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
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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 additon 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 additon 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^+ strains were examined with the electron microscope. Sex pili were observed on P^+ strains but not on P^- strains (Bhaskaran, Dyer and Rogers, 1969). Hence genetic recominbation between P^+ strains and P^- 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 CHOLERAE*.

Laboratory designation	Source
HVc 41	W. Burrows, *V. comma*, C-281
HVc 49	W. Burrows, *V. comma*, C-291
HVc 250	Y. Watanabe, *V. cholerae*, type El Tor, 17
HVc 300	ATCC, *V. comma*, 14033, El Tor
HVc 301	ATCC, *V. comma*, 14734, El Tor
HVc 307	Y. Watanabe, *V. cholerae*, type El Tor, 86
HVc 322	S. Mukerjee, *V. cholerae*, non-agglutinable
HVc 323	S. Mukerjee, *V. cholerae*, non-agglutinable
HVc 330	S. Mukerjee, *V. cholerae*, 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 03 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 03 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_4Cl	1.1
$CaCl_2$	0.011
$MgCl_2$	0.095
KH_2PO_4	0.087
Na_2SO_4	0.023
$FeCl_3$	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 Selas 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_2HPO_4	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 03 Selas 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 asceptically on to the plate: aureomycin 30 μg, chloramphenicol 30 μg, dihydrostreptomycin 10 μg, erythromycin 15 μg, lincomycin 2 μg, nalidixic acid 30 μg, neomycin 30 μg, penicillin 10 units, polymyxin B 300 units, tetracycline 30 μg, and triple sulfa 300 μg. 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 X 10^9 to 1 X 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 X 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 X 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.

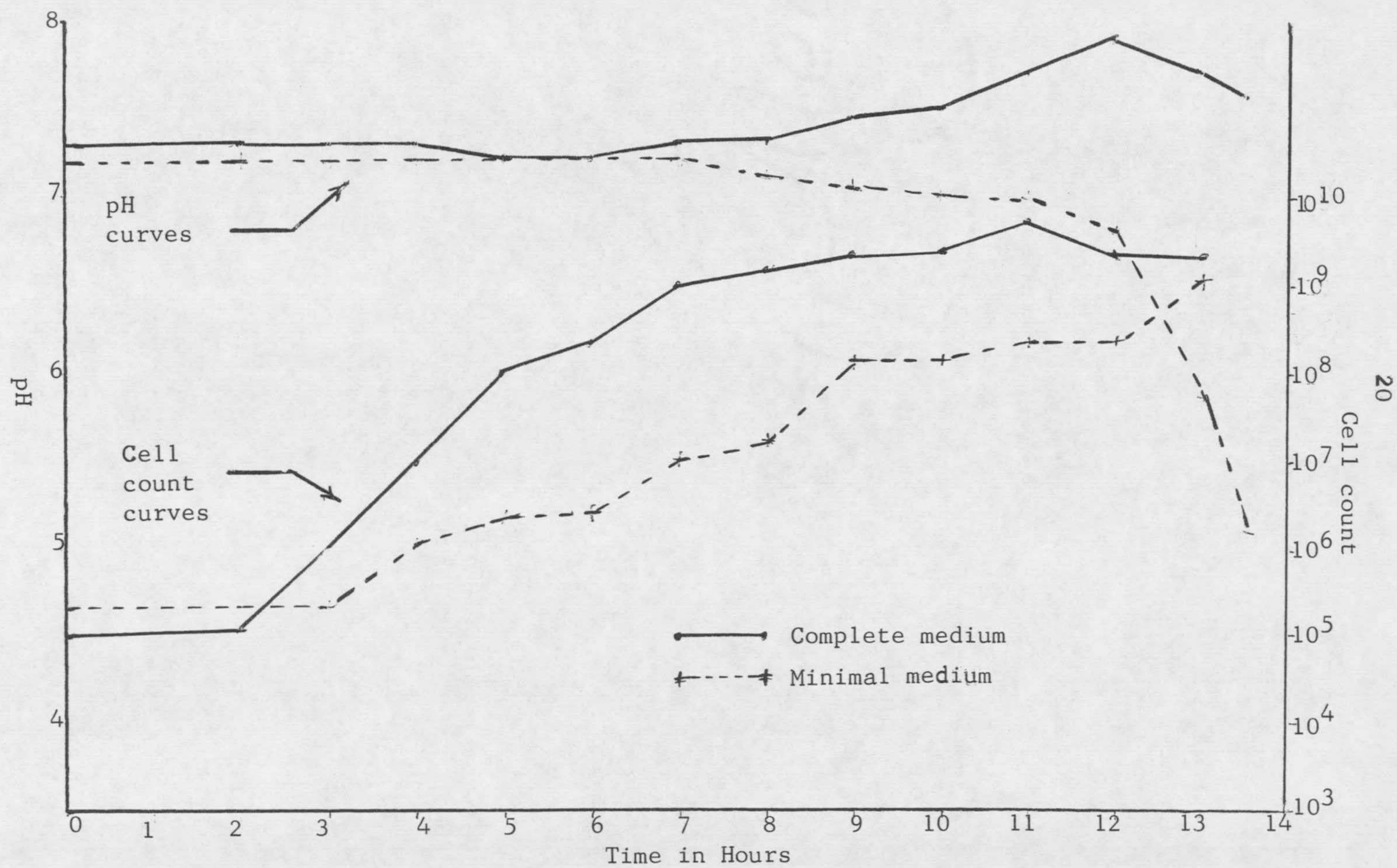

Figure 1. The pH and cell count with respect to time of HVc 49 grown in complete and minimal media.