over a slightly longer fraction of the growth cycle in 569B. (iii) The other strains do not produce PF in TCY, even at an initial *p*H of 6.5 which yields excellent growth (7), whereas high PF titers can be achieved in several other media, such as 2% peptone and completely synthetic media, when 569B is employed as the biological agent.

Thus, it appears that one or more physiological deviations resulting in a loss of control of biosynthesis or increased permeability, or both, have made 569B ideally suited for in vitro PF production with TCY as the choice medium.

DISCUSSION

Throughout this investigation, a basic assumption has been made, namely, that PF is a valid measure of the cholera enterotoxin responsible for the fluid accumulation in cholera. Partial, though indirect, justification for this assumption comes from the work of Finkelstein (9) who has shown that choleragen (a concentrated semipurified filtrate from syncase-grown 569B) which produces cholera in humans (1) elicits the PF reaction when injected intradermally in amounts below 1 μg. Further data linking PF activity with the actual disease was supplied by Craig (4), who not only demonstrated the presence of PF in stool filtrates from actively purging cholera cases, but also found that the skin reaction was neutralized by convalescent sera from these same patients.

Evidence more directly referable to TCY filtrates is presented in work recently reported from this laboratory (17), in which it was shown that a direct correlation exists between PF activity and fluid accumulation in infant rabbits injected intraintestinally with sterile TCY filtrates and with concentrated material derived from these filtrates by dextran sulfate precipitation. Crude TCY filtrates and other more highly purified, PF preparations have also been found to elicit extensive fluid accumulation in adult rabbit ligated ileal loops and in jejunal loops in dogs (S. H. Richardson, 1969).

The answer to the question of whether PF and cholera enterotoxin are identical will have to await the complete purification of the toxin. It can be stated, however, that thus far preparations which cause fluid accumulation always have high PF titers and that preparations at all stages of purity which possess high PF titers potentiate fluid accumulation in all of the currently accepted animal models (S. H. Richardson, 1969).

The data obtained in this study indicate that the use of a Casamino Acids-containing medium for

high-titer PF production offers a number of distinct advantages over the use of the complex media commonly employed for toxin production. The main advantage is that of reproducibility, since casein hydrolysates are relatively more consistent in their composition and stimulation of microbial activities, such as PF production, than are the various tissue digests. A second point favoring the use of the simpler TCY medium is that the resultant culture filtrates are essentially free of nondialysable medium-derived components (or antigens) which interfere with toxin purification.

It should be pointed out (Tables 4 and 5) that TCY does not favor PF formation from vibrio strains other than 569B even when excellent growth occurs and the temperature and *p*H are optimal (7). However, addition of 0.25% KCl and 0.02% Na_2HPO_4 modifies this response and elicits PF titers 50 to 75% of those of 569B from several other vibrio strains (S. H. Richardson, 1969).

Starting with the original studies of De (6), it has been repeatedly pointed out that cholera enterotoxin production in vitro is a direct function of the oxygen tension of the medium (2, 3, 7, 13, 15, 16). In contrast to these reports, Craig (5) showed that under rather special conditions maximal PF titers were obtained after 48 hr in stationary cultures. Craig employed a strain of vibrio (B1307) differing from 569B in several respects (*see* Table 5). His data also indicate that PF elaboration under these conditions is dependent on autolysis since viable cell counts are inversely proportional to PF titers. In Craig's medium (which consists only of mineral salts and sucrose), it appears that the cells are undergoing metabolic lysis of the type described by Gallut (11) in his studies on the release of vibrio 0 antigens.

The data presented here (Tables 1-3) are all consistent with the conclusion that maximal PF activity, minimally contaminated with cellular exoenzymes or autolysis products, is best achieved after relatively short growth periods (8 to 16 hr) in fully oxygenated cultures.

Kinetics of in vitro PF elaboration. Close inspection of Fig. 3 and 4 reveals a distinct pattern regarding the increase in PF activity in culture supernatant fluids. With 569B, the first detectable PF appears at about 3 hr. After an additional 2 to 4 hr of exponential growth, there is an abrupt change in the rate of growth which coincides with a switch from exponential to linear growth and a "burst" of PF titer increase.

The burst of PF is detectable by titration of closely spaced sequential samples or by recording the increasing area of blueing elicited by constant dilutions (1:10) of the supernatant fluid. There are at least two models which might explain the observed kinetics. Cell-associated PF could be continuously synthesized and accumulated throughout the entire growth cycle and suddenly released as a result of the switch from balanced to unbalanced growth, or de novo PF synthesis and elaboration could be triggered by the change in growth mode. The observation that PF titers appear to be independent of the initial inoculum size tends to suggest that late synthesis is occurring; i.e., 30 cell doublings with the constant synthesis of PF should yield more total PF than eight cell doublings, but in fact they do not.

The further fact that PF is not detectable in lysed or sonic-treated material (Table 1) from cells which have not passed through the transitional phase of growth also tends to argue against steady accumulation and sudden release as a possible mechanism, although more extensive experiments (such as pulse and chase studies with media containing a single, radioactive amino acid)

TABLE 5. *Comparison of optimal conditions for permeability factor (PF) production by Strains 569B and VC12 of V. cholerae*[a]

Vibrio	Medium[b]	Molarity and initial *p*H of buffer[c]	Temp and temp range[d]	PF avg[e] and hr of first PF detected
569B	TCY	0.01 M Tris, 6.5–8.5	25 C, (25–40)	250,000 (2 hr)
VC12[f]	2% peptone, 0.5% NaCl	0.001 M Tris, 6.5	25 C, (25–30)	150,000 (6–8 hr)

[a] At 250 2-inch rev/min.

[b] A 50 ml amount of autoclaved media/500-ml conical baffle flask.

[c] Phosphate buffer (0.01 M) can be substituted with 569B; phosphate is inhibitory to PF production by VC 12.

[d] Maximal PF titer achieved at 10 to 14 hr; stable for an additional 8 hr with 569B 4–5 hr with VC12. Titers as high as 400,000 BD/ml have been achieved with 569B; titers of 250,000 BD/ml have been achieved with other strains.

[e] Expressed as blueing doses per ml.

[f] Applies to NIH 41, NIH 35A3, and B1307 inoculated to 10^8 CFU/ml from overnight slant. No PF in TCY, even at *p*H 6.5.

will have to be carried out before a definite answer can be obtained.

The data presented here and other information which will be treated elsewhere have led to a working hypothesis regarding PF formation by *V. cholerae*. It is postulated that growing cells after attaining a minimal population density (OD 1.0, or greater; 10^9 CFU/ml) are somehow induced to enter a phase of unbalanced growth. It is not possible at the present time to define the trigger mechanism(s) which initiates unbalanced growth; however, since its onset appears to be relatively independent of cell density (above the minimal level), accumulation of toxic metabolites is probably not a major factor.

This sudden shift in growth rate might be likened to "step down" experiments in which shifts from a rich or complete medium to a poor nutritionally deficient medium often result in the establishment of linear growth. When this critical balance is achieved, the cells apparently react either by synthesizing an altered surface component (or its precursor) which is not capable of fulfilling its normal structural function (e.g., a cytoplasmic membrane lipoprotein) or by elaborating or releasing a protein or peptide which is normally a part of the periplasmic complex. Changes of this sort associated with alterations in cellular permeability have been repeatedly observed in studies on microbial excretion of macromolecules.

The accumulated material(s) could then indirectly affect its response in the host by being converted to a pharmacologically active peptide, such as secretin, which was recently shown by Leitch and Burrows (14) to elicit fluid accumulation in ligated ileal loops, or it could be rapidly bound per se and exert a direct effect on the host's tissue.

In support of this hypothesis, Richardson and Kennedy (S. H. Richardson and J. R. Kennedy, 1968) have shown that there is, in fact, a transient accumulation of amorphous material on the surface of 569B immediately prior to and during the linear growth phase and its concomitant burst of PF release. Proceeding or planned experiments involving radioactive substrates and ferritin-labeled antibody against highly purified PF may help to elucidate the nature of this extracellular substance; perhaps, they will furnish direct evidence concerning its mechanisms of interaction with the host's tissues.

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LITERATURE CITED

1. Benyajati, C. 1966. Experimental cholera in humans. Brit. Med. J. 1:140–142.
2. Burrows, W. 1968. Cholera toxins. Ann. Rev. Microbiol. 22:245–268.
3. Coleman, W. H., J. Kaur, M. E. Iwert, G. J. Kasai, and W. Burrows. 1968. Cholera toxins: purification and preliminary characterization of ileal loop reactive type 2 toxin. J. Bacteriol. 96:1137–1143.
4. Craig, J. P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature 207:614–616.
5. Craig, J. P. 1966. Preparation of the vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. 92:793–795,
6. De, S. N., M. L. Ghose, and J. Chandra. 1962. Further observations on cholera enterotoxin. Trans. Roy. Soc. Trop. Med. Hyg. 56:241–245.
7. Evans, D. J., Jr., and S. H. Richardson. 1968. *In vitro* production of choleragen and vascular permeability factor by *Vibrio cholerae*. J. Bacteriol. 96:126–130.
8. Finkelstein, R. A., H. T. Norris, and N. K. Dutta. 1964. Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intraintestinal infection and experimental cholera produced with cell-free products. J. Infect. Dis. 114:203–216.
9. Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biologic activities of purified procholeragen. Amer. J. Immunol. 96:440–449.
10. Finkelstein, R. A., S. W. Nye, P. Atthasampunna, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: effect of choleragen on vascular permeability. Lab. Invest. 15:1601–1609.
11. Gallut, J., and J. Giuntini. 1963. Etude de *Vibrio cholerae* au microscope electronique et relation entre l'aspect morphologique et l'agglutination "O." Bull. World Health Organ. 29:767–771.
12. Kasai, G. J., and W. Burrows. 1966. The titration of cholera and antitoxin in the rabbit ileal loop. J. Infect. Dis. 116: 606–614.
13. Lankford, C. E., and U. Legsomburana. 1965. Virulence factors of choleragenic vibrios. Proc. Cholera Res. Symp. Honolulu, p. 109–120. (U.S. Govt. Printing Off., Washington, D.C.).
14. Leitch, G. J., and W. Burrows. 1968. Experimental cholera in the rabbit ligated intestine: ion and water accumulation in the duodenum, ileum, and colon. J. Infect. Dis. 118: 349–359.
15. Richardson, S. H., and D. J. Evans, Jr. 1965. The effects of *Vibrio cholerae* extracts on membrane transport mechanisms. Proc. Cholera Res. Symp. Honolulu, p. 139–144. (U. S. Govt. Printing Off., Washington, D.C.).
16. Richardson, S. H. 1966. Inhibition of intestinal ion translocase enzymes by culture filtrates of *Vibrio cholerae*. J. Bacteriol. 91:1384–1386.
17. Richardson, S. H., and D. J. Evans, Jr. 1968. Isolation of cholera toxins by dextran sulfate precipitation. J. Bacteriol. 96:1443–1445.
18. Sack, R. B., C. C. J. Carpenter, R. W. Steenberg, and N. F. Pierce. 1966. Experimental cholera: a canine model. Lancet 7456:206–207.