



A comparative study of enzymes and other characteristics of *Vibrio cholerae* grown in diverse media  
by Bruce Albert Braaten

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY in Genetics

Montana State University

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Abstract:

Representative strains of *Vibrio cholerae* (including "classical", El Tor and non-agglutinable biotypes) were studied. Optimal conditions for growth in minimal medium were determined. Certain strains required adenosine 5' monophosphoric acid, and all strains required repeated adjustments of pH for prolonged growth. Motility, major somatic antigens and the ability to support phage growth were essentially the same in minimal and complete media. On the other hand, hemolysin destructive factor (HDF), and the enzymes hemolysin and protease were not produced in minimal medium by those strains otherwise capable of their production in complete medium. Hemolysin production is elicited by the addition to minimal medium of concentrations of sodium cholate which do not spontaneously lyse cells. A cell-bound hemolysin was discovered by virtue of the fact that it is produced in minimal as well as complete media. Efforts to demonstrate transduction and conjugation were unsuccessful.

A COMPARATIVE STUDY OF ENZYMES AND OTHER CHARACTERISTICS OF VIBRIO  
CHOLERAE GROWN IN DIVERSE MEDIA

by

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## ABSTRACT

Representative strains of Vibrio cholerae (including "classical", El Tor and non-agglutinable biotypes) were studied. Optimal conditions for growth in minimal medium were determined. Certain strains required adenosine 5' monophosphoric acid, and all strains required repeated adjustments of pH for prolonged growth. Motility, major somatic antigens and the ability to support phage growth were essentially the same in minimal and complete media. On the other hand, hemolysin destructive factor (HDF), and the enzymes hemolysin and protease were not produced in minimal medium by those strains otherwise capable of their production in complete medium. Hemolysin production is elicited by the addition to minimal medium of concentrations of sodium cholate which do not spontaneously lyse cells. A cell-bound hemolysin was discovered by virtue of the fact that it is produced in minimal as well as complete media. Efforts to demonstrate transduction and conjugation were unsuccessful.

## INTRODUCTION

Vibrio cholerae, the etiological agent of the disease cholera, was first isolated in 1883 by Robert Koch. The cholera vibrio is a gram-negative, short, comma-shaped bacterium which may occur singly or in short chains. The organism is motile and has a single polar flagellum. V. cholerae is rapidly killed in a medium with a pH lower than 6.0 but grows profusely in an alkaline medium.

The cholera vibrios can be divided into three groups on the basis of their serologic specificity, their ability to produce hemolysin and their sensitivity to polymixin B (Felsenfeld, 1967). Both "classical" and El Tor vibrios are agglutinated with O-I group antisera whereas a third group of cholera vibrios is not. Members of the latter group are known as the nonagglutinable vibrios. The "classical" vibrios are separated from the El Tor vibrios on the basis of hemolysin production and resistance to polymixin B. Most of the El Tor vibrios produce hemolysin and are polymixin B resistant, while the "classical" vibrios do not produce hemolysin and are polymixin B sensitive.

The cholera vibrios produce a wide variety of exoenzymes (Felsenfeld, 1967). For example, they have been found to produce collagenase, elastinase, nucleotidases, decarboxylases, lipase, protease, lecithinase, mucinase, hemolysin and hemodigestive enzyme. Little is known about the inducible or constitutive nature of the above enzymes. Nothing is known about the transfer and recombination

of genetic information, for the above enzymes, between strains of cholera vibrios. Hence, it was of interest to study the physiological genetics of one or more of the above enzymes, and to explore the possibility of transferring genetic information for an enzyme between cholera vibrio strains. Hemolysin was chosen for the major part of the study because of the relative ease and sensitivity of detecting it by the spot plate method (Kalsow, Newman and Rutherford, 1967). Protease and hemolysin destructive factor (HDF) were also studied because of their ease of detection. In addition to the study of exoenzymes, it was of interest to examine the stability of the phenotypic expression of some other characteristics of the cholera vibrios. For this study, it was decided to examine motility, major somatic group antigens and the ability of the cholera vibrios to support phage growth.

In 1905 Kraus and Pribram found strains of cholera, isolated from the El Tor quarantine camp, which were hemolytic (Feeley and Pittman, 1963). The hemolytic property of the El Tor vibrios has been reported by some to be stable (Greig, 1914; Meinicke, 1905; Snapper, 1921) and by others to be variable (de Moor, 1949; Heiberg, 1935; Roy and Mukerjee, 1962). Roy and Mukerjee (1962) reported that an El Tor vibrio strain became non-hemolytic after it had been subcultured 12 times in synthetic medium. The organism remained hemolytic after passage in nutrient broth or passage in synthetic

medium with 0.25% sheep erythrocytes. Roy and Mukerjee (1962) argue that this is evidence that hemolysin is an adaptive enzyme system which is induced by a specific factor present in red blood cells. Watanabe and Seaman (1962) found that the El Tor vibrio hemolysin molecule was 95% lipid and also contained 14 of the common amino acids. Kalsow and Newman (1968) found that the El Tor hemolysin was destroyed within 15 minutes at 56 C and was stable between the pH range of 5 to 9. There is little further information about the biochemical and genetic basis of the hemolysin of the El Tor vibrios.

There is also very little biochemical and genetic information on protease production by V. cholerae. Liu and Hsieh (1969) observed that 4% ammonium sulfate in trypticase soy broth inhibited protease production by V. cholerae. Some biochemical information is available on hemolysin destructive factor (HDF). On the observation of a decrease in hemolysin titer in old cultures, Feeley and Pittman (1963) were the first to speculate that there was an enzyme or factor which destroyed hemolysin. Wake and Yamamoto (1966) worked out an in vitro test for HDF and found that HDF was thermolabile, resistant to 0.2% formalin and was active in a pH range of 6.9 to 8.6. Kalsow and Newman (1968) studied the time sequence of the production of hemolysin and HDF in four strains of El Tor vibrios. They found that

hemolysin was produced within four hours, while HDF was not produced until about 20 hours after incubation of the cultures.

The observation of genetic recombination in V. cholerae was first made by K. Bhaskaran (1958). Bhaskaran mixed a lysogenic purine dependent strain with a nonlysogenic methionine dependent strain in the same flask. After incubation, the mixed culture, plated under selective conditions, gave rise to a greater number of colonies than did either strain plated alone. However, the above method did not elucidate whether recombination was mediated via conjugation, transduction or transformation. Later it was found that for recombination to occur one of the parent strains had to have a fertility factor, designated the P factor (Bhaskaran, 1959). Strains possessing the P factor ( $P^+$  strains) produced plaque-like clearings when tested on strains which did not have the P factor ( $P^-$  strains). It was possible that the P factor was a bacteriocin as no phage could be recovered from the plaques. The  $P^+$  character behaved like  $F^+$  character in that the  $P^+$  character was infective for  $P^-$  strains (Bhaskaran, 1959). However the  $P^+$  strains differ from  $F^+$  strains in that  $F^+$  strains do not produce plaques on  $F^-$  strains. Hence the  $P^+$  character behaved like a fertile bacteriocin. Crosses between two  $P^-$  strains of V. cholerae are infertile (Bhaskaran, 1959). Recombination between two strains of V. cholerae, one requiring

valine and isoleucine and the other arginine and purine, was observed by Bhaskaran (1960). The role of the fertility factor P was further elucidated when P<sup>+</sup> strains were examined with the electron microscope. Sex pili were observed on P<sup>+</sup> strains but not on P<sup>-</sup> strains (Bhaskaran, Dyer and Rogers, 1969). Hence genetic recombination between P<sup>+</sup> strains and P<sup>-</sup> strains appears to involve a type of conjugation mechanism.

Another form of episomal recombination, from Salmonella typhosa to V. cholerae, has been reported by Baron and Falkow (1961). These workers transferred the F-Lac episome of S. typhosa to V. cholerae. Once possessing the episome, V. cholerae could act as a donor for the episome in crosses with Salmonella typhimurium as a recipient. Transmission of multiple drug-resistance from an El Tor vibrio to Shigella and Salmonella via conjugation has been reported by Goto and Kuwahara (1969).

Although lysogenicity has been demonstrated in the cholera vibrios (Newman, 1960; Newman and Eisenstark, 1964), there is nothing known about the transmission of genetic information within or between strains of cholera vibrios via transduction.

The present study was undertaken with the following major objectives in mind. First, it was necessary to find a minimal medium for V. cholerae and to develop a method of culturing the organism in the minimal medium for an extended period of time.

The above objective was needed so that well defined physiological genetic experiments could be carried out. Second, enzymes and other characteristics of V. cholerae would be compared for differences in their phenotypic expression when the bacteria were grown in complete versus minimal media. Third, if the phenotypic expression of a trait was found to be lacking in minimal medium, an attempt would be made to find the chemical or chemicals needed for production of the trait in minimal medium. If such chemicals were found, an attempt would be made to find if the chemicals worked by induction of genes or if the chemicals were necessary building blocks for a gene product. Fourth, a study of genetic recombination of selected markers within and between strains of V. cholerae would be carried out. Both transduction and conjugation experiments would be utilized.

## MATERIALS AND METHODS

### A. Organisms

The strains of Vibrio cholerae used were maintained on slants of tryptose agar and stored at 4 C. Table I lists the strains and sources of V. cholerae used in this study. The phage used in the transduction experiments were PVc 117 and PVc 301 which are lysogenic in the V. cholerae strains HVc 117 and HVc 301 respectively.

TABLE I. STRAINS AND SOURCES OF VIBRIO CHOLERAЕ.

Laboratory designation	Source
HVc 41	W. Burrows, <u>V. comma</u> , C-281
HVc 49	W. Burrows, <u>V. comma</u> , C-291
HVc 250	Y. Watanabe, <u>V. cholerae</u> , type El Tor, 17
HVc 300	ATCC, <u>V. comma</u> , 14033, El Tor
HVc 301	ATCC, <u>V. comma</u> , 14734, El Tor
HVc 307	Y. Watanabe, <u>V. cholerae</u> , type El Tor, 86
HVc 322	S. Mukerjee, <u>V. cholerae</u> , non-agglutinable
HVc 323	S. Mukerjee, <u>V. cholerae</u> , non-agglutinable
HVc 330	S. Mukerjee, <u>V. cholerae</u> , El Tor

B. Media

Heart infusion broth (Difco) was used for complete medium. Minimal medium was tris-glucose broth described by A. D. Hershey (1955). Table II gives the composition of the tris-glucose broth (TG broth). Phenol red (5 mg) was added to each liter of minimal broth as a pH indicator. Certain strains of V. cholerae required the addition of adenosine 5' monophosphoric acid to the minimal medium (see Results). Minimal broth without glucose was autoclaved at 121 C for 20 minutes. After the minimal broth cooled, 1 ml of a .20% glucose solution, which had been filter sterilized through an O3 Selas filter, was added to each 100 ml of minimal broth. In certain experiments, additional chemicals were added to the minimal medium. After addition of some chemicals, the pH of the medium had to be readjusted to 7.4 with 2.5 M NaOH. The new medium was sterilized by autoclaving at 121 C for 20 minutes or, if the added chemicals were heat labile, filtering the medium through a O3 Selas filter. In the transduction experiments tryptose agar (Difco) was used as the base agar and semisolid heart infusion agar (BBL) was used for the overlay. Tryptose agar (Difco) with various antibiotics added was used in both transduction and conjugation experiments.

TABLE II. COMPOSITION IN GRAMS PER LITER OF TG BROTH.

---

Glucose	2.0
NaCl	5.4
KCl	3.0
NH <sub>4</sub> Cl	1.1
CaCl <sub>2</sub>	0.011
MgCl <sub>2</sub>	0.095
KH <sub>2</sub> PO <sub>4</sub>	0.087
Na <sub>2</sub> SO <sub>4</sub>	0.023
FeCl <sub>3</sub>	0.00016
*Tris buffer	12.1

Add one liter of double distilled water and enough concentrated HCl to adjust the broth to a pH of 7.4 at 21 C.

---

\*trishydroxymethylaminomethane (Sigma Chemical Co., St. Louis, Missouri)

### C. Cultures

Broth cultures were grown in 250 ml quantities in 500 ml Erlenmyer flasks and incubated on a shaker at 37 C. In one experiment, broth cultures were incubated at 25 C on a shaker. The inoculum was taken either directly from a tryptose agar slant or from a turbid suspension of the bacteria grown in 50 ml of minimal. Minimal medium cultures required an occasional pH adjustment after about 8 hours of incubation (see Results). In conjugation experiments cultures were incubated without shaking to prevent the breaking apart of any possible mating pairs.

### D. Assay for hemolysin

Hemolysin produced by the hemolytic vibrios was assayed by the spot plate method (Kalsow, Newman and Rutherford, 1967). This technique involved filtering culture fluid through a 03 Sela filter (approximately 0.25 micron pore size), and spotting one drop of filtrate on Petri plates containing 10 ml of 3% suspension of goat red blood cells (Colorado Serum Company) in tris-salt agar (Table III). The plates were incubated at 37 C overnight and a zone of lysis was observed if hemolysin was present. The hemolysin in the filtrate could be quantitated by making two-fold dilutions of the filtrate in tris buffered salt solution (Table III) and spotting

a drop of each dilution. The titer of hemolysin was taken as the highest dilution showing a zone of lysis on the goat blood plate after incubation.

The hemolysin of the non-agglutinable vibrio HVC 323 is not filterable through an 03 Selas filter. Hence, cultures of this organism were centrifuged for 20 minutes at 15000 rpm in a Sorvall SS-3 centrifuge using a SS-34 head. Supernatant fluid was treated with 0.5 ml of 2500 µg/ml stock chloramphenicol and incubated for two hours at 37 C to kill any residual bacteria. After incubation the supernatant fluid was spotted in the manner described above.

TABLE III. COMPOSITION IN GRAMS PER LITER OF TRIS BUFFERED SALT SOLUTION AND AGAR.

---

NaCl	8.0
Na <sub>2</sub> HPO <sub>4</sub>	0.1
KCl	2:23
Glucose	1.0
*Tris buffer	1.0

Add one liter of double distilled water and enough concentrated HCl to adjust the broth to a pH of 7.4 at 21 C.

For tris agar add 15 grams of Bacto-agar (Difco).

---

\*trishydroxymethylaminomethane (Sigma Chemical Co., St. Louis, Missouri)

#### E. Assay for hemolysin destructive factor

Hemolysin destructive factor (HDF) was assayed as follows. One ml of filtrate from El Tor strain Hvc 300, known to contain hemolysin, was mixed in tube A with one ml of culture filtrate suspected to contain HDF. A control tube B was made by mixing one ml of the filtrate containing hemolysin with a ml of tris buffered salt solution. The two tubes were incubated in a water bath at 37 C for two hours, and after incubation a series of two-fold dilutions in tris buffered salt solution were made for each tube. Each series of dilutions were spotted on Petri plates containing 3% goat red blood cells suspended in tris-salt agar and incubated overnight. If tube A contained HDF, the resulting titer of hemolysin from that tube was not as high as that of the control tube B as measured by the spot plate technique (see previous section).

#### F. Assay for protease

Protease was assayed on Petri plates of milk-tryptose agar. The plates were made by mixing one ml of powdered, defatted milk to each two ml of melted tryptose agar. The plates were then poured and allowed to solidify. Culture fluid thought to contain protease was filtered and a drop of the filtrate was spotted on the milk-tryptose agar plates. The plates were incubated overnight at 37 C, and if protease was present, a zone of clearing was observed.

#### G. The agglutination test

Serological typing of cholera vibrios was done by the slide agglutination test (Humphrey and White, 1964). The antisera used were obtained from Difco and were of the Ogawa, Inaba and Hikojima types. These antisera react with major somatic antigens found in both the "classical" and El Tor vibrios. A drop of saline and a drop of antiserum were placed on a microscope slide. Care was taken that the drops did not intermix. A loopful of bacterial cells from a centrifuged, log phase culture was added to each of the two drops. The slide was gently rocked over a light source. In this test if the bacteria are of the type that the antiserum was specific for, clumping is observed within two minutes in the drop of antiserum but not in the drop of saline. If agglutination occurs in both drops the test is invalid. If clumping does occur in either drop the bacteria are not of the type that the antiserum was specific for or the antiserum is not good.

#### H. Preparation of phage stocks

For the transduction experiments phage stocks were made by the overlay technique. Usually about 40 tryptose agar Petri plates were poured so that each plate contained at least 30 ml of medium. The plates were used within a few hours after pouring so that they

would not be too dry. Three ml of melted overlay agar (semi-solid heart infusion agar) were placed into each of several tubes and the tubes were placed in a 45 C water bath. A log phase culture of donor bacteria, containing about  $10^8$  to  $10^9$  bacteria per ml, was used as the host for the phage. A phage dilution was prepared from a previously titered phage stock so that each Petri plate would have about  $10^4$  to  $10^5$  plaques per plate after incubation. After the tubes containing the overlay agar cooled to the temperature of the water bath, 0.5 ml of donor bacteria and one ml of the phage dilution were added to each of five tubes. Only five tubes were done at a time to prevent heat killing of the bacteria and virus. Each tube was mixed and poured on to a tryptose agar plate. The overlay agar was allowed to solidify and the plates were incubated for about eight hours at 37 C.

After incubation the phage were eluted from the plates by adding three ml of heart infusion broth to each plate and eluting for four hours at room temperature or two hours at 37 C. The broth containing the phage was then poured off each plate and pooled. In some experiments, in an unsuccessful attempt to increase the titer, rather than elute the phage, the overlay from each plate was scraped into a Servall omni-mixer (Sorvall). The overlay was broken up by turning the mixer on for three consecutive five second periods. After mixing, the agar was separated by centrifuging the

the material in a Sorvall SS-3 centrifuge at 9000 rpm for 20 minutes using a GSA head. The supernatant containing the phage was poured off and pooled.

To eliminate donor bacteria from the phage lysate, the lysate can be filter sterilized through an O3 Sela filter or treated with chloroform. Because of the possibility that the phage could be filtered out or that it might be chloroform sensitive, both methods were used to sterilize the lysate in order to find if one method would give a higher phage titer than the other. Chloroform treatment gave a 10 fold higher titer of phage than the filtration method. After sterilization, the lysate was tested for viable bacterial cells and stored at 4 C.

#### I. Phage titer procedure

Phage stocks were titered by making 100-fold dilutions of the phage in heart infusion broth. A half ml of the appropriate indicator bacteria from a log phase culture was added to three ml of overlay agar at 45 C. One-tenth ml of a given phage dilution was added to the tube, mixed and plated in duplicate on a base of tryptose agar. The overlay was allowed to solidify and the plates were incubated overnight at 37C. After incubation the plates were examined and the plaques counted.

J. Antibiotic sensitivity test

The strain of bacteria to be tested was inoculated into heart infusion broth and grown to log phase at 37 C. One-tenth ml of the culture was spread on to a tryptose agar plate. The following antibiotic sensitivity disks (Difco) were placed aseptically on to the plate: aureomycin 30  $\mu$ g, chloramphenicol 30  $\mu$ g, dihydrostreptomycin 10  $\mu$ g, erythromycin 15  $\mu$ g, lincomycin 2  $\mu$ g, nalidixic acid 30  $\mu$ g, neomycin 30  $\mu$ g, penicillin 10 units, polymyxin B 300 units, tetracycline 30  $\mu$ g, and triple sulfa 300  $\mu$ g. The plate was incubated overnight at 37 C and examined for bacterial growth. Bacteria were considered sensitive if a clear zone was observed around the antibiotic disk.

## RESULTS

### A. The growth of cholera vibrios in minimal medium.

#### 1. Nutritional requirements.

To study the physiological genetics of an organism a defined growth medium is needed. Furthermore, to study the constitutive or inducible nature of enzymes produced by an organism, it is desirable to find a medium containing only the chemicals needed for growth. Hence, experiments were carried out to find the minimal growth requirements for the cholera vibrios.

All V. cholerae strains used grew to a concentration of  $1 \times 10^9$  to  $1 \times 10^{10}$  bacteria/ml within 4 to 8 hours when incubated on a shaker in complete medium (heart infusion broth) at 37 C. The number of bacteria was determined by viable cell counts. Cholera strains HVc 41, HVc 49, HVc 301 and HVc 307 also grew well in minimal medium (tris-glucose broth) even after they had been subcultured three times in minimal medium to eliminate contaminating nutrients which may have been introduced into the minimal medium via the original inoculum.

V. cholerae strains HVc 300, HVc 322 and HVc 323 did not grow in tris-glucose medium. In a first step to identify the required growth factor or factors, all strains were inoculated into minimal medium containing tissue culture concentrations of one of the following: amino acids (Eagle tissue culture concentrate, Hyland Laboratories), vitamins (Eagle tissue culture concentrate, BBL) or

purines and pyrimidines. All grew only in the minimal medium with purines and pyrimidines added. The three strains of bacteria were then tested for their ability to grow in minimal medium plus one of the following: adenine, guanine, thymine, cytosine or uracil. Only the purines, adenine or guanine, promoted growth. However, the growth obtained was slow and the final concentration of bacteria was only  $1 \times 10^7$ /ml after 36 hours of incubation at 37 C. The addition of both amino acids and vitamins to minimal medium with purines failed to enhance cell growth beyond that obtained in minimal medium containing only adenine or guanine. Tris-glucose medium containing the nucleoside, adenosine or guanosine, or the nucleotide, adenylic acid or guanylic acid, gave better cell growth, as measured by the time required for the cultures to become acid, than tris-glucose medium containing the purine base adenine or guanine. The best cell growth was obtained in tris-glucose medium with adenosine 5' monophosphoric acid (AMP) added at a concentration of 1 mg per 100 ml of tris-glucose medium. All three strains grew to a concentration of  $1 \times 10^9$  bacteria/ml in this medium within 12 hours at 37 C provided the pH was adjusted (see below). Consequently, tris-glucose medium with AMP added was used as a minimal medium for the above three strains of bacteria.

## 2. The effect of pH.

Both uninoculated minimal and complete media have a pH of about 7.4, but the buffering capacity of minimal medium was not as great as that of complete medium. As can be seen in Figure 1, there is a precipitous decrease in the pH of a culture grown in minimal medium after 12 hours, whereas in complete medium the pH was slightly higher than 7.4. It has long been known that alkalinity favors the multiplication of the cholera vibrios and a pH of 6.0 is about the lower limit for the growth of the organism (Felsenfeld, 1967). In order to detect the rapid decrease in pH of the cholera vibrios grown in minimal medium, a pH indicator, phenol red, was added to the medium. The pH was adjusted with 2.5 M NaOH whenever the medium became slightly orange in color. This technique was successful in extending both the log phase and the stationary phase of the cholera vibrios grown in minimal medium (Figure 2). By use of the above technique, the cholera vibrios could be grown for 16 hours in minimal medium in a comparable number to growth in complete medium. After 16 hours additional glucose as well as pH adjustment was necessary to extend the stationary phase in minimal medium.

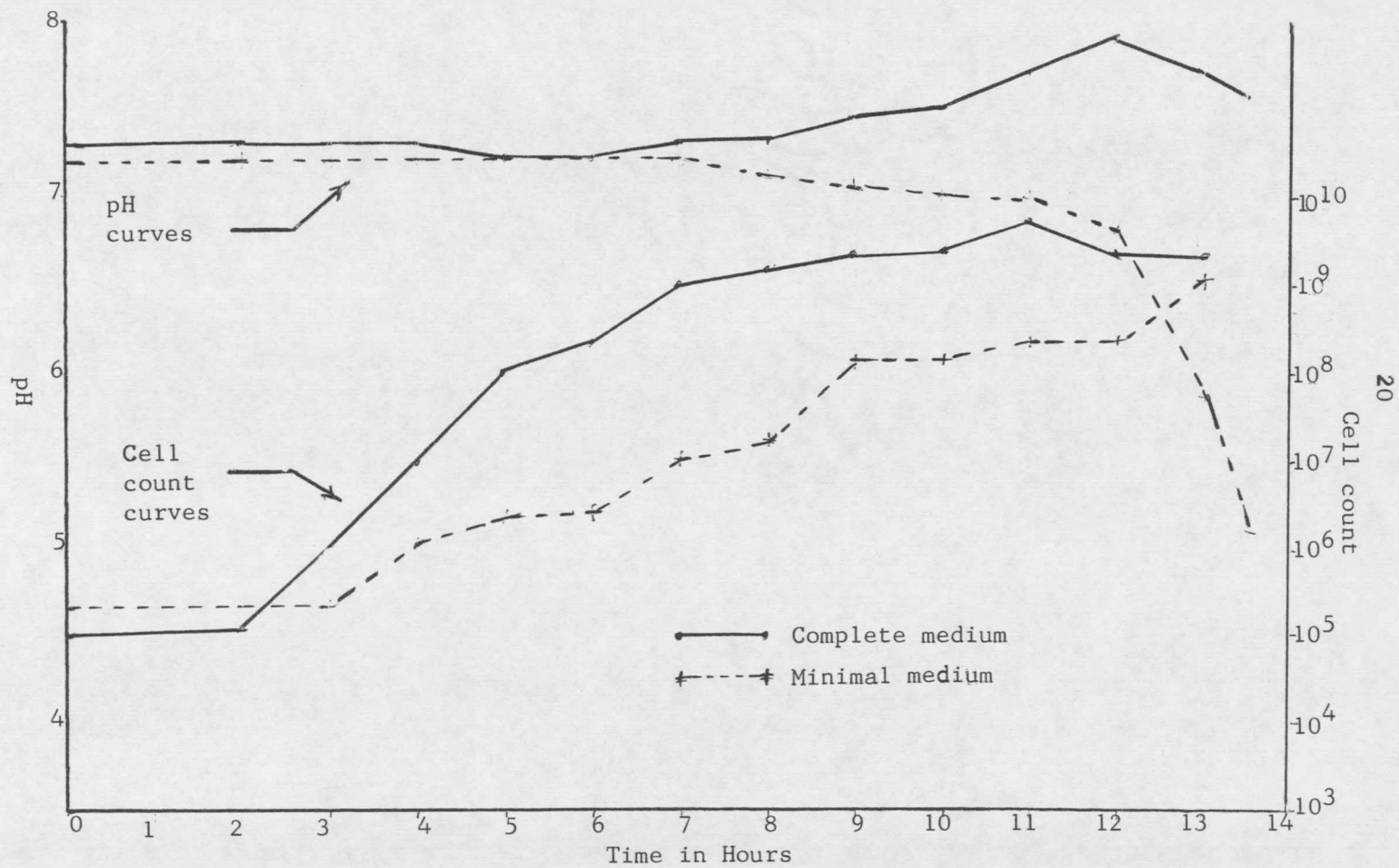


Figure 1. The pH and cell count with respect to time of HVC 49 grown in complete and minimal media.

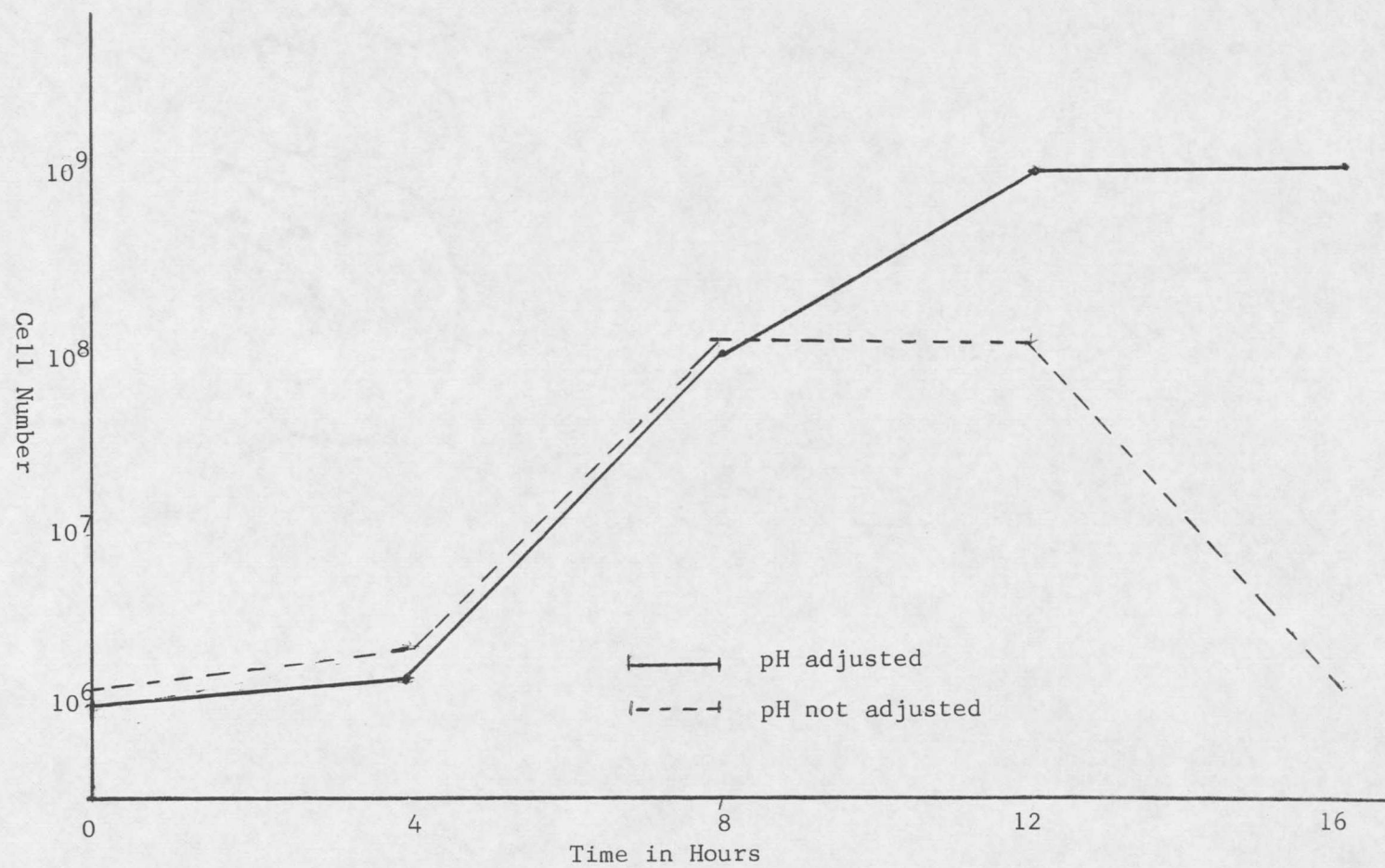


Figure 2. Cell number with respect to time of Hvc 301 grown in minimal medium with and without the pH adjusted.

B. The effects of growth in minimal medium on various enzymes and characteristics of the cholera vibrios.

Experiments were carried out to detect differences in the phenotypic expression of certain enzymes and characteristics of V. cholerae grown in complete versus minimal media. The rationale was to find if certain enzymes or enzyme systems were constitutive. If enzymes were found not to be constitutive, experiments were conducted to determine what chemicals were needed for their induction and/or production.

1. Studies on hemolysin.

a. Hemolysin production by the El Tor vibrios cultured in complete medium versus minimal medium.

Hemolysin production was studied because of the ease and sensitivity of detecting it by the spot plate method (Kalsow, Newman, and Rutherford, 1967). The El Tor vibrios may be subdivided on the basis of hemolysin production in complete medium. Many of the El Tor strains produce hemolysin in increasing amounts until a peak titer is reached and then, as the culture incubates, the hemolytic activity disappears due to the production of hemolysin destructive factor (HDF). The above description is characteristic of the El Tor strains HVc 250, HVc 301, HVc 307 and HVc 324. In contrast, El Tor strain HVc 300 does not produce HDF and a high hemolysin titer is reached which does not disappear as the culture

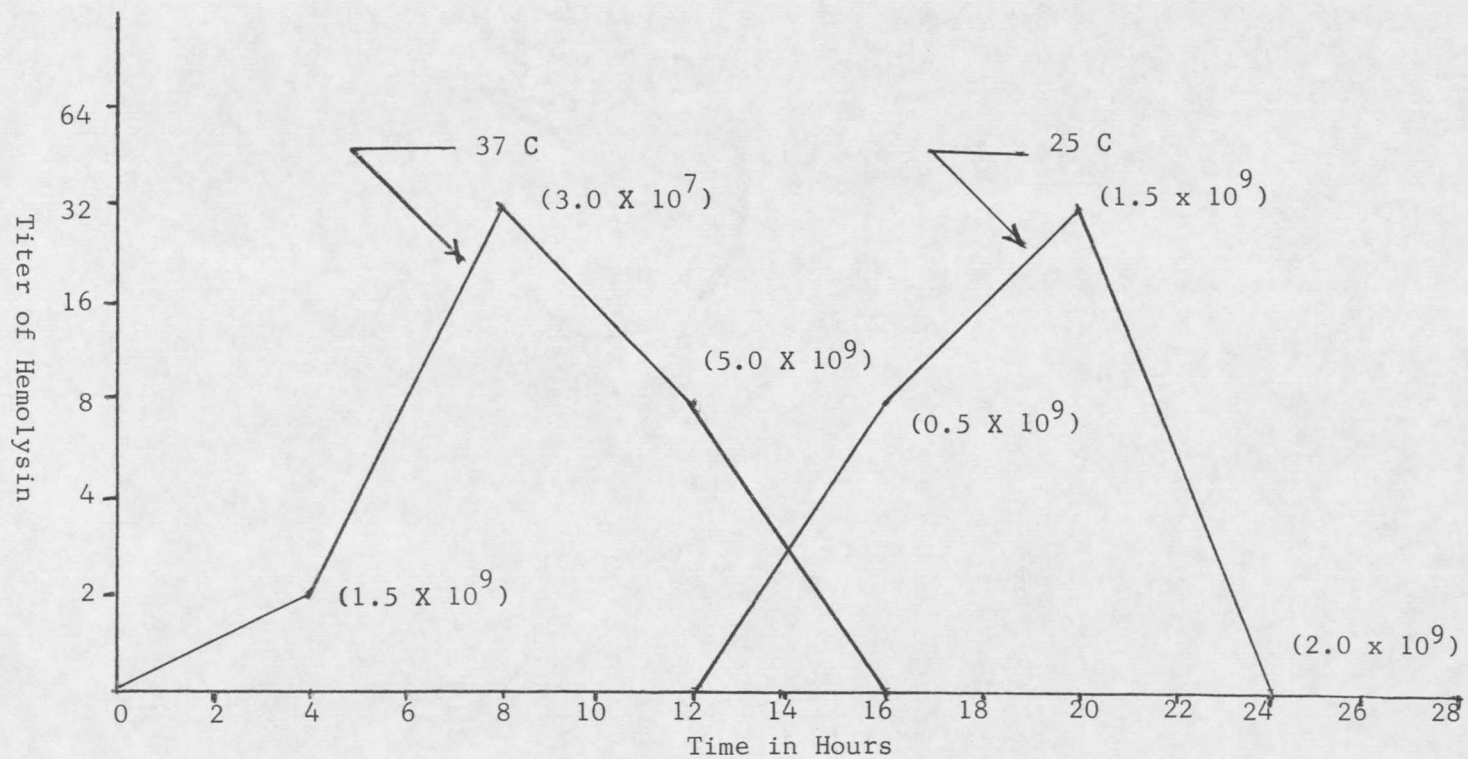
incubates. Finally, the El Tor strain HVc 330 does not produce hemolysin in complete medium.

The appearance of hemolysin in complete medium by the hemolytic El Tor vibrios is affected by the cultural conditions. Figure 3 shows that both hemolysin and HDF are later in appearing in a culture of HVc 301 grown at 25 C than at 37 C. Of course, this is to be expected due to the slower growth of the organism at 25 C.

Hemolysin was not detected when the hemolytic El Tor vibrios were cultured in minimal medium at 37 C. The cell numbers of the minimal medium cultures were comparable to those of the complete medium culture controls. El Tor strains HVc 300 and HVc 301 were carried in minimal medium at 37 C for up to 40 hours and at no time was hemolysin demonstrable. Both of these strains show high titers of hemolysin within 8 hours when grown in complete medium at 37 C.

- b. Hemolysin production by the hemolytic El Tor vibrios in minimal medium with sodium cholate added.

In an attempt to identify the factor necessary for hemolysin production, several chemicals were added to flasks of minimal medium and inoculated with the El Tor vibrio HVc 301. The following chemicals gave negative results: amino acids (Eagle tissue culture concentrate, Hyland Laboratories), vitamins (Eagle tissue culture concentrate, BBL), purines (adenine and guanine), pyrimidines



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Figure 3. Hemolysin production with respect to time by HVC 301 grown in complete medium at 25 C and 37 C. Cell numbers are given in parentheses.

(thymine, cytosine and uracil), lecithin, cholesterol and short chained saturated fatty acids. A positive result was obtained when HVc 301 was inoculated into a flask of minimal medium containing sodium choleate (NBCo, Cleveland, Ohio). Sodium choleate is a crude Ox-bile extract which contains sodium cholate as well as the sodium salts of taurocholic, glycocholic and desoxycholic acids. The individual bile salts were obtained from Sigma Chemical Company and added to flasks of minimal medium. Each of the bile salts was capable of stimulating hemolysin production by HVc 301 when added to minimal medium. However, hemolysin production by HVc 301 in minimal medium with taurocholic acid was very poor. Uninoculated minimal medium, containing the same concentration of bile salts, did not cause any lysis on goat blood plate controls.

A concentration of 0.1 gram of sodium cholate (99% chromatographic purity) per 100 ml of minimal medium (2.3 mM) was sufficient to stimulate hemolysin production. A concentration of 0.23 mM would not stimulate hemolysin production, and 23 mM caused some inhibition of cell growth although hemolysin was produced. High concentrations of sodium cholate (230 mM) in uninoculated minimal medium caused the spontaneous lysis of goat red blood cells. Hemolysin production was not maintained by the cholera vibrios when they were transferred from minimal medium

containing 2.3 mM sodium cholate to a flask of minimal medium containing a 10 fold lower concentration of sodium cholate.

All of the hemolytic El Tor strains produced hemolysin in minimal medium with 2.3 mM of sodium cholate added. The El Tor strains HVc 301 and HVc 307 were subcultured 20 times in minimal medium and then inoculated into a flask of minimal medium with 2.3 mM sodium cholate added. Hemolysin was produced indicating that there was no selection for a mutant nonhemolytic El Tor after several passages in minimal medium.

c. Hemolysin production by the non-agglutinable vibrios.

Both of the non-agglutinable vibrio strains HVc 322 and HVc 323 produced hemolysin when grown 12 hours at 37 C in complete medium. However, the hemolysin produced by HVc 323 is not filterable through an O3 Sela filter although it is present in the supernatant fluid of a centrifuged culture. Like the El Tor vibrios, the non-agglutinable vibrios did not produce hemolysin in minimal medium. The non-agglutinable vibrios did produce hemolysin in minimal medium with 2.3 mM sodium cholate added. As in complete medium, the hemolysin produced by HVc 323 in minimal medium with 2.3 mM sodium cholate added was not filterable through an O3 Sela filter. The nonfilterable nature of the hemolysin of HVc 323 represents a genetic difference of this organism from that of the El Tor strains. The El Tor strains produce a filterable hemolysin.

- d. Discovery of a cell associated hemolysin in the El Tor vibrio HVc 301.

The following technique was developed to obviate the necessity of filtering culture fluid to check for hemolysin production by the El Tor vibrios. The El Tor vibrio strain HVc 301 was grown in tris-glucose broth and a loopful of the culture was streaked on tris-glucose agar plates and on heart infusion agar plates. The plates were incubated overnight and the resulting colonies were killed with chloroform vapor. The plates were then overlaid with 5 ml of a 3% suspension of goat red blood cells in semisolid agar. Zones of hemolysis were observed within 30 minutes after overlaying on both the complete and minimal agar plates. This observation was not expected as hemolysin was never found to be produced in liquid minimal medium.

It was possible that the Bacto agar (Difco) used as a solidifying agent was contaminated with nutrients which caused the production of hemolysin on the minimal agar plates. Hence the following more purified agars were used as solidifying agents: Purified agar (Difco), Ionagar #2 (Colab Laboratories Inc.) and Agarose (Marine Colloid, Inc.). Experiments repeated on minimal plates using the above agars all gave positive results for zones of hemolysis around the bacterial colonies. Positive results were also obtained when an inorganic silica gel colloid (Dupont, Ludox) was used to solidify the minimal

medium. The latter experiment appeared to rule out the possibility that contaminating nutrients in the solidifying agent were causing the production of hemolysin.

An experiment was then designed to determine if there was a cell associated hemolysin. V. cholerae strain HVc 301 was cultured in complete and minimal media for 10 hours at 37 C on a shaker. An aliquot of fluid from each culture was filtered through an O3 Selas filter and the filtrate was tested for hemolysin. The remainder of each culture was treated with chloroform to kill the bacteria and the culture fluid was centrifuged at 7000 rpm (Sorvall GSA head) for 30 minutes. Supernatant fluid and cells were spotted on goat blood plates. The chloroform treated cells were streaked on to complete agar plates to check for death of the bacteria. No growth occurred. The results of the experiment are presented in Table IV.

It can be seen from Table IV that HVc 301 cells possess hemolytic activity regardless of whether they are cultured in complete or minimal media. This is in contrast to the hemolytic activity present in the filtrates and supernates of HVc 301 grown in complete or minimal media. It appears that V. cholerae strain HVc 301 produces two hemolysins. One is filterable, not cell associated and stimulated by sodium cholate for its production or release, while the other hemolysin is not filterable, cell associated and not dependent on sodium cholate for its production.

TABLE IV. THE PRESENCE OR ABSENCE OF HEMOLYTIC ACTIVITY IN THE FILTRATE, SUPERNATE OR CELLS OF V. CHOLERAE STRAIN Hvc 301 GROWN IN COMPLETE OR MINIMAL MEDIA.

Type of Medium Used to Culture Bacteria	Culture Material Tested	Chloroform Treatment	Hemolysis + or -
Complete	Filtrate	No	+
Complete	Supernate	Yes	+
Complete	Cells	Yes	+
Minimal	Filtrate	No	-
Minimal	Supernate	Yes	-
Minimal	Cells	Yes	+

2. Studies on other enzymes and properties of V. cholerae.

a. Growth characteristics of the cholera vibrios cultured in minimal medium versus complete medium.

A culture of V. cholerae grown in minimal medium has a 1 to 2 hour longer lag phase than a culture grown in complete medium (Figure 1). Microscopic examination suggests that cholera vibrios grown in minimal medium are slightly larger and that there is a greater tendency for chain formation than is seen when the organisms are grown in complete medium.

- b. The production of hemolysin destructive factor (HDF) by the El Tor vibrio Hvc 301 in complete medium versus minimal medium containing 2.3 mM sodium cholate.

The hemolysin produced by Hvc 301 cultured in complete medium was destroyed by HDF after 16 hours of growth at 37 C (Figure 3). In contrast, Hvc 301 cultured in minimal medium containing 2.3 mM sodium cholate produced a hemolysin titer which was found to remain high for as long as 36 hours on incubation at 37 C (Figure 4). Filtrates of the above 36 hour cultures contained no detectable HDF. It was concluded that the production of HDF was delayed or that HDF was not produced at all in minimal medium containing 2.3 mM sodium cholate. The organism did produce HDF after 20 hours at 37 C when cultured in tissue culture medium 199 (Difco) with 2.3 mM sodium cholate added. It was then attempted to further define the chemicals necessary for HDF production. Amino acids (Eagle tissue culture concentrate, Hyland Laboratories), vitamins (Eagle tissue culture concentrate, BBL) or purines and pyrimidines failed to stimulate HDF production in minimal medium with 2.3 mM sodium cholate added.

- c. The production of protease by the cholera vibrio strains Hvc 41 and Hvc 301 in complete medium versus minimal medium.

V. cholerae strains Hvc 41 and Hvc 301 produce protease in complete medium. The enzyme is not present in the filtrate of a 20 hour minimal medium culture of either of the above strains.

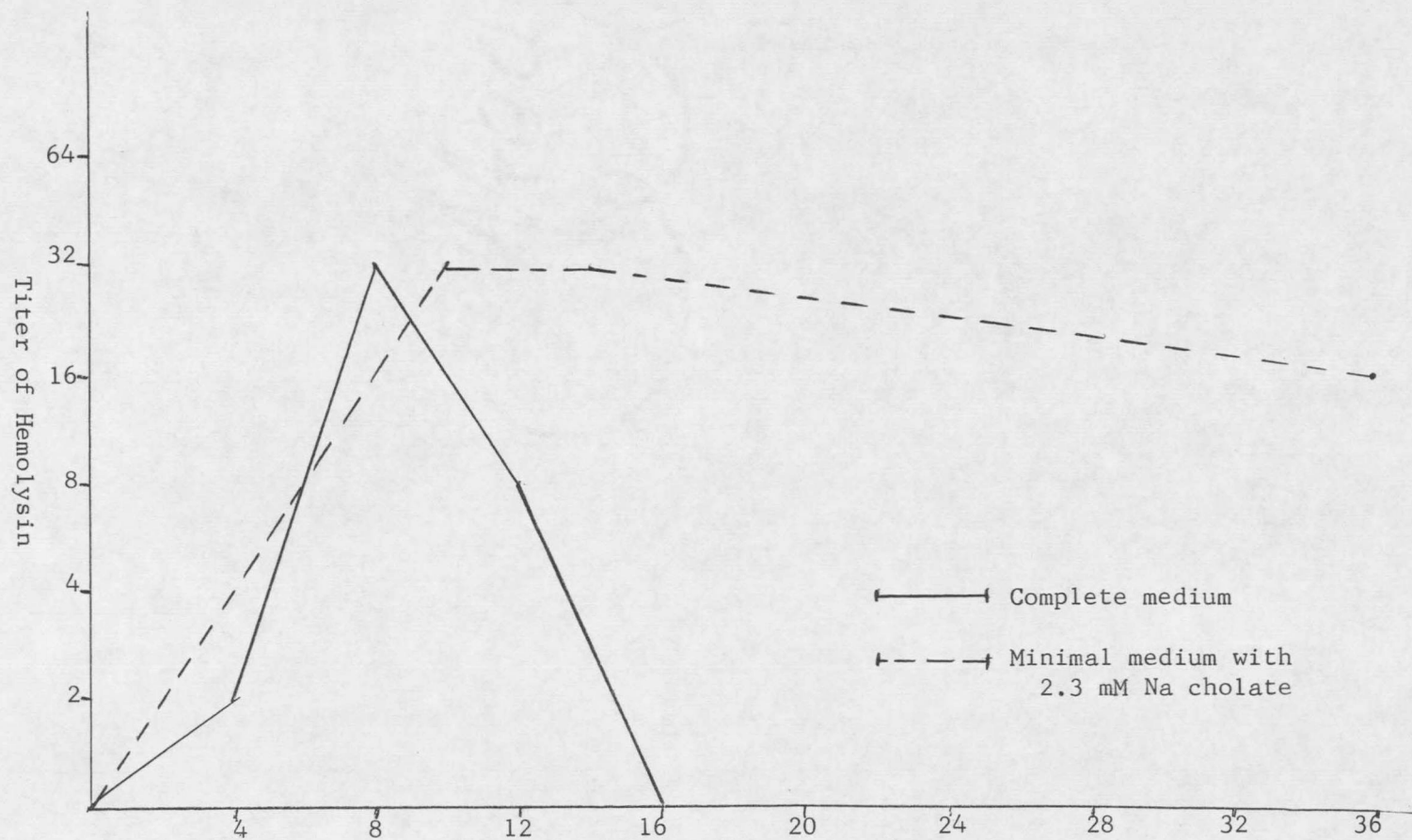


Figure 4. Hemolysin production with respect to time by HVC 301 grown in complete and minimal media at 37 C. The minimal medium contained 2.3 mM Na cholate.

Nor was protease produced by the above strains when inoculated into minimal medium containing tissue culture concentrations of one of the following: amino acids (Eagle tissue culture concentrate, Hyland Laboratories), vitamins (Eagle tissue culture concentrate, BBL) or purines and pyrimidines. Negative results were also obtained in minimal medium containing 2.3 mM sodium cholate and minimal medium containing crystalline bovine albumin (Sigma Chemical Co.) at a concentration of 0.1 g of protein per 100 ml of minimal.

- d. Antigenic characteristics of certain strains of cholera vibrios cultured in complete medium versus minimal medium.

It is known that environmental changes can sometimes affect the phenotypic expression of antigenic cell surface structures (Braun, 1965). Both "classical" and El Tor vibrios belong to the O-1 serologic group (Felsenfeld, 1967). This group consists of organisms containing one or both of two major somatic antigens. Cholera vibrio strains containing only one of the major somatic antigens are either of the Ogawa type or the Inaba type. Those strains containing both major somatic antigens are of the Hikojima type. Three Hikojima type strains of cholera vibrios, HVc 41, HVc 250 and HVc 301, were checked for their serologic type when grown in minimal medium. As when grown in complete medium, strains HVc 41 and HVc 250 agglutinated with Ogawa, Inaba and

Hikojima antisera when grown in minimal medium. Thus, the minimal medium environment did not alter the major somatic antigens of the above two strains. The El Tor strain Hvc 301 produced a granular growth in minimal medium and agglutinated spontaneously in the saline control. Consequently, the serologic type of Hvc 301 grown in minimal medium could not be ascertained by the agglutination test.

- e. The motility of certain strains of cholera vibrios cultured in minimal medium.

Flagellated species of bacteria can display environmentally induced phenotypic modifications with respect to motility (Braun, 1965). Hence, it was of interest to check the motility of the cholera vibrios grown in minimal medium. The cholera vibrios have a single polar flagellum and are actively motile organisms. V. cholerae strains Hvc 41, Hvc 250 and Hvc 301 were grown in minimal medium. A wet mount of each culture was made and examined microscopically. In each case motility was observed including one strain, Hvc 301, which had been subcultured 22 times in minimal medium. It was concluded that the phenotypic expression of the genes controlling motility was not affected by the minimal medium.

- f. A comparison of phage growth on a cholera vibrio host grown on complete agar plates versus minimal agar plates.

V. cholerae strain HVc 41 was grown to log phase in complete medium. The culture was centrifuged at 15000 rpm for 10 minutes in a Sorvall SS-3 centrifuge using a type SS-34 head and the supernatant was discarded. The cell pellet was washed several times with tris buffered saline, resuspended in minimal medium and used as inoculum for the phage host bacterial lawn. A phage stock of PVc 301 was diluted 100 fold in minimal medium and 0.1 ml of that was taken as a  $10^{-1}$  dilution of phage in minimal medium. Further dilutions of the phage were made in minimal medium. The phage was titered on a lawn of HVc 41 grown on complete medium agar plates and minimal medium agar plates. The resultant titer of phage was  $12 \times 10^7$  on the complete medium plates versus  $7 \times 10^7$  on the minimal medium plates. The plaques on the minimal plates were smaller but this was probably due to the medium which became too acid and killed the host bacteria which, of course, inhibited further phage growth. Thus, the phage PVc 301 was able to replicate itself with almost the same efficiency in V. cholerae HVc 41 grown under the two differing environmental conditions.

- g. Some miscellaneous observations on the inhibition of the growth of the cholera vibrio HVc 301 by ascorbic acid and certain fatty acids.

In the course of finding what chemicals might stimulate hemolysin production in minimal medium, it was noted that certain chemicals inhibited the growth of V. cholerae strain HVc 301. Ascorbic acid (Sigma Chemical Co.) delayed cell growth in minimal medium at a concentration as low as 0.005 mg of ascorbic acid per ml of minimal medium. A higher concentration of ascorbic acid (1 mg per ml of medium) was needed to delay the growth of the organism in complete medium. Increased concentrations of ascorbic acid in the two media led to an increase in the time between inoculation of the cultures and the observation of turbid growth. Growth curves were not run.

Various saturated organic acids (Sigma Chemical Co.) were added to minimal medium at a concentration of 1 ml or 1 mg of acid per 100 ml of medium. The medium was readjusted to a pH of 7.4 with 2.5 M NaOH before inoculation. Growth was obtained in flasks containing one of the first five saturated organic acids, i.e. formic acid through pentanoic acid. Hexanoic, octanoic and decanoic acids prevented cell growth. Growth was observed in flasks of minimal medium containing dodecanoic acid or higher chained fatty acids. However, the solubility of the higher chained saturated fatty acids in minimal medium is slight.

C. Attempts to detect genetic recombination.

1. Transduction within a single strain.

Several attempts were made to transfer dihydrostreptomycin resistance via transduction within a strain of cholera vibrio. It was of interest to make the attempts because there have been no reports of transduction in the cholera vibrios. V. cholerae phage strain Pvc 117 was grown on V. cholerae substrain Hvc 41<sub>r</sub> which was resistant to 100 µg of dihydrostreptomycin but not dihydrostreptomycin dependent. The titer of the phage stock obtained by the overlay technique (see Methods) was  $3 \times 10^{10}$ . A dihydrostreptomycin sensitive substrain of Hvc 41<sub>s</sub> was grown to a concentration of  $5.0 \times 10^8$  viable cells and 1 ml of the culture was added to 9 ml of phage stock or to 9 ml of diluted phage stock. The phage stock was diluted in heart infusion broth in a series of 10 fold dilutions in order to find the best ratio of phage to bacteria for transduction of dihydrostreptomycin resistance. The phage and bacteria were incubated at 37 C together for 2.5 hours and the bacteria were centrifuged at 15000 rpm for 10 minutes in a Sorvall SS-3 centrifuge using a type SS-34 head. The bacteria were resuspended in heart infusion broth and incubated for 12 hours at 37 C. After incubation the cells were centrifuged as described above and the pellet of bacteria was spread on to plates of tryptose agar containing 100 µg of dihydrostreptomycin per ml of agar. The plates were incubated

for 72 hours at 37 C and observed at various intervals for colonies. No transduction of dihydrostreptomycin resistance into a sensitive HVc 41<sub>s</sub> substrain was observed. The experiment was repeated three times with the same result. Three controls were run with each experiment. First, 2.5 ml of undiluted phage stock was added to 50 ml of fresh heart infusion broth and incubated on a shaker at 37 C for 72 hours. Growth was never observed indicating that there were no donor bacteria in the phage stock. Second, the sensitive substrain, HVc 41<sub>s</sub> was grown in heart infusion broth with no phage added and treated as the cultures containing phage. This was used to check for the mutation of the sensitive substrain to dihydrostreptomycin resistance. In one experiment a mutation to resistance did occur. Finally, a few tryptose agar Petri plates containing dihydrostreptomycin were streaked with the resistant substrain. Growth of the resistant substrain was observed in each experiment indicating that the dose of antibiotic in the tryptose agar was not too high.

## 2. Transduction between strains of cholera vibrios.

The rationale for this experiment was to find if transduction would occur using a dihydrostreptomycin resistant El Tor vibrio for the donor strain and a sensitive "classical" vibrio for the recipient strain. If transduction was observed, an attempt would be made to

transfer the hemolysin marker from an El Tor strain to a "classical" strain via transduction,

First, it was desirable to find a strain of phage which would grow in both El Tor and "classical" vibrio strains but not be virulent for the "classical" strain. Of eight phage strains tested, one, Pvc 301, gave temperate growth in both El Tor and "classical" vibrio strains. Phage stocks were made using the overlay technique (see Methods) on the El Tor strain Hvc 250<sub>r</sub> which was resistant to 100 µg of dihydrostreptomycin. Transduction of the antibiotic resistance marker to the sensitive "classical" strain Hvc 41<sub>s</sub> was attempted seven times in the manner described in the preceding section. No transduction was observed. One reason may have been that it was very difficult to obtain high titers of phage stock. In order to raise the titer, the phage was passed several times through the donor bacteria to try to adapt the phage to its host. However, titers above  $1 \times 10^7$  were seldom obtained. Other methods, such as varying the time of phage incubation or varying the phage inoculum on the host bacteria, also failed to raise the titer.

### 3. Conjugation

It was of interest to see if genetic recombination would be observed after incubating an El Tor vibrio strain with a "classical"

vibrio strain. Four El Tor and 27 "classical" vibrio strains were tested for their sensitivity to 11 antibiotics. Antibiotic sensitivity disks were used in the test (see Methods). All the El Tor vibrios tested were resistant to 300 units of polymixin B whereas all the "classical" vibrios tested were not. One of the "classical" vibrios, HVc 49, was resistant to seven antibiotics while the rest were resistant to only two. It was thought that this strain, HVc 49, might contain a resistance transfer factor. Hence it would be a good strain for mediating conjugation. Four El Tor strains and strain HVc 49 were incubated in a shaker at 37 C in 50 ml of heart infusion broth for eight hours. The cultures were diluted with heart infusion broth so they contained about  $5 \times 10^8$  cells per ml. Five ml from each El Tor strain was mixed with 5 ml of HVc 49 in a 25 by 200 mm culture tube and incubated for 4 hours without shaking at 37 C. Control tubes containing 5 ml of each culture separately were made, diluted with 5 ml of heart infusion broth and incubated at 37 C for four hours without shaking. After incubation the cells were centrifuged at 15000 rpm for 10 minutes in a Sorvall SS-3 centrifuge using a type SS-34 head. The cell pellet from each tube was spread on Petri plates of tryptose agar containing 30  $\mu$ g nalidixic acid, 30  $\mu$ g chloramphenicol and 300 units of polymixin B. Strain HVc 49 was resistant to the former two antibiotics and sensitive to polymixin B. The reverse was true

for the El Tor vibrios. The plates were incubated for 24 hours at 37 C and the colonies were counted. No colonies were found on the El Tor control plates. Colonies were present on the plates of the mixed cultures as well as on plates of the HVc 49 control. Hence the colonies probably did not represent recombinants but rather represented mutants of HVc 49 to polymixin B resistance.

## DISCUSSION

Cholera is an infectious disease which still provokes the sensation of panic in many developing countries throughout the world. The control of the high death loss due to cholera is theoretically available, but it cannot be practically implemented due to lack of proper sanitary measures, lack of suitably trained medical personnel, insufficient electrolyte replacement fluids and poor means of transporting patients to treatment centers. Over the past ten years considerable emphasis has been placed on increased research on the cholera vibrio. The goals of this research have been, and are, primarily to develop vaccines which give a high degree of protection with a longer duration of immunity and to improve methods of therapy.

The approach which was used as a theme for the research in this dissertation is to learn more about the basic physiology, biochemistry and genetics of the cholera vibrios. The various metabolic products which are produced by the cholera vibrios may be of significance in the total disease picture. In particular, the toxins, including hemolysin, have been studied because of the probability that the diarrhea of cholera is dependent on one or more of the toxins elaborated by the cholera vibrios during growth in the small intestine.

Most of the V. cholerae strains are not fastidious organisms. The majority of those available to this laboratory are either

prototrophs or purine auxotrophs. Hence, the bacteria and their metabolic products can be studied under chemically defined conditions. This characteristic is of singular importance in the study of the physiological genetics of an organism. Finkelstein and Lankford (1955) devised a chemically defined medium for the growth of some strains of V. cholerae. Bhaskaran and Rowley (1956) reported a purine requirement for certain strains of V. cholerae. Aside from the work conducted in the laboratory of Dr. K. Bhaskaran, little research has dealt with the genetic characteristics of the cholera vibrios.

Of the V. cholerae strains used, some required only glucose and inorganic salts (TG minimal medium) for growth while other strains required the addition of purines to the minimal medium. However, growth of the purine requiring strains was always sparse. Better growth of the purine requiring strains was obtained with adenosine 5' monophosphoric acid which added to the minimal medium for these strains in later experiments.

The primary difficulty in growing V. cholerae in minimal medium was one of pH. V. cholerae is rapidly killed at a pH lower than 6.0. The organism readily ferments glucose to acid under aerobic conditions and a culture in minimal medium dies after only a few hours of incubation. The problem of acidity was avoided by adding phenol red to the minimal medium and adjusting the pH

when necessary with NaOH. When this was done, the growth in minimal medium was comparable to growth in complete medium (heart infusion broth). The availability of a minimal medium in which the growth of the cholera vibrios can be followed for extended periods of time provides a method for the comparative analysis of growth and enzyme synthesis. V. cholerae can be grown in complete medium without pH adjustment. In fact, the pH of complete medium raises as the culture incubates.

Motility, major somatic antigens, and the ability to support phage growth were essentially the same for V. cholerae grown in minimal and complete media. It cannot be assumed that these characteristics would be expressed in both types of media. Members of normally flagellated species such as Salmonella typhimurium or Listeria monocytogenes, can display environmentally induced, temporary phenotypic modifications in the presence, motility and morphology of flagella (Braun, 1965). Since motility was observed in minimal medium, the genes controlling motility and the presence of flagella for V. cholerae were still operative under minimal growth conditions.

Braun (1965) also cites examples of environmental effects that alter other antigenic cell surface structures. Two examples are the destruction of M antigen of streptococci by a protease formed during growth in medium of low oxidation-reduction potential

and the interfering effects of sublethal concentrations of penicillin on the development of certain O antigens (somatic antigens). Somatic antigenic variation was not observed with two strains of V. cholerae grown under minimal conditions. There may have been some antigenic change in a third strain. This strain produced a granular growth in minimal medium and agglutinated spontaneously in saline. In contrast, the growth in complete was not granular and the organism was serologically of the Hikojima type. Because different growth patterns sometimes reflect underlying antigenic changes, the latter strain may have changed antigenically when grown in minimal medium. This was not proved conclusively because of the spontaneous agglutination of the organism in saline. Hence, any agglutination observed in antiserum would be meaningless. In any case, for two V. cholerae strains, the phenotypic expression of the genes controlling the major somatic antigens was not affected by growth in minimal medium.

In view of the fact that V. cholerae grows well in minimal medium, most of the enzyme systems involved in reproduction must be operating at about their normal efficiency. As a means of determining possible alterations in surface structure, the ability of phage to attach and replicate on cells growing in minimal medium was assayed. No difference in the attachment and replication was observed, suggesting that the phage attachment sites were not

altered and that the necessary building blocks for phage protein and nucleic acid were present in the host bacteria.

It was postulated that hemolysin production represented an inducible enzyme system and that this could be demonstrated by growth of V. cholerae under minimal conditions. It was shown that hemolysin was not synthesized in minimal medium or in minimal medium supplemented with amino acids, vitamins or purines and pyrimidines. It was, however, produced in minimal medium when sodium choleate was added. Sodium choleate contains sodium cholate as well as the sodium salts of taurocholic, glycocholic and desoxycholic acids. Each of the individual salts, except taurocholic, stimulated hemolysin by the El Tor vibrios in minimal medium. The hemolysis observed on goat blood plates tested with filtrates from cultures containing taurocholic acid was very slight and may not have been caused by the presence of hemolysin. Sodium cholate was the salt available with the greatest purity (99% chromatographic purity), so most further experimentation was done with it. It should be stressed that 230 mM of sodium cholate in minimal medium will lyse goat red blood cells spontaneously. A concentration of 23 mM sometimes gave a slight zone of lysis. The occurrence of lysis at this concentration probably depended on the condition of the goat blood cells used at the time of the test.

The concentration of sodium cholate (2.3 mM) used to produce hemolysin by the El Tor vibrios in minimal medium never produced hemolysis by itself.

It was of interest to find if sodium cholate worked by the induction of the gene or genes which produce hemolysin, or if the sodium cholate was a necessary building block in the hemolysin molecule. In the beta-galactosidase system it is known that after induction has occurred a 1000 fold lower concentration of an unmetabolizable inducer, too low to initiate induction, can maintain the production of the enzyme (Davis, Dulbecco, Eisen, Ginsberg and Wood, 1967). The inducer in the above case was methyl thio-galactoside. The compound is an inducer but not a substrate for the enzyme. An experiment was run to find if El Tor cells producing hemolysin in minimal medium with 2.3 mM sodium cholate would continue to produce hemolysin at a lower concentration of sodium cholate. The bacteria did not continue to produce hemolysin in minimal medium containing a 10 fold lower concentration of sodium cholate. Therefore, sodium cholate may be a necessary building block in the hemolysin molecule and not an inducer. The fact that lipid accounts for 95% of the molecule supports the building block theory. However, induction cannot be absolutely ruled out because sodium cholate may be both an inducer and building block for the hemolysin molecule. In this case the sodium cholate

would be metabolized to hemolysin and a maintenance concentration for the induction of hemolysin could not be attained.

One might argue that the El Tor vibrios produce bile salts in minimal medium but not at levels which are hemolytic. Then, when the El Tor vibrios were grown in minimal medium with 2.3 mM sodium cholate, the additional bile salt produced by the bacteria would provide a total amount sufficient to render the culture hemolytic. Evidence that this is not the case is provided by the non-agglutinable vibrio HVC 323. This organism produces hemolysin but the hemolysin is not filterable through an 03 Selas filter. The hemolysin produced by HVC 323 grown in minimal medium with 2.3 mM sodium cholate added is also not filterable. If bile salts alone were causing the hemolysis, the latter observation would not occur because the bile salts are filterable through an 03 Selas filter.

The bile salts are metabolic excretion products of cholesterol (Harrow and Mazur, 1958). Hence cholesterol was tested in minimal medium for the ability to stimulate hemolysin production by the El Tor vibrios. No hemolysin was produced. Since the hemolysin molecule is 95% lipid some of the low chained saturated fatty acids were also tested in minimal medium. Again, hemolysin was not produced.

Two other products (HDF and protease) known to be released by certain strains of V. cholerae grown in complete medium, were analysed

for their production in minimal medium. These two substances were not synthesized under minimal conditions. The chemicals needed to stimulate the production of HDF and protease in minimal medium were not found. Protease and HDF were not produced in minimal medium with amino acids, sodium cholate, vitamins or purines and pyrimidines added. HDF was produced in tissue culture medium 199 (Difco) with 2.3 mM sodium cholate added. Medium 199 contains several amino acids, vitamins, purines, pyrimidines and salts. It is probable that some combination of chemical factors are needed for HDF production. Protease was not produced in minimal medium when crystalline bovine albumin was added. Hence the presence of that particular protein was not enough to stimulate protease production. The results may provide the basis for further research on the various metabolic products produced during growth of cholera vibrios. It is postulated that one of the difficulties in demonstrating vibriocine production by the cholera vibrios is due to the production of protease by the organisms. Protease may destroy most of the vibriocines that were released into the growth medium. The growth of vibriocinogenic strains in minimal medium would possibly be a good system for the study of vibriocine production in the absence of protease synthesis.

A cell associated, nonfilterable hemolysin was discovered by virtue of the fact that it was produced in minimal medium. What

relation, if any, the cell associated hemolysin has with the filterable hemolysin is not known. It is possible that the cell associated and the filterable hemolysin are the same and that sodium cholate is needed for the release of the hemolysin from the cell. Since sodium cholate is an emulsifying agent for lipid, and the filterable hemolysin is 95% lipid, it may be that sodium cholate in some way promotes the release of the cell associated hemolysin in to the broth of the culture. Once in the broth, the cell associated hemolysin would become the filterable hemolysin. However, if this were true, all the bile salts should do the same thing. This was not observed in that hemolysin production by the El Tor vibrios grown in minimal medium with sodium taurocholate was extremely slight. Furthermore, the slight hemolysis observed with sodium taurocholate may have been caused by the bile salt because a relatively high concentration was used.

Transduction of genetic markers within or between strains of V. cholerae has never been reported. Several experiments were run to transfer dihydrostreptomycin resistance within or between strains of V. cholerae via transduction. All attempts were unsuccessful. Transduction from an El Tor strain to a "classical" strain was unsuccessful, however, only low titers of phage were obtained from El Tor hosts. Of several different methods, none was found to increase the titer of phage grown on El Tor hosts. Transduction of

dihydrostreptomycin resistance within a "classical" strain of cholera vibrio was also unsuccessful. High titers of phage were obtained from dihydrostreptomycin resistant hosts. The probable number of transducing phage should have been relatively high, however, transduction was not observed. One explanation may be that the phage used is only a specialized transducer and that it cannot pick up the genetic marker for dihydrostreptomycin resistance.

Attempts to transfer genetic markers within and between strains of cholera vibrios via conjugation were also unsuccessful. It is probable that the multiple antibiotic resistant strain of cholera vibrio used did not really carry a resistance transfer factor. Hence, conjugation would not occur.

## SUMMARY

Representative strains of Vibrio cholerae (including "classical", El Tor and non-agglutinable biotypes) were studied. Optimal conditions for growth in minimal medium were determined. Certain strains required adenosine 5' monophosphoric acid, and all strains required repeated adjustments of pH for prolonged growth. Motility, major somatic antigens and the ability to support phage growth were essentially the same in minimal and complete media. On the other hand, hemolysin destructive factor (HDF), and the enzymes hemolysin and protease were not produced in minimal medium by those strains otherwise capable of their production in complete medium. Hemolysin production is elicited by the addition to minimal medium of concentrations of sodium cholate which do not spontaneously lyse cells. A cell-bound hemolysin was discovered by virtue of the fact that it is produced in minimal as well as complete media. Efforts to demonstrate transduction and conjugation were unsuccessful.

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