The microaerophilic nature of Vibrio Fetus
by Lewis K May

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

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A historical review "of the early- work on -the cultivation of Vibrio fetus is presented with special emphasis :being--placed~on the solid media and the conditions used for the cultivation of the organism. In the present investigation, attempts 'were made to cultivate the organism on Difco's brain liver heart semisolid medium which had been modified by the addition of sufficient agar to raise the total agar content to one per cent. ,Since these ,attempts were unsuccessful. this medium was enriched by the addition of 10 per cent sterile-yeast autolysate, 10 per cent sterile horse serum* or 0.2 per cent soluble starch and the organisms were Incubated in an atmosphere of increased carbon dioxide* ,.inconsistent results were obtained with these methods so a search for a more satisfactory medium was undertaken. This search resulted in the selection of thiol medium' which was used as the solid medium in the remainder of the work. Since this medium is also manufactured as a semi solid medium, its cpmposition was altered by the addition of sufficient agar to raise the total agar content to 1.0 per cent. During the subsequent investigations a large number of subsurface colonies were observed on a deep pour plate which had been incubated under atmospheric conditions. This observation was taken as an indication that the organism perhaps preferred a lowered oxygen tension and this indication stimulated the investigation of this possibility. The investigation was conducted by incubating the organism under various atmospheric pressures. From the data obtained in these investigations, it was decided that the atmospheric pressure, as found at the Bozeman altitude of 4,700 ft, should be decreased about 50 per cent in order to have conditions suited to the cultivation of V. fetus as surface colonies.

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ABSTRACT

A historical review of the early work on the cultivation of Vibrio fetus is presented with special emphasis being placed on the solid media and the conditions used for the cultivation of the organism. In the present investigation, attempts were made to cultivate the organism on Difco's brain liver heart semisolid medium which had been modified by the addition of sufficient agar to raise the total agar content to one per cent. Since these attempts were unsuccessful, this medium was enriched by the addition of 10 per cent sterile yeast autolysate, 10 per cent sterile horse serum, or 0.2 per cent soluble starch and the organisms were incubated in an atmosphere of increased carbon dioxide. Inconsistent results were obtained with these methods so a search for a more satisfactory medium was undertaken. This search resulted in the selection of thiol medium which was used as the solid medium in the remainder of the work. Since this medium is also manufactured as a semisolid medium, its composition was altered by the addition of sufficient agar to raise the total agar content to 1.0 per cent. During the subsequent investigations a large number of subsurface colonies were observed on a deep pour plate which had been incubated under atmospheric conditions. This observation was taken as an indication that the organism perhaps preferred a lowered oxygen tension and this indication stimulated the investigation of this possibility. The investigation was conducted by incubating the organism under various atmospheric pressures. From the data obtained in these investigations, it was decided that the atmospheric pressure, as found at the Bozeman altitude of 4,700 ft, should be decreased about 50 per cent in order to have conditions suited to the cultivation of V. fetus as surface colonies.

THE MICROAEROPHILIC NATURE OF *VIBRIO FETUS*

INTRODUCTION

A study of *Vibrio fetus* was undertaken in the hope of finding a suitable laboratory medium and the proper conditions for cultivating isolated colonies of this pathogenic organism from small inocula. The nature of the investigation was prompted by the need for a medium which so favored the cultivation of *V. fetus* that studies might be made which would lead to a disclosure of its natural habitat. This information is needed so that efforts can be directed toward solving the factors involved in the transmission of *V. fetus*. The lack of a suitable solid medium for the cultivation of isolated colonies of this organism complicates the problem of obtaining pure cultures from contaminated pathological specimens and impedes studies of variation and serological changes.

An examination of the literature revealed that earlier attempts to cultivate *V. fetus* as isolated colonies on solid media were successful only when an inoculum of millions of cells was employed. The growth of 1-5 isolated colonies from an inoculum of this size illustrates the inadequate nature of the existing media. In addition to nutritional requirements, other important conditions such as temperature, time of incubation, atmosphere, and pH have not been thoroughly investigated.

The widespread occurrence of *V. fetus* and the pathological conditions (vibriosis) which it imposes cause serious losses in the cattle and sheep industries. These losses gave an added impetus to work in this field.

HISTORICAL REVIEW

The first workers to associate vibrios with infectious abortion were McFadyean and Stockman. In 1913 these two investigators isolated the organism from the fetuses of aborting ewes and also succeeded in producing infectious abortion by injecting cattle with the exudate from aborting ewes. Seven head of cattle were infected, two of which aborted, and in one of the aborted fetuses vibrios were detected. Brief mention is also made of the isolation of vibrios in cattle in Ireland and Wales in 1911 (Smith, 1918).

In the United States, Smith (1918, 1919a, 1919b, and 1923), Smith and Taylor (1919), and Smith, Little, and Taylor (1920) made a detailed study of a spirillum isolated in pure culture by Smith (1918) from aborting cattle. This study established the facts that the spirillum existed in the comma form in young cultures, hence the name Vibrio fetus, and that there was an etiological relationship between this organism and bovine abortion.

Smith and Taylor (1919) described V. fetus as fine wavy sinuous organisms of various lengths. The smallest forms appeared as minute curved S-shaped lines; the longest stretched nearly across the field of the microscope. The short forms were actively motile, usually possessing a single polar flagellum. The long forms moved sluggishly or were quiescent. As the cultures grew older, deeply stained granules appeared on or within the organism. The longer forms usually contained granules which were arranged along the filament at fairly regular intervals. When

present in the short forms these granules were located terminally. The organism was gram negative.

After this work, reports of vibrionic abortion in both cattle and sheep came from many places in the United States and abroad (Schroeder, 1920; Traum, 1923; Welch and Marsh, 1924; Barger, 1928; Graham and Thorp, 1930; Fritz and Barnes, 1935; Ryff, 1940; Plastridge, 1941; Olson, 1946; Canham, 1948; and Stegenga, 1951).

Various types of semisolid media have been reported to support the growth of V. fetus with the initial growth appearing in a thin layer from 0.5 to 1.0 cm beneath the surface and then extending upward. McFadyean and Stockman in 1913 cultivated the organism on the agar, gelatin, serum mixture as used by Bang for the cultivation of Brucella abortus (Smith, 1918). Slight growth of V. fetus in a simple beef peptone bouillon was obtained by Smith and Taylor (1919) with strains which had been maintained for some time in artificial media. Plastridge and Williams (1943) and Bell (1950) succeeded in cultivating the organism by adding 0.3 per cent agar to liver infusion broth and heart infusion broth respectively. Huddleson (1948) found that thiol medium would support the growth of V. fetus.

The search for a solid medium which would support the growth and continued viability of V. fetus was started by Smith (1918). By adding bits of tissue, particles of meconium, or drops of stomach contents containing the organism to slanted nutrient agar and hermetically closing the tubes with sealing wax, growth was obtained. Transfers to fresh

tubes, however, were apt to fail. The quest for a more suitable medium led to the addition of a few drops of defibrinated horse blood to the condensation water of ordinary nutrient agar slants. This medium supported a feeble growth of this organism through continued transfers. The growth occurred over the sedimented corpuscles in the condensation water and as a thin gray film between the glass and agar. Barger (1928) replaced the defibrinated horse blood used by Smith with 10 per cent sterile horse serum and reported that the nutrient agar-serum combination was a better medium from the standpoint of the amount of growth obtained. Plastridge and Williams (1948) obtained growth on blood agar by streaking amniotic fluid, stomach fluid, and suspensions of ground cotyledons over the surface of blood agar plates and incubating them in an atmosphere of 10 per cent carbon dioxide for 3 to 6 days. In 1949, Plastridge, Williams, and Roman prepared a medium for the cultivation of V. fetus by modifying Difco's thiol medium with the addition of 19 grams of agar and 0.05 gram of glutathione (not essential for all strains of V. fetus) per liter. The medium was adjusted to a pH of 6.8, tubed in test tubes, sterilized by autoclaving, and allowed to cool in the slanted position. The slants were inoculated with four 4mm loopsful of a V. fetus culture grown in a semisolid medium. One to five colonies per slant were usually obtained after 3 to 4 days incubation at 37 C in an atmosphere containing 5 to 10 per cent carbon dioxide.

MATERIALS AND METHODS

Cultures

The *V. fetus* culture used in this investigation was originally isolated in 1942 by Dr. H. S. Cameron, California State College, Davis, California. The organisms were obtained by the author from the Veterinary Research Laboratory, Montana State College, Bozeman, Montana, which in turn had obtained it in 1950 from the Animal Disease Station, United States Bureau of Animal Industry, Beltsville, Maryland, where it was carried as ovine strain No. 543.

Media and enrichments

The cultures were maintained in 8 to 10 ml of Difco's brain liver heart semisolid medium. The medium was prepared by dissolving 46g of the dehydrated medium in 1000 ml of distilled water. A liter of the rehydrated medium contained:

Bacto-liver, infusion from	50 g
Calf brains, infusion from	200 g
Beef heart, infusion from	250 g
Proteose-peptone, Difco	10 g
Neopeptone, Difco	3.25 g
Bacto-tryptone	3.25 g
Bacto-dextrose	2 g
Sodium chloride	5 g
Disodium phosphate	2.5 g
Bacto-agar	1.75 g

The medium was then tubed and sterilized in the autoclave at a temperature of 121 C for 15 minutes.

Two solid culture media were used routinely in this research. The first was Difco's brain liver heart semisolid medium and the other was Difco's thiol medium also manufactured as a semisolid medium. For solid

media, the composition of both of these media was altered by the addition of sufficient agar to raise the total agar content to 1.0 per cent. The thiol medium was prepared by dissolving 30g of the dehydrated medium in 1000 ml of distilled water. A liter of the rehydrated medium contained the following ingredients:

Proteose peptone no. 3, Difco . . .	10	g
Bacto-yeast extract	5	g
Bacto-dextrose	1	g
Sodium chloride	5	g
Thiol complex	8	g
Bacto-agar	1	g
p-aminobenzoic acid	0.05	g

In some of the experimental work these media were enriched by the addition of 10 per cent sterile yeast autolysate, sterile horse serum, or 0.2 per cent soluble starch. The yeast autolysate and serum were added to the media when they had cooled to 45 C after sterilization, while the soluble starch was added prior to sterilization. The yeast autolysate was prepared by suspending one pound of Fleischmann's yeast in 1000 ml of distilled water. This suspension was placed in a 45 C incubator and left for 4 days. At the end of this time, the autolyzed yeast cells were removed by filtering the suspension through diatomaceous earth and the filtrate was sterilized by filtering it through a Seitz filter. The horse serum was obtained from the Veterinary Research Laboratory where it had been sterilized by filtration. Both the yeast autolysate and the serum contained a few drops of chloroform which had been added as a preservative. The soluble starch was a Difco product.

The diluent

Sterile nutrient broth was used as the diluent throughout this investigation. The growth was removed from the cultures by suspending it in 1 ml of nutrient broth and the desired dilutions were obtained by adding 1 ml of this suspension to appropriate volumes of the diluent. The number of organisms contained in the suspension of nutrient broth was determined by direct microscopy and found to be approximately 700,000,000 organisms per ml.

Preparation of the streaked and poured plates

The streaked and poured plate methods for obtaining isolated colonies were employed in these experiments. The streaked plates were inoculated with a 1:10 dilution of the growth from the cultures and the poured plates were inoculated with a 1:100 dilution of this same growth. These dilutions were used as the inoculum throughout this research unless otherwise specified. The cultures were incubated for 4 days at 37 C before the growth was used as inoculum and the inoculated plates were incubated for 4 days at the same temperature before the colonies were counted.

Generation of the carbon dioxide

In part of the experiments, the plates were incubated under an atmospheric pressure increased 10 per cent by the addition of carbon dioxide. This atmosphere was attained by placing the inoculated plates in Brewer anaerobic jars, sealing the jars with plasticine, and generating the desired amount of carbon dioxide within the jar. Generation of the carbon dioxide in the anaerobic jar was achieved by placing a gelatin capsule

containing the required amounts of sodium bicarbonate and tartaric acid in a test tube which had been partly filled with cold water. Before sealing the anaerobic jars, one of these tubes was placed on the inside. The incubation temperature warmed the water to a sufficient degree to melt the gelatin, allowing the water to mix with the sodium bicarbonate and the tartaric acid. The chemical reaction which followed this mixing generated the carbon dioxide.

The removal of the condensation water

Since the solid media contained only 1.0 per cent agar there was a large amount of condensation water which had to be removed from the inoculated plates. Two methods were employed for its removal: The first of these was to replace the regular petri plate covers with aluminum covers which were equipped with cardboard liners for absorbing the moisture. The other method involved placing a piece of filter paper, on which had been deposited 4 drops of glycerine, between the top and bottom of the inverted petri plate.

EXPERIMENTAL RESULTS

Studies made using brain liver heart medium

Since growth of V. fetus had been obtained in semisolid media by other workers, an attempt was made to cultivate the organism in Difco's brain liver heart semisolid medium. The tubed medium was inoculated with a loopful of the organisms and then incubated at 37 C. A distinct zone of growth appeared about 0.5 cm beneath the surface of the medium on the second day of incubation. This zone of growth extended upward on continued incubation forming, by the fourth day, a thick mucoid mass of cells at the surface.

The fact that Difco's brain liver heart semisolid medium supported the growth of V. fetus suggested that with additional agar it might also serve as a solid medium for obtaining isolated colonies of this organism. To check the growth-supporting qualities of this solid medium, streaked plates were inoculated from a culture and poured plates were inoculated with a 1:10 dilution of the same. Patches of isolated surface growth were started on the streaked plates by inoculating them with large masses of the organisms. This growth especially favored small spots on the surface of the medium that had been damaged during the course of the inoculation. The organism seemed capable of establishing itself in these scarred areas and producing this surface growth. By heavily inoculating a small area on a fresh plate, successful transplants of this surface growth could be made. No growth was obtained on the poured plates.

The effect of yeast autolysate and carbon dioxide

The limited amount of surface growth obtained on this solid medium led to the belief that the medium and the conditions for obtaining the growth of isolated colonies were not optimal. For this reason, a means of enriching the medium and altering the conditions was sought. It was decided to enrich the solid medium with sterile yeast autolysate and to increase the atmospheric pressure 10 per cent by the addition of carbon dioxide. An increased amount of carbon dioxide necessitated adjusting different portions of the medium to various pH values in order to determine the optimum pH for these conditions. A range of pH values from 6.0 to 8.0 was checked by adjusting the first portion to pH 6.0 and increasing the pH of each succeeding portion by 0.4 until a value of pH 8.0 had been reached.

Duplicate sets of streaked and poured plates were inoculated for each pH value. The inoculum was a 1:10 dilution of the growth from a culture. After 4 days incubation, the anaerobic jars were opened and the plates were examined. The streaked plates of pH values 7.2, 7.6, and 8.0 all had between 35 and 60 surface colonies and the poured plates in these pH ranges had too many subsurface colonies to count. The surface colonies were circular, convex, smooth, and of a very sticky consistency. In size, the colonies varied from 2 to 4 mm in diameter and were a light brown or buff color. The subsurface colonies were very small circular colonies ranging in size from less than 1.0 mm to 1.5 mm in diameter and were characterized by dark brown centers which were

surrounded by a lighter brown border. Stained smears made from several different colonies, both surface and subsurface, revealed that the majority of the cells were of the comma form with some spirilla of 2 to 3 turns being observed. Since there was no significant difference in the amount of growth obtained in the pH range of 7.2 to 8.0, the pH of 7.6 was selected and used in all subsequent experiments involving an atmosphere containing an increased carbon dioxide content.

In this experiment, the petri plates had been inverted during incubation allowing the condensation fluid to collect on the top of the petri plate. Mold spores found this fluid to be a suitable medium for growth and were a cause of trouble. The mold started growing at the junction between the bottom and top of the petri plate and progressed between these two surfaces to the interior of the petri plate. Large areas on some of the plates were contaminated in this way.

Since the number of colonies obtained on the poured plates could not be determined, serial dilutions were made. The plates were incubated in an atmosphere of increased carbon dioxide content but were not inverted during incubation. The plates inoculated with a 1:10 dilution had too many colonies to be counted, those inoculated with a 1:100 dilution had 150 to 300 colonies per plate, and those inoculated with a 1:1000 dilution had 5 to 20 colonies per plate. The subsurface colonies were of 2 types. About half of them were very small circular colonies ranging in size from less than 1.0 mm to 1.5 mm in diameter; the others displayed a bursting grenade effect with many small circular colonies radiating

out from a large central colony. The large central colonies reached a diameter of from 5 to 6 mm.

A direct microscopic count of the inoculum was made at this point. This count showed that the plates inoculated with the highest dilution had received an inoculum in the neighborhood of a million cells. A comparison of the number of cells in the inoculum with the number of colonies on the plates established that less than 0.001 per cent of the cells in the inoculum were producing colonies. This was particularly true in the case of the surface growth. Consequently, another method of enriching the medium was tried in an attempt to increase the number of colonies returned.

The effect of serum and carbon dioxide

Since blood serum is known to contain many growth factors, an experiment was carried out to compare its effectiveness as a nutritional enrichment with the yeast autolysate. To make the comparison 2 sets of streaked and poured plates were inoculated. One set contained the solid medium enriched with sterile yeast autolysate, in the other set the solid medium was enriched with sterile horse serum. Both sets of plates were placed in anaerobic jars in the upright position, the jars were sealed, and the carbon dioxide content of the atmosphere was increased. At the end of the incubation period, the jars were opened and the plates were checked for growth. The streaked plates produced no isolated colonies so no plate counts could be made from them. Careful examination of the plates, however, revealed a thin gray film of growth over the entire surface of

the medium. It covered the top of the plate, extended down the sides of the plate -- between the medium and the glass -- and across the bottom of the plates. Since the organisms were motile and the medium was kept very moist by the condensation water, the organisms swarmed over the entire surface of the medium. The poured plates produced isolated subsurface colonies of the 2 types previously described. The plate counts ranged from 100 to 150 colonies on both sets of plates. These counts indicated that as nutritional enrichments, the yeast autolysate and the serum were about of the same value. Stained smears were made and disclosed that the cells were predominantly of the comma shape. An occasional cell was observed with a granule at one end. When a means of removing the excess amount of condensation water had been found, the streaked plate portion of this experiment was repeated and plate counts ranging between 10 and 25 colonies per plate were obtained.

The effect of soluble starch and carbon dioxide

Because both the yeast autolysate and the serum were inadequate nutritional enrichments, an experiment was conducted to determine the effect of soluble starch upon the medium and to compare this effect with that produced by the serum and the yeast autolysate. Three sets of streaked and poured plates were prepared with the plates in each of the sets receiving medium which contained one of the nutritional enrichments. The plates were inoculated and incubated in an atmosphere of increased carbon dioxide content for 4 days. After the fourth day of incubation, the plates were examined for growth. The streaked plates produced no

growth nor did those poured plates which had been enriched with soluble starch. A 76 per cent decrease in the number of subsurface colonies on the other plates was also noted. The average plate count was about 60 and all colonies were of the small circular type. Stained smears made of the seed cultures and the subsurface colonies revealed that the organisms were still of the comma form and apparently had undergone no degenerative morphological change such as was frequently mentioned in the literature concerning this organism. Therefore, additional experiments were carried out to obtain surface growth on these 3 media but all efforts ended in failures. These failures prompted attempts to re-establish subsurface growth but these were unsuccessful also.

An investigation of the brain liver heart medium

Since these attempts to obtain growth were unsuccessful, an investigation of the brain liver heart solid medium was undertaken. This investigation resulted in a comparison of the growth supporting qualities of the jar of brain liver heart medium currently in use with a fresh jar of the medium. Two sets of plates were prepared with one set containing the medium currently in use and the other set containing the fresh medium. The medium in both sets of plates had been enriched with sterile serum. The plates were inoculated and incubated in an atmosphere of increased carbon dioxide for 4 days. At the end of the incubation period, the plates were checked for growth. The plates containing the fresh medium supported the growth of both surface and subsurface colonies, while the plates containing the other medium produced no colonies. This indicated

that the brain liver heart medium had undergone a progressive deterioration on being exposed to the atmosphere which rendered it unsuitable for use as a solid medium. Therefore, it was decided to search for a more satisfactory solid medium.

Studies made using thiol medium

Various prepared media, which had been recommended for the cultivation of fastidious organisms, were examined by inoculating streaked and poured plates of each. The plates were incubated under an atmosphere of increased carbon dioxide for 4 days. Of the various media examined Difco's thiol medium most consistently produced the largest number of colonies. Consequently, it was selected as the medium to be used routinely as the solid medium and the search for an improved method of cultivating the organism was again undertaken.

Since maintaining an atmosphere of increased carbon dioxide content was rather inconvenient, it was decided to try substituting sodium bicarbonate for the carbon dioxide. Two portions of the thiol medium were prepared. In one portion the pH was adjusted to 7.6, and in the other the pH was adjusted to 7.0. After sterilization, the medium of pH 7.0 was modified by the addition of sufficient sodium bicarbonate to make the final concentration 0.1 per cent. Two sets of streaked and poured plates were made using these two media. All plates were inoculated and those plates containing the medium of pH 7.6 were incubated in an atmosphere of increased carbon dioxide content, while those plates containing medium of pH 7.0 were incubated under atmospheric conditions. After a

4 day incubation period, the streaked and poured plates incubated under an atmosphere of increased carbon dioxide produced the usual amount of growth. Only one of the poured plates incubated under atmospheric conditions produced growth and there were too many colonies to be counted on it. An examination of the plate revealed that it was the last plate to be poured and had received all the excess medium. Therefore, it was a deep pour plate in that it contained approximately 35 ml of the medium. The subsurface growth on this plate was taken as an indication that the organisms perhaps preferred a lowered oxygen tension and this indication stimulated further study of this possibility.

The investigation was carried out by preparing the required amount of thiol medium and adjusting its pH to 6.8. A series of plates were poured containing different amounts of medium. Some plates received 10 ml, some 25 ml, and some 50 ml. Two sets of plates were made for each amount of medium. One set received an inoculum from a 1:1000 dilution and the other set received an inoculum from a 1:10,000 dilution. The plates were incubated for 4 days under atmospheric conditions. At the end of the incubation period, the plates were examined for growth. Those plates containing 10 and 25 ml of the medium produced no growth, while those plates containing 50 ml of the medium had too many colonies to be counted. The results in this experiment supported the hypothesis that the organisms required semi-anaerobic conditions, but since the number of colonies produced per plate was still unknown, that portion of the experiment involving 50 ml of medium per plate was repeated. Half the plates

were inoculated with a 1:100,000 and the other half were inoculated with a 1:1,000,000 dilution. After 4 days incubation, all plates showed subsurface colonies with those receiving an inoculum from the 1:100,000 dilution producing too many to be counted, while those receiving an inoculum from the 1:1,000,000 dilution had counts which averaged 65 colonies per plate.

The effect of lowering the atmospheric pressure

Since it seemed that the organism preferred a reduced oxygen tension, additional experiments were conducted to determine the effect of lowering the atmospheric pressure. In these experiments, both streaked and poured plates were prepared with each plate receiving 25 ml of the medium and were inoculated with a 1:1,000,000 dilution. In order to attain the necessary atmospheric conditions, some of the plates were incubated in Brewer anaerobic jars under measured atmospheric pressures. In the first of these experiments, four sets of plates were made and three of these sets were placed in anaerobic jars, the jars were sealed, and the various atmospheric pressures were adjusted. The first jar had 12 per cent of the air removed, the second had 31 per cent, and the third had 54 per cent. The fourth set of plates was incubated under normal atmospheric conditions. The counts obtained after four days incubation are as shown in Table I. One of the poured plates incubated under normal atmospheric conditions produced 113 subsurface colonies. An error in technique is the only explanation that can be offered for this. From an examination made at the time the plate was counted, it appeared that the plate had

TABLE I

The number of *V. fetus* colonies obtained under normal atmospheric pressure, and under a 12, 31, and 54 per cent reduction in atmospheric pressure.

Poured plate counts (Millions)	Streaked plate counts (Millions)
Normal atmospheric pressure	
0	0
113*	0
0	0
0	0
12 per cent reduction	
232	0
223	0
186	0
211	0
31 per cent reduction	
116	0
167	0
178	0
181	2
54 per cent reduction	
158	61
123	60
130	33
115	46

The uninoculated controls produced no growth.

* See text, page 21

received more than the specified 25 ml of medium. The excess medium probably produced the necessary conditions for obtaining growth under atmospheric conditions. All of the surface colonies but two were produced on those streaked plates incubated in the jar in which there was a 54 per cent reduction of atmospheric pressure, while the largest number of subsurface colonies were produced in the jar in which there was a 12 per cent reduction. There was a gradual decrease in the number of subsurface colonies produced with each increase in the amount of air removed. These observations prompted another experiment to determine the atmospheric conditions best suited to surface growth.

Five sets of streaked and poured plates were made and four of these sets were incubated in anaerobic jars under various atmospheric conditions. In one jar, 20 per cent of the air was removed, in another jar 40 per cent was removed, in a third jar 80 per cent was removed, and a fourth jar was completely evacuated. The fifth set of plates was incubated under atmospheric conditions. After four days incubation, the jars were opened and the plate counts were made. The results are shown in Table II. An examination of these data reveal some inconsistencies which probably were caused by an unequal removal of the air from the plates. Since the condensation water was not removed from the plates, a moisture lock formed between the tops and bottoms of the petri plates trapping air and excess moisture within them. The trapped air and excess moisture allowed thin films of growth to form over the surface of the streaked plates and an inconsistent number of colonies in some of the

TABLE II

The number of V. fetus colonies obtained under normal atmospheric pressure, and under a 20, 40, 80, and 100 per cent reduction in atmospheric pressure.

Poured plate counts (Millions)	Streaked plate counts (Millions)
Normal atmospheric pressure	
0	0
0	0
0	0
0	0
20 per cent reduction	
55	Thin film of growth over entire surface
43	
39	
42	
40 per cent reduction	
53	Thin film of growth over entire surface
49	
45	
40	
80 per cent reduction	
35	Thin film of growth over entire surface
38	
191	
207	
100 per cent reduction	
264*	Thin film of growth over entire surface*
135*	
0	
0	

The uninoculated controls produced no growth.

*See text, page 23

plates. However, no colonies developed under normal atmospheric pressure, but subsurface colonies were produced on the poured plates incubated under reduced atmospheric conditions.

After a means of removing the moisture was devised, another experiment was conducted to determine the effect of serum on surface and subsurface growth, and the effect of a 10 per cent increase in the carbon dioxide pressure in an atmosphere in which 50 per cent of the air had been removed. Five sets of plates were prepared with half of the plates in each set containing medium that had been enriched with 10 per cent sterile horse serum. Four sets of these plates were incubated in anaerobic jars under reduced atmospheric conditions. The first set was incubated in a jar in which 50 per cent of the air had been removed, the second set was incubated in a jar in which 80 per cent of the air had been removed, the third set was incubated in a jar in which all of the air had been removed, and the fourth set was incubated in a jar in which 50 per cent of the air had been removed and in which the carbon dioxide content was increased. The fifth set of plates was incubated under atmospheric conditions.

The results obtained in this experiment are in Tables III and IV. There were no colonies produced under normal atmospheric conditions and except for the fifty surface colonies obtained on one streaked plate no colonies were produced when 100 per cent of the air had been removed. The production of these fifty colonies was thought to be due to incomplete evacuation of the plate. From the data obtained in these three experiments, it was decided that the atmospheric pressure, as found at the

TABLE III

The number of V. fetus colonies obtained with and without serum under normal atmospheric pressure, and under a 50, 80, and 100 per cent reduction in atmospheric pressure.

Poured plate counts (Millions)		Streaked plate counts (Millions)	
Plain	Serum	Plain	Serum
Normal atmospheric conditions			
0	0	0	0
0	0	0	0
50 per cent reduction			
101	193	66	205
71	190	88	191
80 per cent reduction			
10	24	56	180
17	31	72	201
100 per cent reduction			
0	0	0	0
0	0	50*	0

The uninoculated controls produced no growth.

*See text, page 25

TABLE IV

The number of V. fetus colonies obtained with and without serum under a 50 per cent reduction in atmospheric pressure and under a 50 per cent reduction in atmospheric pressure plus a 10 per cent increase in the carbon dioxide content.

Poured plate counts (Millions)		Streaked plate counts (Millions)	
Plain	Serum	Plain	Serum
50 per cent reduction			
101	193	66	205
71	190	88	191
50 per cent reduction plus 10 per cent carbon dioxide			
46	76	0	0
54	61	0	0

The uninoculated control produced no growth.

Bozeman altitude of 4700 ft, should be decreased about 50 per cent in order to have conditions suited to the cultivation of V. fetus as surface colonies. This decrease would be somewhat less for subsurface colonies and would be determined by the amount of media in the plates. The decrease in the number of subsurface colonies and the absence of surface colonies on those plates incubated in an atmosphere in which half of the air had been removed and the carbon dioxide increased indicated that the organism did not require carbon dioxide in increased concentration and that it might be slightly inhibitory.

The surface colonies on thiol medium without serum were light yellow, circular, convex, smooth, and very small in size reaching a maximum diameter of 3 mm. Two types of subsurface colonies were obtained on this medium. The first type was a small, circular, grayish colony ranging in size from less than 1.0 mm to 1.5 mm in diameter. The second type was a large, white, fluffy colony from 4 to 5 mm in diameter. The large fluffy colonies were obtained only when the condensation water was not removed from the plates. Hence, the bursting grenade-like colonies obtained on the brain liver heart medium and the large fluffy colonies obtained on the thiol medium were attributed to the very moist state of the medium. The size of the colonies was increased slightly by the addition of serum to the medium; otherwise their appearance was unchanged. In stained smears made from the growth on thiol medium, colonies were found that consisted primarily of cells in the long spiral form, as well as colonies where the cells were predominantly comma shaped.

DISCUSSION

The search for a suitable laboratory medium and the proper conditions for the cultivation of V. fetus led to the use of a variety of cultural enrichments and conditions. The course of the investigation was determined, in part, by the results obtained by earlier workers. These workers had found it necessary to inoculate their solid media heavily and to enrich them with bits of tissue, blood, serum, etc. In addition to this, a number of the investigators reported the need of a 5 to 10 per cent increase in the carbon dioxide content of the atmosphere. The microaerophilic nature of this organism was discovered by the author when a large number of subsurface colonies were observed on a deep pour plate which had been incubated under atmospheric conditions. Following this discovery it was found that surface colonies of V. fetus could be produced by incubating the organisms under a reduced atmospheric pressure. About a 50 per cent reduction in atmospheric pressure was found to be optimal for surface colonies at the Bozeman altitude of 4700 ft. The decrease for subsurface colonies would be determined by the depth of the media in the plates.

The microaerophilic nature of the organism explains the difficulties encountered by other workers in cultivating the organism on solid media and explains, to some extent, the beneficial effect of the modifications they found it necessary to adopt. The possible respiration of the added bits of tissues or of blood cells would tend to make conditions more suitable for the organism; probably the same end is served by an extra

heavy inoculation. The characteristic growth pattern formed by the organism on brain liver heart semisolid medium also supports this view, for there is an initial zone of subsurface growth which on continued incubation, and the corresponding increase in the number of cells, moves upward, eventually forming a thick mucoid mass of cells at the surface. The replacement of some of the air by carbon dioxide would also aid a microaerophilic organism in growing.

It seems that the organism does not require carbon dioxide in increased concentration; for in jars from which about half of the air was removed, the carbon dioxide content was only about 0.013 per cent, and the growth was satisfactory. The addition of one tenth atmosphere of carbon dioxide to a jar from which half of the air had been removed may even have been slightly inhibitory as is illustrated by the data shown in Table IV. However, the reduction in the number of colonies obtained under these conditions may have been due to a pH effect as no work was done to determine a suitable pH for these conditions.

Thiol medium appeared not to be the best possible medium for the cultivation of V. fetus since the size and number of the colonies could be increased when it was enriched with serum, as is illustrated by the data shown in Table III. It was true that the increase in numbers was only twofold and the increase in size was only slight but it was consistent. This and a number of other physiological examinations are left for future investigators.

SUMMARY

Difco's brain liver heart medium in the semisolid state was found to be a satisfactory medium for the cultivation of Vibrio fetus. However, when this medium was modified by the addition of sufficient agar to raise the total agar content to 1.0 per cent, it would support only a very limited growth of the organism and efforts directed towards improving its growth-supporting qualities were unsuccessful. A satisfactory solid medium was prepared by altering the composition of Difco's thiol medium with sufficient agar to raise the total agar content to 1.0 per cent.

About a fifty per cent decrease in the atmospheric pressure, as found at the Bozeman altitude of 4700 ft, was needed in order to have conditions suited to cultivation of V. fetus as surface colonies from small inocula. For subsurface growth, the necessary reduction in atmospheric pressure was found to be a function of the depth of the medium in the plates. Apparently the organism does not need carbon dioxide in increased concentration.

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