THE STIMULATION OR INHIBITION OF GROWTH AND ACID PRECIPITABLE MATERIAL PRODUCTION OF *VIBRIO FETUS* BY KREB'S CYCLE INTERMEDIATES, FATTY ACIDS, AMINES, AND MISCELLANEOUS COMPOUNDS

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To Albert, Carol, and Amy

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INTRODUCTION AND REVIEW OF LITERATURE

Vibrios were associated with abortion in sheep by McFadyean and Stockman in 1913. Subsequently, vibrios were found to be causatively associated with infectious abortion in cattle by Smith and Taylor in 1919. They named the organism Vibrio fetus.

Alexander (1957) studied the energy sources of ovine strains of V. fetus by the addition of various substrates to a peptone medium which was diluted so that it permitted only minimal growth. He found that lactate, pyruvate, α-ketoglutarate, succinate, fumarate, malate, aspartate, asparagine, glutamate, and proline served as energy sources. However, glucose, hexosediphosphate, glycerol, glycerophosphate, oxalacetate, citrate, isocitrate, oxalosuccinate, tartarate, glycine, alanine, threonine, phenylalanine, histidine, lysine, arginine, cysteine, methionine, leucine, and ornithine did not serve as energy sources. He also showed that lactate, pyruvate, isocitrate, and fumarate were oxidized by resting cell suspensions of V. fetus.

Kuzdas and Morse (1956), while developing a selective medium for V. fetus, found that vibrios grow in a medium containing 1% oxbile. They also stated that nicotinic

acid, thiamine, calcium pantothenate, pyridoxal-HCl, and biotin were needed for growth of V. fetus.

Trueblood and Tucker (1957) presented evidence indicating that amniotic fluid added to the medium enhanced growth of V. fetus.

Lecce (1958), using a total of 27 strains of V. fetus from a variety of sources, studied the oxidative capability of resting cells against 30 substrates. He used the Thunberg technique, and used triphenyl tetrazolium chloride as the electron acceptor. He reported that lactate, formate, pyruvate, α-ketoglutarate, and succinate were used as electron donors. Although formate was the most active electron donor, this substrate completely inhibited growth of vibrios at a concentration of 0.5% and delayed growth at a concentration of 0.2%. There was selective inhibition of growth of vibrios based on the species of animal from which the organism was isolated when glycine was incorporated into the medium. Growth of all five sheep strains tested was not inhibited by 0.8% glycine. Glutamate was only slightly active with four of 27 strains of V. fetus.

Kiggens and Plastridge (1958), using the Warburg technique, studied the oxidative activity of two strains of V. fetus against various substrates using whole cells

and cell-free extracts. None of 16 carbohydrates tested was oxidized, and with the exception of pyruvate, there was no significant oxygen uptake with glycolytic intermediates. Although there was rapid oxygen uptake with all members of the tricarboxylic acid cycle when a cell-free extract was employed, whole cells showed slight or no oxygen uptake with citrate, cis-aconitate, isocitrate, and α-ketoglutarate. Glutamate, glutamine, aspartate, asparagine, proline, and cysteine were the only amino acids oxidized of the 23 which were tested. Acetate was the only fatty acid oxidized.

Zemjanis and Hoyt (1960a) compared the sensitivity of V. fetus, Pseudomonas aeruginosa, and Proteus vulgaris to various enzyme inhibitors. All of the strains of V. fetus employed in the study were found to be more sensitive than Ps. aeruginosa or P. vulgaris to the 19 enzyme inhibitors tested. Therefore, these compounds were eliminated as potential additional materials to be included in a selective medium for V. fetus.

Zemjanis and Hoyt (1960b), by adding various substrates to a peptone semisolid medium, reported that certain concentrations of cysteine, lactate, α-ketoglutarate, glutamate, glutamine, uracil, thymine, p-aminobenzoic acid,

and 17-β-estradiol enhanced the growth of V. fetus. They also stated that the addition of magnesium, manganese, and iron salts stimulated the growth of 22 strains of V. fetus. Molybdenum trioxide caused slight stimulation of growth of V. fetus when cultures were incubated aerobically, but growth in the presence of this compound was inhibited in an environment containing 15% carbon dioxide. Cobalt acetate at a concentration below 0.05% neither inhibited nor increased growth of V. fetus, while higher concentrations were bactericidal. Pyruvate at a concentration of 1% completely inhibited growth of V. fetus, while lower levels of pyruvate neither inhibited nor increased growth. These workers reported that the growth of 22 strains of V. fetus studied was unaffected by aspartate, adenine, and urea; however, all concentrations of fumarate investigated caused inhibition of growth.

Smibert (1963) reported the development of a chemically defined medium for V. fetus. He found that a liquid or semisolid medium containing 18 amino acids, B-vitamins, and minerals supported growth of all 87 strains of V. fetus tested. The addition of spermine, cadavarine, putrescine, betaine, thioglycolate, thiosulfate, cystine,

and ascorbic acid to the liquid defined medium had no effect on growth of the slower growing strains. Purines and pyrimidines were not required. Sodium carbonate and sodium thiosulfate enhanced growth of a few strains. Magnesium and ferrous salts were the only major mineral requirements. The only vitamin required by most strains was nicotinic acid, although additional B-vitamins stimulated growth. Smibert also reported that the amino acid requirements were heterogenous. The organisms were divided into five nutritional groups on the basis of their amino acid requirements. Of 87 strains tested, 26 grew in medium A containing glutamate, proline, aspartate, and leucine; nine, which did not grow in A grew in medium B which contained glutamate, proline, aspartate, leucine, and methionine; 15, which did not grow in A or B grew in medium C which contained glutamate, proline, aspartate, leucine, methionine, arginine, and alanine; and 34, which did not grow in A, B, or C grew in medium D which contained glutamate, proline, aspartate, leucine, methionine, arginine, alanine, serine, hydroxyproline, valine, glycine, threonine, tryptophan, tyrosine, and cystine; three of 87 strains grew in medium E but not in A, B, C, or D. Medium E is listed in Table 1. All 87 strains grew in Medium E. Smibert

found that amino acid requirements were related to neither biochemical activity nor origin of the strains of V. fetus studied.

Fletcher and Plastridge (1963) studied the growth requirements of 26 catalase-positive, H_2S-negative microaerophilic strains of V. fetus. They found that a chemically defined medium that would support the growth of 22 of 26 strains tested consisted of aspartate, cysteine, glutamate, sodium acetate, $MgCl_2 \cdot 6H_2O$, NaCl, K_2HPO_4, KH_2PO_4, and niacin. Growth of vibrios was compared on the defined agar medium, yeast extract agar, blood agar, and Bacto-Thiol agar. On the defined medium growth of four of ten glycine-intolerant strains equaled or exceeded growth obtained on the yeast extract agar. Two of ten strains yielded less growth in the defined medium, and four of ten strains gave little or no growth. All of the 16 glycine-tolerant strains grew on the defined medium. Niacin appeared to stimulate growth of four strains, although it did not stimulate those strains which failed to grow on the defined medium. Biotin, pyridoxine, riboflavine, and thiamine added to the defined medium did not stimulate growth of the vibrios. Members of the glycine-tolerant group were able to grow in the presence of 1% glycine in

Bacto-Thiol medium. However, growth of both groups was inhibited by 1% glycine in the defined medium. The 26 strains of vibrios tested were divided into two groups on the basis of their ability to grow in a concentration of 0.01% glycine in the defined medium.

Hoff (1956) developed a chemically defined medium which supported growth of two strains of V. fetus and two strains of Vibrio spp.. The chemically defined medium consisted of 19 amino acids, 8 B-vitamins, 4 purines and pyrimidines, 4 salts, a reducing agent, trace minerals, sodium pyruvate, and 1.5% agar. Experiments in which amino acids were deleted by groups and then singly indicated that the strains of V. fetus studied were more demanding in their amino acid requirements than the strains of Vibrio spp.. V. fetus failed to grow in a medium in which all amino acids except glutamate and aspartate were omitted, although the Vibrio spp. strains grew in this medium. Glutamate and tryptophan were required by both strains of V. fetus. Isoleucine, leucine, lysine, methionine, and valine were stimulatory for growth of one strain of V. fetus, while alanine, glycine, histidine, phenylalanine, proline, and serine were stimulatory for growth of the other strain of V. fetus. Hoff reported that his results indicated the

ability of Vibrio spp. to synthesize, to some extent, the amino acids they required. Arginine, aspartate, glutamate, leucine, lysine, methionine, serine, and tryptophan were stimulatory for one strain of Vibrio spp., while arginine, glutamate, lysine, methionine, valine, and cystine were stimulatory for the other strain of Vibrio spp.. Hoff studied the oxidative activity of one strain each of V. fetus and Vibrio spp. against various amino acids. He also investigated the ability of these strains to deaminate amino acids. Vibrio spp. and V. fetus both oxidized asparagine, aspartate, glutamine, glutamate, and cysteine. In addition, V. fetus oxidized proline. Both V. fetus and Vibrio spp. deaminated asparagine, aspartate, and glutamine. However, aspartate and glutamine were deaminated by V. fetus in only one of two experiments. Vibrio spp. only deaminated glutamate in one of two experiments.

Reich, Morse, and Wilson (1956) studied the effect of 10% carbon dioxide on the growth of V. fetus. They reported that carbon dioxide only serves as a diluent to lower the concentration of oxygen in the environment. They found that as much growth occurred in an atmosphere of nitrogen or helium as in an atmosphere of carbon dioxide. This showed that carbon dioxide was not required for growth.

Kiggens and Plastridge (1956) reported that the optimum atmosphere for the growth and isolation of V. fetus was provided by reducing the oxygen concentration to 5% and increasing the carbon dioxide concentration to 10%.

Fletcher and Plastridge (1964) studied the gaseous requirements for growth of 49 strains of microaerophilic vibrios from various sources. They used 36 catalase-positive and H_2S-negative strains regarded as V. fetus which were divided into two groups on the basis of glycine tolerance. Members of group I were glycine-negative, while members of group II were glycine-positive. These workers reported that on yeast extract agar and in the presence of 10% carbon dioxide, the optimal oxygen level for the growth of organisms belonging to group I was 2.5%, while the optimal amount of oxygen for the growth of group II organisms was 5%. Fletcher and Plastridge stated that in the presence of 10% carbon dioxide, a group I strain from man and a group I strain from sheep gave significantly larger amounts of growth than did group I strains from cattle in the presence of 12.5% oxygen, 15% oxygen, and 17.5% oxygen. In the presence of 2.5% oxygen, the optimal carbon dioxide level for growth of all strains tested on yeast extract agar was 10%. Members

of group I were unable to tolerate 50% to 70% carbon dioxide, while members of group II grew in an atmosphere containing these high concentrations of carbon dioxide. In the presence of 50% to 70% carbon dioxide, group I vibrios isolated from man and sheep yielded larger amounts of growth than the group I vibrios isolated from cattle. Group I and group II strains which were able to grow on the defined medium, yielded maximal growth in an atmosphere containing 1% oxygen and 10% carbon dioxide. The vibrios were less tolerant to high concentrations of carbon dioxide and oxygen when grown on the defined medium than when grown on the yeast extract agar. These workers stated that the effect of carbon dioxide concentrations exceeding 10% was a lowering of pH for group I vibrios grown on the defined medium, while more than 10% carbon dioxide was toxic for group II vibrios. Fletcher and Plastridge stated that the growth of group I strains of vibrios isolated from cattle differed from the growth of group II strains isolated from cattle, chickens, man, and sheep. They concluded that strains of V. fetus associated with bovine infertility differed from strains of V. fetus involved in disease of chickens, man, and sheep.

Nageswararao and Blobel (1963) isolated an antigenic substance from broth culture filtrates of 21 catalase-positive strains of V. fetus. Broth cultures were centrifuged and the supernatant fluid filtered by means of filter candles with an average capillary radius of 300 mμ. The filtrate was adjusted to a pH of 3.2, stored overnight at 4 C and centrifuged. The precipitate was washed twice in 0.01 M citrate at a pH of 3.2, and dissolved in 0.01 M phosphate buffer (pH 7.0). Following clarification by centrifugation, the solution was dialyzed against distilled water for 18 hr. These workers designated the final product an acid precipitable material (APM). Cell extracts were prepared by sonic oscillation of V. fetus cell suspensions. The cell extracts were centrifuged, the supernatant fluid filtered, and the filtrate dialyzed against distilled water. These workers reported that protein per unit weight of carbohydrate generally was higher in the APM than in the cell extract filtrates. The carbohydrate content of APM was lower than that of the cell extracts. Therefore, the differences in the chemical compositions of the APM and of the cell extracts indicated that the major part of the APM was not a cellular component liberated into the culture

fluid by lysis of cells. Nageswararao and Blobel demonstrated that the APM was antigenic. When antiserums against APM from three strains were absorbed with homologous cell extracts and whole cell suspensions, the agglutinin titers of two of three strains were decreased. The titer of the third strain was completely adsorbed. By the use of immunoelectrophoretic analyses and cross-precipitin tests, these workers reported some serologic heterogeneity of APM isolated from different strains of V. fetus. The APM of two strains retained antigenicity after heating at 60 C for 30 minutes as well as after boiling for 5, 15, and 30 minutes. Antigenicity was lost after boiling for two hours and after autoclaving at 121 C for 15 minutes. On electrophorograms, APM from three strains was stained with protein stains but not with glycoprotein and lipid stains.

Osborne and Smibert (1962) reported that the bovine was hypersensitive to a material produced by V. fetus when grown in the chemically defined broth. The toxic material was found in the cell-free supernatant fluid of three to five day old cultures. Twelve calves and six adult cows showed clinical symptoms of anaphylactoid shock following intravenous inoculation with a broth

culture or the cell-free supernatant fluid from a broth culture. Calves were refractory to a second dose of supernatant fluid for at least two days. The supernatant fluid inoculated intradermally into the cervical skin of 36 cows, calves, and young adult cattle gave a positive skin reaction in all animals. Osborne and Smibert suggested that the bovine becomes hypersensitive to V. fetus either passively during prenatal development or by early post-natal contact with vibrios or other microorganisms sharing common antigens with V. fetus.

Osborne and Smibert (1964) conducted an extensive study of the hypersensitivity and abortifacient action of V. fetus whole cell cultures and cell-free supernatant fluid in the bovine, caprine, ovine, porcine, and lapine species. These workers reported that all strains of V. fetus used produced toxicity signs following a single intravenous inoculation, although a second inoculation 24 or 48 hr later produced slight or no shock symptoms. Whole cell V. fetus cultures were highly toxic as were V. fetus cell-free supernatant fluids. Washed whole cells were only slightly toxic. A more severe shock reaction was noted in calves older than one month and up to sexual maturity than in calves that were younger than one month

or beyond sexual maturity. A moderate degree of hypersensitivity to V. fetus whole cell cultures and supernatant fluids was reported in colostrum deprived pigs and calves. After receiving colostrum for one day, hypersensitivity was not enhanced. Osborne and Smibert reported on the abortifacient action of V. fetus supernatant fluids and whole cell cultures in the bovine, caprine, and ovine species. Two of four pregnant cows aborted following inoculation of V. fetus (bovine origin) whole cell cultures. Necropsy of a third pregnant cow revealed a dialated cervix and impending abortion. One of two goats inoculated with V. fetus supernatant fluids showed a reversible shock reaction followed by abortion in two days. A larger dose of V. fetus supernatant fluid was given a second goat and an irreversible shock syndrome occurred. Viable V. fetus cell culture inoculated into two pregnant goats resulted in reversible shock reactions followed by abortions. In one goat the inoculation of V. fetus whole cell culture followed with V. fetus supernatant fluid four days later resulted in reversible shock after both inoculations and abortion following the inoculation of the supernatant fluid. These workers inoculated five ewes with whole

cell cultures or cell-free supernatant fluid. However, only four ewes were pregnant. The non-pregnant ewe died within 24 hours. Another pregnant ewe aborted after 18 days, and died the following day. A severe reversible anaphylactoid shock occurred in two ewes inoculated with V. fetus supernatant fluids. One of the ewes aborted three days after inoculation. A mild anaphylactoid shock occurred in a pregnant sow inoculated with V. fetus supernatant fluid. Three pregnant rabbits inoculated with V. fetus supernatant fluid in the marginal ear vein exhibited a severe irreversible shock reaction. One unbred female rabbit had no reaction when inoculated intravenously with a bovine strain of V. fetus whole cell culture which was lethal for the two pregnant rabbits. It was therefore suggested that pregnancy increased the sensitivity of the rabbit to V. fetus toxin. However, a larger dose of a bovine strain of V. fetus whole cell culture produced severe reversible anaphylactoid shock in another unbred female rabbit. In all animals, the severity of the shock reaction seemed to bear a linear relationship to the size of the inoculum. These workers stated that the nature of abortion involving V. fetus appeared to be allergic since a bacteremia was not

demonstrable in the bovine following intravenous inoculation with viable V. fetus cells and since pregnant goats aborted within 48 hours after V. fetus inoculation, and a single inoculation of V. fetus cell-free supernatant fluid could produce a severe anaphylactoid shock reaction and abortion of pregnant animals.

The purpose of this study was to test the stimulatory or inhibitory effect of Kreb's cycle intermediates, fatty acids, amines, and miscellaneous compounds on the growth and the production of acid precipitable material by Vibrio fetus when grown in a chemically defined medium.

MATERIALS AND METHODS

Seven strains of V. fetus (3530, 085, 277, 279, Kohler, S_2 and 30) isolated from cases of bovine abortion were used in the study. Strains 085, 277, 279, Kohler, and 30 grew well in Brucella broth (Albimi) containing 0.15% agar, while strains 3530 and S_2 grew poorly. Stock cultures were maintained in Brucella broth (Albimi) containing 0.15% Bacto-agar and were transferred weekly. Stock cultures of each strain were also stored under mineral oil.

Biochemical and physiological characteristics of each strain were determined by inoculating one loopful of culture of each strain into Brucella semisolid media (Albimi) containing 3.5% sodium chloride, 1% glycine, and 1% oxbile. Hydrogen sulfide production was determined using Brucella semisolid media (Albimi) containing 0.02% cysteine. Strips of filter paper impregnated with lead acetate were used as the detection system for H_2S. Catalase activity was determined by the addition of 3 ml of 3% hydrogen peroxide to cultures of each strain.

Cultures used to inoculate the experimental media were maintained in the chemically defined liquid medium

of Smibert (1963) and incubated at 37 C in an atmosphere of 85% nitrogen, 10% carbon dioxide, and 5% oxygen. The composition of the medium is shown in Table 1. Seven ml of experimental media were placed in 16 mm by 150 mm screw cap culture tubes. The number of cells inoculated into experimental broth media was determined by using a Petroff-Hauser counting chamber and a phase contrast microscope. Five day old broth cultures were diluted 1:100 with 0.1% formalized 0.85% sodium chloride solution. The Petroff-Hauser counting chamber was filled, and the number of vibrio cells in 48 squares counted. After determining the average number of cells per square, the number of bacteria per ml was calculated. Appropriate dilutions were made so that a known number of cells was inoculated into each tube of experimental media.

The smallest number of cells of V. fetus that could grow in the chemically defined medium was investigated as well as the length of time for growth to be seen visibly. An inoculum containing 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 cells of V. fetus was inoculated into duplicate tubes each containing seven ml of the chemically defined medium. The cultures were checked for visible growth

Table 1. Composition of chemically defined medium for *Vibrio fetus*

Amino Acids	Amount mg	Salts	Amount mg	Vitamins	Amount µg
L-Glutamic acid	200	$(NH_4)_2SO_4$	300	Nicotinic acid	1 mg
L-Proline	120	K_2HPO_4	400	Pyridoxal-HCl	200
L-Aspartic acid	70	NaCl	5	Pyridoxine-HCl	200
L-Leucine	70	Salt solution*	3 ml	Pyridoxamine·2HCl	100
L-Methionine	50	Sodium thiosulfate	80	p-Aminobenzoic acid	400
L-Arginine	40	Sodium carbonate	40	Biotin	10
Ornithine	20			Folic acid	100
DL-α-Alanine	30			Calcium pantothenate	500
DL-Serine	30			Thiamine-HCl	400
L-Lysine HCl	20			Riboflavine	100
Hydroxy-L-proline	20			B_{12}	10
DL-Valine	10			i-Inositol	200
Glycine	30			Choline chloride	400
L-Threonine	30			Ascorbic acid	500
L-Tryptophan	30			Water	100 ml
L-Phenylalanine	20				
L-Tyrosine	30				
DL-Isoleucine	50				
L-Cystine	40				

*Salt solution: 800 mg of $MgSO_4$ and 40 mg of $FeSO_4 \cdot 7H_2O$ in 100 ml of distilled water, pH adjusted to 7.1, medium filtered and sterilized by autoclaving. Semisolid medium contained 0.15% agar.

at 24 hr, 48 hr, 72 hr, 96 hr, and 144 hr. After seven days incubation, each culture was mixed on a Rotary Evapo-Mix, diluted 1:6 with 0.1% formalized 0.85% sodium chloride solution, and the optical density read at 540 mμ in a Model 14 Coleman Universal Spectrophotometer equipped with a flow through cuvette. The experiment was replicated at least one other time.

Experimental media consisted of the chemically defined medium containing various concentrations of the test compounds. Tricarboxylic acid cycle intermediates, lactic acid, pyruvic acid, propionic acid, and butyric acid were added to the chemically defined medium at concentrations of 30 mg, 100 mg, and 400 mg per 100 ml. Spermidine, cadavarine, putrescine, aminobutyric acid, glutathione, glutamine, asparagine, and hydroxybutyric acid were added to the medium at a concentration of 20 mg per 100 ml. Surface-active agents (tween 80, tween 40, and tergitol 7) were incorporated into the medium at a concentration of 0.01 ml per 100 ml. Phenylacetyl chloride was added at a concentration of 10 mg per 100 ml. Sodium bicarbonate was incorporated into the medium at a concentration of 710 mg per 100 ml. The control for each experiment consisted of a tube of chemically defined media inoculated with each strain.

Cultures were observed daily for visible growth. After five days incubation, each control culture and each test culture in experimental media was mixed on a Rotary Evapo-Mix, diluted 1:6 with 0.1% formalized 0.85% sodium chloride solution, and the optical density read at 540 mμ in the spectrophotometer. Slightly turbid cultures were not diluted before reading in the spectrophotometer.

After incubation of all cultures and prior to reading culture densities, wet mounts of all cultures were checked for non-vibrio cells using a phase contrast microscope.

The usefulness of the method described by Zemjanis and Hoyt (1960b) was investigated. Stimulation or inhibition of growth by experimental compounds was determined by an increase or decrease in the depth of growth of vibrios in 7 ml of semisolid media contained in 16 mm by 150 mm screw cap culture tubes. The chemically defined medium was used as the control. A depth of growth of vibrios of 0-5 mm was recorded as +, 5-10 mm as ++, 10-15 mm as +++, and 15-20 mm as ++++. A double loop was used for inoculation of cultures into semisolid experimental media.

The concentration of acid precipitable material (APM) produced by each strain of V. fetus grown in experimental medium was determined, as well as the concentration of APM produced by each strain of V. fetus grown in the liquid chemically defined medium. Experimental media consisted of the chemically defined medium containing the above mentioned compounds. The stimulation or inhibition of APM production by the experimental compounds was determined for each strain of V. fetus. Five day old cultures were centrifuged at 18,000 x g for 30 minutes. Three ml of the supernatant fluid were mixed with 3 ml of 20% trichloroacetic acid. The optical density of the mixture was read at 380 mμ in the spectrophotometer after 10-15 minutes at room temperature. The control was 3 ml of uninoculated chemically defined medium plus 3 ml 20% trichloroacetic acid.

All cultures in liquid media were incubated at 37 C in desiccator jars in an atmosphere containing 85% nitrogen, 10% carbon dioxide, and 5% oxygen. All cultures in semisolid media were incubated aerobically at 37 C.

RESULTS

The biochemical and physiological characteristics of all strains of *V. fetus* used in this study are shown in Table 2. All strains were catalase-positive. The catalase reaction was weak with strains 3530 and S_2. Strains 277, 279, Kohler, and 085 produced H_2S, while strains 3530, S_2, and 30 did not produce H_2S. None of the strains grew in a medium containing 3.5% sodium chloride. All strains grew in the presence of 1% glycine, and all grew in a medium containing 1% oxbile. Strains 3530, S_2, and 30 were *V. fetus* var. *venerealis*, while strains 085, 277, 279, and Kohler were identified as *V. fetus* var. *intestinalis*.

Strains 085, 277, 279, and Kohler grew in the chemically defined medium when inoculated with only 10^1 cells (Table 3); however, strain 3530 required at least 10^3 cells for the initiation of growth in the chemically defined medium. The number of cells in the inoculum did not appreciably affect the total cell crop for any of the strains. The number of hours required for visible growth of *V. fetus* to be observed is shown in Table 4. Strain 3530 required a longer time for visible growth to occur than strains 085, 277, 279, and Kohler.

Table 2. Biochemical and physiological characteristics of seven strains of *Vibrio fetus*

	Strains of *Vibrio fetus*						
	3530	085	277	279	Kohler	S_2	30
Catalase	+	+	+	+	+	+	+
H_2S	-	+	+	+	+	-	-
3.5% NaCl	-	-	-	-	-	-	-
1.0% Glycine	+	+	+	+	+	+	+
1.0% Oxbile	+	+	+	+	+	+	+

Results expressed as plus (+) for positive test or growth and minus (-) for negative test or growth.

Table 3. Influence of inoculum size on total growth of five strains of *Vibrio fetus*

Number cells/inoculum	Strains of *Vibrio fetus*				
	3530	085	277	279	Kohler
10^6	0.24	0.26	0.21	0.30	0.29
10^5	0.22	0.23	0.23	0.27	0.23
10^4	0.21	0.26	0.22	0.28	0.26
10^3	0.19	0.21	0.22	0.25	0.24
10^2	0.02	0.27	0.21	0.32	0.32
10^1	0.02	0.25	0.15	0.21	0.26

Results expressed as optical density at 540 mμ. Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Table 4. Number of hours required for visible growth of five strains of *Vibrio fetus*

Number cells/inoculum	Strains of *Vibrio fetus*				
	3530	085	277	279	Kohler
10^6	72	48	24	24	24
10^5	96	48	24	24	24
10^4	96	48	48	24	24
10^3	144	48	48	48	48
10^2	-	48	48	48	48
10^1	-	48	72	48	48

Results expressed as number of hours required for visible growth to be seen.

Table 5 shows that 30 mg, 100 mg, and 400 mg of fumarate and oxalacetate increased the growth of strain 3530 in the chemically defined medium. The 100 mg and 400 mg concentrations of succinate and lactate also stimulated growth of this strain. The 30 mg and 100 mg concentrations of citrate increased the growth of strain 3530. Tween 80 and tween 40 increased growth of strain 3530.

The 400 mg concentrations of lactate and butyrate stimulated growth of strain 085 (Table 6). Glutathione, glutamine, asparagine, and tergitol 7 inhibited the growth of this strain.

Table 7 shows that lactate at concentrations of 100 mg and 400 mg increased the growth of strain 277. Oxalacetate at a concentration of 400 mg also stimulated growth. Propionate at the 400 mg level was inhibitory. Spermidine, glutathione, glutamine, asparagine, and tergitol 7 were also inhibitory.

Growth of strain Kohler was increased slightly by succinate at the 400 mg concentration (Table 8). Growth of strain 279 was neither increased nor decreased by the compounds tested.

The 100 mg concentration of citrate stimulated growth of strain 30 (Table 9). Malate at a concentration of

Table 5. Influence of various test compounds on total growth of *Vibrio fetus* strain 3530

Compound	mg/100 ml						
	10 mg	20 mg	30 mg	100 mg	200 mg	400 mg	710 mg
Phenylacetyl chloride	-0.02	-	-	-	-	-	-
Spermidine	-	-0.01	-	-	-	-	-
Cadavarine	-	-0.03	-	-	-	-	-
Putrescine	-	-0.03	-	-	-	-	-
Aminobutyric acid	-	-0.04	-	-	-	-	-
Hydroxybutyric acid	-	-0.06	-	-	-	-	-
Glutathione	-	-0.08	-	-	-	-	-
Glutamine	-	-0.03	-	-	-	-	-
Asparagine	-	-0.04	-	-	-	-	-
Tween 80	-	+0.22	-	-	-	-	-
Tween 40	-	+0.15	-	-	-	-	-
Tergitol 7	-	-0.03	-	-	-	-	-
Oxalacetic acid	-	-	+0.21	+0.19	+0.19	-	-
Fumaric acid	-	-	+0.61	+0.68	-	+0.92	-
Succinic acid	-	-	+0.09	+0.17	-	+0.32	-
α-Ketoglutaric acid	-	-	0	-0.01	-	-0.07	-
Malic acid	-	-	+0.01	+0.06	-	+0.09	-
Citric acid	-	-	+0.14	+0.13	-	0	-
Acetic acid	-	-	+0.02	+0.02	-	+0.01	-
Lactic acid	-	-	+0.05	+0.10	-	+0.16	-
Pyruvic acid	-	-	+0.04	+0.12	-	-0.01	-
Propionic acid	-	-	-0.01	0	-	-0.04	-
Butyric acid	-	-	-0.01	+0.02	-	-0.03	-
Sodium bicarbonate	-	-	-	-	-	-	-0.01

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Dash (-) indicates that no test was run at this level.

Table 6. Influence of various test compounds on total growth of *Vibrio fetus* strain 085

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	-0.09	-	-	-	-	-
Spermidine	-	-0.07	-	-	-	-
Cadavarine	-	-0.08	-	-	-	-
Putrescine	-	-0.05	-	-	-	-
Aminobutyric acid	-	-0.03	-	-	-	-
Hydroxybutyric acid	-	-0.02	-	-	-	-
Glutathione	-	-0.24	-	-	-	-
Glutamine	-	-0.26	-	-	-	-
Asparagine	-	-0.25	-	-	-	-
Tween 80	-	-0.02	-	-	-	-
Tween 40	-	-0.01	-	-	-	-
Tergitol 7	-	-0.28	-	-	-	-
Fumaric acid	-	-	-0.06	-0.12	-0.05	-
Succinic acid	-	-	0	+0.06	+0.08	-
α-Ketoglutaric acid	-	-	-0.02	-0.01	0	-
Malic acid	-	-	+0.01	+0.01	+0.05	-
Oxalacetic acid	-	-	+0.01	-0.10	+0.03	-
Citric acid	-	-	+0.02	+0.03	-0.05	-
Acetic acid	-	-	+0.04	+0.02	+0.02	-
Lactic acid	-	-	+0.04	+0.09	+0.16	-
Pyruvic acid	-	-	+0.04	+0.06	+0.07	-
Propionic acid	-	-	+0.03	+0.02	+0.02	-
Butyric acid	-	-	+0.02	+0.01	+0.15	-
Sodium bicarbonate	-	-	-	-	-	-0.07

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Dash (-) indicates that no test was run at this level.

Table 7. Influence of various test compounds on total growth of *Vibrio fetus* strain 277

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	-0.02	-	-	-	-	-
Spermidine	-	-0.13	-	-	-	-
Cadavarine	-	-0.09	-	-	-	-
Putrescine	-	-0.08	-	-	-	-
Aminobutyric acid	-	-0.08	-	-	-	-
Hydroxybutyric acid	-	-0.01	-	-	-	-
Glutathione	-	-0.20	-	-	-	-
Glutamine	-	-0.15	-	-	-	-
Asparagine	-	-0.15	-	-	-	-
Tween 80	-	-0.09	-	-	-	-
Tween 40	-	-0.05	-	-	-	-
Tergitol 7	-	-0.20	-	-	-	-
Fumaric acid	-	-	-0.01	0	+0.08	-
Succinic acid	-	-	+0.03	+0.03	+0.02	-
α-Ketoglutaric acid	-	-	+0.03	+0.04	+0.04	-
Malic acid	-	-	+0.01	-0.01	+0.07	-
Oxalacetic acid	-	-	0	+0.02	+0.10	-
Citric acid	-	-	+0.03	0	+0.02	-
Acetic acid	-	-	-0.02	+0.02	+0.04	-
Lactic acid	-	-	+0.06	+0.23	+0.15	-
Pyruvic acid	-	-	+0.03	+0.01	-0.02	-
Propionic acid	-	-	-0.01	-0.09	-0.11	-
Butyric acid	-	-	+0.02	+0.04	-0.05	-
Sodium bicarbonate	-	-	-	-	-	-0.06

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Dash (-) indicates that no test was run at this level.

Table 8. Influence of various test compounds on total growth of Vibrio fetus strain Kohler and strain 279

Compound	mg/100 ml 30 mg		100 mg		400 mg	
	Kohler	279	Kohler	279	Kohler	279
Fumarate	+0.02	+0.03	+0.03	0	+0.05	+0.02
Succinate	+0.01	+0.03	+0.03	+0.03	+0.13	+0.05
α-Ketoglutarate	-0.02	-0.01	+0.02	+0.02	+0.08	+0.04
Malate	+0.01	+0.01	0	-0.11	+0.05	+0.06
Oxalacetate	+0.01	+0.04	+0.03	+0.06	-	-
Citrate	-0.05	+0.02	+0.02	-0.01	0	-0.03

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium. Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Table 9. Influence of various test compounds on total growth of Vibrio fetus strain 30

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	0					
Spermidine	-	-0.13	-	-	-	-
Cadavarine	-	-0.11	-	-	-	-
Putrescine	-	-0.08	-	-	-	-
Aminobutyric acid	-	-0.10	-	-	-	-
Hydroxybutyric acid	-	-0.06	-	-	-	-
Glutathione	-	-0.10	-	-	-	-
Glutamine	-	-0.07	-	-	-	-
Asparagine	-	-0.07	-	-	-	-
Tween 80	-	-0.13	-	-	-	-
Tween 40	-	-0.08	-	-	-	-
Tergitol 7	-	-0.22	-	-	-	-
Succinic acid	-	-	-0.09	-0.07	0	-
α-Ketoglutaric acid	-	-	+0.02	+0.05	+0.09	-
Malic acid	-	-	-0.04	-0.04	-0.10	-
Citric acid	-	-	+0.06	+0.12	+0.05	-
Acetic acid	-	-	-0.04	-0.07	-0.05	-
Lactic acid	-	-	0	+0.01	+0.01	-
Pyruvic acid	-	-	+0.01	+0.04	+0.03	-
Propionic acid	-	-	+0.03	+0.02	+0.02	-
Butyric acid	-	-	+0.06	+0.05	+0.05	-
Sodium bicarbonate	-	-	-	-	-	-0.09

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Dash (-) indicates that no test was run at this level.

400 mg inhibited the growth of this strain. Other compounds which inhibited growth of strain 30 were spermidine, cadavarine, aminobutyrate, glutathione, tween 80, and tergitol 7.

The 400 mg concentrations of succinate, α-ketoglutarate, and pyruvate stimulated growth of strain S_2 (Table 10). The 100 mg and 400 mg levels of lactate were also stimulatory. Citrate and butyrate at a concentration of 30 mg stimulated the growth of strain S_2. Cadavarine, tween 80, and tween 40 were also stimulatory. However, glutathione, glutamine, and asparagine inhibited the growth of strain S_2.

The production of APM by V. fetus strain 3530 in the chemically defined medium is shown in Table 11. The amount of APM produced by strain 3530 was increased by the 100 mg and 400 mg concentrations of lactate. These levels of lactate also increased growth. Tween 80 and tween 40 increased APM production as well as growth. APM production was decreased by the presence of tergitol 7 in the medium. However, tergitol 7 did not decrease the growth of strain 3530.

Table 12 shows that the 100 mg and 400 mg concentrations of α-ketoglutarate increased the amount

Table 10. Influence of various test compounds on total growth of Vibrio fetus strain S_2

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	0	-	-	-	-	-
Spermidine	-	0	-	-	-	-
Cadavarine	-	+0.12	-	-	-	-
Putrescine	-	+0.09	-	-	-	-
Aminobutyric acid	-	+0.09	-	-	-	-
Hydroxybutyric acid	-	-0.07	-	-	-	-
Glutathione	-	-0.12	-	-	-	-
Glutamine	-	-0.12	-	-	-	-
Asparagine	-	-0.10	-	-	-	-
Tween 80	-	+0.14	-	-	-	-
Tween 40	-	+0.19	-	-	-	-
Tergitol 7	-	-0.09	-	-	-	-
Succinic acid	-	-	0	+0.05	+0.10	-
d-Ketoglutaric acid	-	-	+0.01	+0.02	+0.11	-
Malic acid	-	-	+0.01	+0.01	-0.08	-
Citric acid	-	-	+0.11	0	0	-
Acetic acid	-	-	+0.01	+0.07	+0.02	-
Lactic acid	-	-	+0.04	+0.10	+0.22	-
Pyruvic acid	-	-	0	+0.08	+0.23	-
Propionic acid	-	-	0	0	0	-
Butyric acid	-	-	+0.13	+0.04	-0.01	-
Sodium bicarbonate	-	-	-	-	-	-0.04

Results expressed as the difference in optical density (540 mμ) between the growth of the cultures in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

Cultures were diluted 1:6 with formalized 0.85% physiological sodium chloride solution.

Dash (-) indicates that no test was run at this level.

Table 11. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain 3530

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	+0.03	-	-	-	-	-
Spermidine	-	-0.01	-	-	-	-
Cadavarine	-	+0.04	-	-	-	-
Putrescine	-	-0.02	-	-	-	-
Aminobutyric acid	-	-0.01	-	-	-	-
Hydroxybutyric acid	-	-0.03	-	-	-	-
Glutathione	-	-0.09	-	-	-	-
Glutamine	-	+0.06	-	-	-	-
Asparagine	-	+0.04	-	-	-	-
Tween 80	-	+0.29	-	-	-	-
Tween 40	-	+0.10	-	-	-	-
Tergitol 7	-	-0.14	-	-	-	-
Succinic acid	-	-	+0.04	-	+0.09	-
α-Ketoglutaric acid	-	-	-0.04	+0.01	+0.07	-
Malic acid	-	-	+0.07	-	-	-
Acetic acid	-	-	+0.02	0	+0.03	-
Lactic acid	-	-	+0.01	+0.18	+0.14	-
Pyruvic acid	-	-	+0.22	-0.12	-0.22	-
Propionic acid	-	-	+0.06	+0.02	-0.03	-
Butyric acid	-	-	-0.01	+0.01	0	
Sodium bicarbonate	-	-	-	-	-	+0.03

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of the culture.

Dash (-) indicates that no test was run at this level.

Table 12. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain 085

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	-0.11	-	-	-	-	-
Spermidine	-	-0.07				
Cadavarine	-	0	-	-	-	-
Putrescine	-	+0.01	-	-	-	-
Aminobutyric acid	-	-0.01	-	-	-	-
Hydroxybutyric acid	-	-0.04	-	-	-	-
Glutathione	-	-0.24	-	-	-	-
Glutamine	-	-0.24	-	-	-	-
Asparagine	-	-0.24	-	-	-	-
Tween 80	-	+0.14	-	-	-	-
Tween 40	-	+0.01	-	-	-	-
Tergitol 7	-	-0.27	-	-	-	-
Succinic acid	-	-	+0.02	+0.01	+0.06	-
α-Ketoglutaric acid	-	-	+0.03	+0.12	+0.26	-
Malic acid	-	-	-0.02	-0.04	+0.07	-
Citric acid	-	-	0	+0.03	+0.01	-
Acetic acid	-	-	+0.04	+0.06	0	-
Lactic acid	-	-	+0.06	+0.07	+0.13	-
Pyruvic acid	-	-	+0.09	+0.03	-0.17	-
Propionic acid	-	-	+0.02	0	-0.06	-
Butyric acid	-	-	+0.06	+0.02	+0.02	-
Sodium bicarbonate	-	-	-	-	-	-0.06

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of the culture.

Dash (-) indicates that no test was run at this level.

of APM produced by strain 085. However, these levels did not show a similar increase in growth. The 400 mg concentration of lactate increased APM production as well as growth of strain 085. The amount of APM was increased by the presence of tween 80 in the medium; however, growth was not increased by tween 80. Glutathione, glutamine, asparagine, tergitol 7, and phenylacetyl chloride all inhibited the production of APM as well as growth of strain 085.

Table 13 shows that the production of APM by strain 277 was stimulated by the 100 mg and 400 mg concentrations of α-ketoglutarate. All levels of malate stimulated APM production. Tween 80 and tween 40 also increased the amount of APM produced. None of the above compounds increased the growth of strain 277. The 400 mg concentration of propionate decreased APM production as well as the growth of strain 277. Spermidine, cadavarine, putrescine, aminobutyrate, glutathione, tergitol 7, and sodium bicarbonate also decreased the amount of APM produced as well as the growth of strain 277.

APM production by strain 30 was increased by the 400 mg concentration of succinate and the 30 mg level of butyrate (Table 14). However, these compounds did

Table 13. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain 277.

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	+0.12	-	-	-	-	-
Spermidine	-	-0.16	-	-	-	-
Cadavarine	-	-0.17	-	-	-	-
Putrescine	-	-0.14	-	-	-	-
Aminobutyric acid	-	-0.14	-	-	-	-
Hydroxybutyric acid	-	+0.03	-	-	-	-
Glutathione	-	-0.12	-	-	-	-
Glutamine	-	-0.04	-	-	-	-
Asparagine	-	-0.06	-	-	-	-
Tween 80	-	+0.10	-	-	-	-
Tween 40	-	+0.11	-	-	-	-
Tergitol 7	-	-0.25	-	-	-	-
Succinic acid	-	-	-0.01	-0.04	-0.04	-
α-Ketoglutaric acid	-	-	+0.09	+0.13	+0.28	-
Malic acid	-	-	+0.13	+0.13	+0.14	-
Citric acid	-	-	-0.04	0	+0.09	-
Acetic acid	-	-	-0.01	+0.02	-0.01	-
Lactic acid	-	-	-0.06	+0.09	+0.05	-
Pyruvic acid	-	-	+0.04	-0.03	-0.13	-
Propionic acid	-	-	-0.03	-0.08	-0.14	-
Butyric acid	-	-	+0.03	+0.06	-0.09	-
Sodium bicarbonate	-	-	-	-	-	-0.10

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of the culture.

Dash (-) indicates that no test was run at this level.

Table 14. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain 30

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	+0.06	-	-	-	-	-
Spermidine	-	-0.13	-	-	-	-
Cadavarine	-	-0.17	-	-	-	-
Putrescine	-	-0.13	-	-	-	-
Aminobutyric acid	-	-0.08	-	-	-	-
Hydroxybutyric acid	-	+0.06	-	-	-	-
Glutathione	-	-0.24	-	-	-	-
Glutamine	-	-0.03	-	-	-	-
Asparagine	-	-0.01	-	-	-	-
Tween 80	-	-0.06	-	-	-	-
Tween 40	-	-0.08	-	-	-	-
Tergitol 7	-	-0.37	-	-	-	-
Succinic acid	-	-	+0.06	+0.07	+0.10	-
α-Ketoglutaric acid	-	-	-0.07	0	+0.07	-
Malic acid	-	-	-0.05	0	-0.08	-
Citric acid	-	-	+0.06	+0.06	0	-
Acetic acid	-	-	-0.01	-0.05	+0.01	-
Lactic acid	-	-	-0.07	-0.03	0	-
Pyruvic acid	-	-	+0.01	+0.07	+0.04	-
Propionic acid	-	-	-0.05	-0.01	-0.05	-
Butyric acid	-	-	+0.12	+0.04	-0.02	-
Sodium bicarbonate	-	-	-	-	-	-0.17

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of the culture.

Dash (-) indicates that no test was run at this level.

Table 15. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain S_2

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	0	-	-	-	-	-
Spermidine	-	0	-	-	-	-
Cadavarine	-	+0.10	-	-	-	-
Putrescine	-	+0.08	-	-	-	-
Aminobutyric acid	-	+0.11	-	-	-	-
Hydroxybutyric acid	-	-0.07	-	-	-	-
Glutathione	-	-0.03	-	-	-	-
Glutamine	-	-0.04	-	-	-	-
Asparagine	-	-0.04	-	-	-	-
Tween 80	-	+0.18	-	-	-	-
Tween 40	-	+0.09	-	-	-	-
Tergitol 7	-	-0.11	-	-	-	-
Succinic acid	-	-	+0.02	+0.15	+0.07	-
α-Ketoglutaric acid	-	-	+0.04	-	+0.40	-
Malic acid	-	-	+0.07	+0.14	-	-
Acetic acid	-	-	0	+0.03	+0.07	-
Lactic acid	-	-	-0.03	+0.18	+0.41	-
Pyruvic acid	-	-	+0.06	+0.13	+0.14	-
Propionic acid	-	-	-0.08	-0.02	-0.03	-
Butyric acid	-	-	+0.07	+0.02	-0.02	-
Sodium bicarbonate	-	-	-	-	-	+0.05

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of culture.

Dash (-) indicates that no test was run at this level.

not increase growth. Spermidine, cadavarine, putrescine, glutathione, tergitol 7, and sodium bicarbonate inhibited production of APM. These compounds also inhibited growth of strain 30.

Table 15 shows that production of APM by strain S_2 was stimulated by the 100 mg and 400 mg concentrations of lactate. The 400 mg concentration of α-ketoglutarate also increased APM. These levels of lactate and α-ketoglutarate also increased the growth of strain S_2. The 100 mg concentrations of succinate and malate increased APM, but growth was not increased by these compounds. The amount of APM produced was increased by the addition of cadavarine, aminobutyrate, and tween 80 to the basal medium. However, these compounds also increased growth of strain S_2. The presence of tergitol 7 in the medium inhibited APM production as well as the growth of strain S_2.

The growth of four of five strains of vibrios tested was increased when lactate was added to the chemically defined medium. Succinate as well as citrate increased the growth of three of five strains.

Glutathione added to the chemically defined medium inhibited the growth of four of five strains of vibrios

Table 15. Influence of various test compounds on the production of acid precipitable material by Vibrio fetus strain S_2

Compound	mg/100 ml					
	10 mg	20 mg	30 mg	100 mg	400 mg	710 mg
Phenylacetyl chloride	0	-	-	-	-	-
Spermidine	-	0	-	-	-	-
Cadavarine	-	+0.10	-	-	-	-
Putrescine	-	+0.08	-	-	-	-
Aminobutyric acid	-	+0.11	-	-	-	-
Hydroxybutyric acid	-	-0.07	-	-	-	-
Glutathione	-	-0.03	-	-	-	-
Glutamine	-	-0.04	-	-	-	-
Asparagine	-	-0.04	-	-	-	-
Tween 80	-	+0.18	-	-	-	-
Tween 40	-	+0.09	-	-	-	-
Tergitol 7	-	-0.11	-	-	-	-
Succinic acid	-	-	+0.02	+0.15	+0.07	-
α-Ketoglutaric acid	-	-	+0.04	-	+0.40	-
Malic acid	-	-	+0.07	+0.14	-	-
Acetic acid	-	-	0	+0.03	+0.07	-
Lactic acid	-	-	-0.03	+0.18	+0.41	-
Pyruvic acid	-	-	+0.06	+0.13	+0.14	-
Propionic acid	-	-	-0.08	-0.02	-0.03	-
Butyric acid	-	-	+0.07	+0.02	-0.02	-
Sodium bicarbonate	-	-	-	-	-	+0.05

Results expressed as the difference in optical density (380 mμ) between APM produced by the cultures grown in the control chemically defined medium and the cultures grown in the experimental chemically defined medium.

APM was precipitated by adding 3.0 ml of trichloroacetic acid to 3.0 ml of culture.

Dash (-) indicates that no test was run at this level.

tested. The growth of three of five strains was inhibited when glutamine, asparagine, or tergitol 7 was added to the chemically defined medium.

Alpha-ketoglutarate added to the chemically defined medium was stimulatory to the production of APM by three of five strains tested. However, growth of only one strain was increased in the presence of α-ketoglutarate. APM production of three of five strains was increased in the presence of lactate. This compound increased the growth of four of five strains. Tween 80 was stimulatory to the production of APM by four of five strains tested. However, there was no increase in growth by any of the strains in the presence of tween 80.

Glutathione added to the chemically defined medium inhibited the production of APM by three of five strains of vibrios tested. This compound inhibited growth of four of five strains. APM production of all strains tested was inhibited by the addition of tergitol 7 to the chemically defined medium. The growth of three of five strains was also inhibited by this compound.

DISCUSSION

This investigation has shown that although the chemically defined medium of Smibert (1963) supported the growth of all strains of V. fetus tested, compounds which stimulated growth of some strains could be added to the medium. Lactate stimulated the growth of four of five strains and did not inhibit the growth of any strains of vibrios tested. Lactate increased APM production due to the increased number of cells produced in its presence. Therefore, the addition of lactate to the chemically defined medium used for growth or APM production would be advantageous. Succinate and citrate stimulated the growth of three of seven strains of vibrios tested and did not inhibit the growth of any strains. Succinate and citrate could also be added to the chemically defined medium used for obtaining maximal growth of V. fetus. The increased APM production of the vibrios in the presence of α-ketoglutarate or tween 80 was due to an increased APM production by each cell, since these compounds neither increased nor decreased growth of the vibrios. Alpha-ketoglutarate would be a desirable addition to the chemically defined medium used

for production of APM and may be useful in studying the biosynthesis of APM. Although tween 80 inhibited growth of one of seven strains of vibrios tested, it could also be added to the chemically defined medium used for production of APM. However, α-ketoglutarate would be a more desirable addition to the medium.

The compounds which increased APM of each cell may have been involved in the biosynthesis of APM. A compound, such as citrate, which increased growth but did not affect APM production, probably was not utilized in the biosynthesis of APM. This compound may have inhibited the synthesis of APM, since an increased growth would normally result in increased APM production.

Nageswararao and Blobel (1963) demonstrated the antigenic nature of APM isolated from broth cultures of V. fetus. The toxicity of APM was shown by Osborne and Smibert (1962). Compounds which increased APM production may be added to the medium when studies involving the antigenic nature and toxicity of APM are conducted. Compounds that increased APM production would facilitate also the study of the biosynthesis of APM.

The study of a specific strain of V. fetus could be facilitated by adding to the chemically defined medium compounds that stimulated the growth of that particular strain. For example, compounds such as fumarate and succinate stimulated growth of strain 3530. These compounds could be added to the chemically defined medium used for growing this particular strain.

None of the compounds stimulated the growth of strain 279. Succinate slightly stimulated the growth of strain Kohler. The chemically defined medium of Smibert supported growth of these strains quite adequately. Therefore, the study of strains 279 and Kohler was discontinued after testing the effect of six compounds on growth of these strains.

Test compounds such as glutathione which inhibited the growth of four of five strains of vibrios tested should not be included in the chemically defined medium of Smibert. Tergitol 7, glutamine, and asparagine all inhibited the growth of three of five strains tested and should, therefore, not be added to the medium. Compounds which inhibited APM production should be avoided as additions to the chemically defined medium. Tergitol 7 inhibited APM production of all strains tested, while

glutathione inhibited APM production of three of five strains.

Alexander (1957) found that citrate did not serve as an energy source of V. fetus. The present investigation has shown that the growth of three of seven strains of V. fetus was increased by the addition of citrate to the medium. The strains used in this investigation differed from those used by Alexander. Different strains of V. fetus may vary in their ability to use some compounds as energy sources. Citrate served as an energy source of three of seven V. fetus strains used in this study, while this compound did not serve as an energy source of the strains used by Alexander. Membrane permeability to various compounds may also differ among strains. Three of seven strains used in this investigation obviously possessed membranes which were permeable to citrate.

Kiggens and Plastridge (1958), using the Warburg technique, reported that there was rapid oxygen uptake with all members of the tricarboxylic acid cycle when a cell-free extract was used. They found, however, that whole cells showed slight or no oxygen uptake with citrate. Cells of the V. fetus strains used by these workers had a permeability barrier to citrate,

cis-aconitate, isocitrate, and α-ketoglutarate. However, the cells of most of the strains used in the present investigation were permeable to citrate.

Alexander (1957) reported that α-ketoglutarate was used as an energy source by the strains of V. fetus he tested. Lecce (1958) studied the oxidative capability of resting cells of 27 strains of V. fetus. He found that α-ketoglutarate was used as an electron donor. Zemjanis and Hoyt (1960b) reported that certain concentrations of α-ketoglutarate enhanced the growth of V. fetus. The present investigation has shown that growth of only one of seven V. fetus strains tested was stimulated by the addition of α-ketoglutarate to the chemically defined medium. The chemically defined medium used in the study may have provided an adequate supply of glutamate for deamination to α-ketoglutarate (Hoff-1956; Smibert-personal communication); therefore, the α-ketoglutarate added to the medium was not needed for growth. As mentioned previously, Kiggens and Plastridge (1958) reported that there was rapid oxygen uptake with all members of the tricarboxylic acid cycle when a cell-free extract was used. However, whole cells showed slight or no oxygen uptake with α-ketoglutarate.

The strains used in this study did not possess a permeability barrier to α-ketoglutarate as evidenced by the fact that the production of APM by three of five strains tested was increased by the addition of α-ketoglutarate to the medium.

Some of the tricarboxylic acid cycle intermediates tested did not stimulate growth of the strains of V. fetus used in this study. The cell membrances may have been impermeable to compounds such as malate which did not stimulate growth or APM production. Some of the strains possibly could have used a shunt of the tricarboxylic acid pathway, thereby eliminating compounds such as fumarate as growth stimulating compounds.

SUMMARY

Lactate, added to the chemically defined medium of Smibert (1963), increased the growth of four of five strains of V. fetus tested and did not inhibit the growth of any strains. The production of APM was increased in the presence of lactate. Succinate and citrate, added to the medium, increased growth of three of five strains of vibrios and did not decrease growth of any strains of V. fetus tested. Alpha-ketoglutarate, added to the chemically defined medium, increased the production of APM by three of five strains of V. fetus. The addition of tween 80 to the medium increased the production of APM by four of five strains of vibrios. The increased APM production by the vibrios in the presence of α-ketoglutarate or tween 80 was not accompanied by a similar increase in growth. Alpha-ketoglutarate and tween 80 increased the production of APM by each vibrio cell. Glutathione, glutamine, asparagine, and tergitol 7 all decreased the growth of V. fetus. Glutathione and tergitol 7 decreased the production of APM by V. fetus.

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ABSTRACT

Title of Thesis: The stimulation or inhibition of growth and acid precipitable material production of Vibrio fetus by Kreb's cycle intermediates, fatty acids, amines, and miscellaneous compounds.

Caroline Amiss Kowalak, Master of Science, 1964

Thesis directed by: Dr. Robert M. Smibert

Using the chemically defined medium designed by Smibert (1963), the stimulatory or inhibitory effect of test compounds on the growth and the production of acid precipitable material by seven strains of Vibrio fetus was determined. The test compounds were Kreb's cycle intermediates, lactate, pyruvate, fatty acids, amines, surface active agents, aminobutyrate, hydroxybutyrate, glutathione, asparagine, phenylacetyl chloride, and sodium bicarbonate. Experimental media consisted of the chemically defined medium containing various concentrations of the test compounds. An exact number of cells of each strain of V. fetus was inoculated into tubes of experimental broth media. The cultures were incubated at 37 C in desiccator jars in an atmosphere

containing 85% nitrogen, 10% carbon dioxide, and 5% oxygen. Cultures were observed daily for visible growth. After five days incubation, the optical density of each culture was read at 540 mμ in a spectrophotometer. The concentration of APM produced by each strain of V. fetus grown in experimental media was also determined. Five day old cultures of each strain were centrifuged, and the APM present in the supernatant fluid was precipitated with trichloroacetic acid. The optical density of the mixture was read at 380 mμ in the spectrophotometer.

Lactate increased the growth of four of five strains of V. fetus tested, and did not inhibit the growth of any strains. Lactate increased APM production of V. fetus due to the increased number of cells produced in its presence. Therefore, the addition of lactate to the chemically defined medium used for growth or APM production would be advantageous. Citrate and succinate, which increased the growth of V. fetus without affecting APM production, could be added to the chemically defined medium used for obtaining maximal growth of vibrios. Studies requiring a large cell crop of vibrios would be facilitated by the use of the chemically defined medium to which lactate, citrate, and/or succinate had been

added. A large cell crop of V. fetus would be useful in preparation of V. fetus cellular antigen. Any study requiring large cell crops of vibrios would be facilitated by the use of a medium which increased the growth of V. fetus.

Alpha-ketoglutarate and tween 80 increased APM production of V. fetus, although these compounds neither increased nor decreased the growth of the vibrios. Therefore, the addition of α-ketoglutarate and tween 80 to the medium used for maximal production of APM would be advantageous. An increased production of APM by V. fetus would aid the study of the biosynthesis of APM. Also, the antigenic nature of APM could be studied more successfully by using a medium which increased the production of APM by vibrios.

Compounds, such as glutathione and tergitol 7, which inhibited the growth and the production of APM of vibrios should not be added to the chemically defined medium. Glutamine and asparagine inhibited the growth of V. fetus and should also be avoided as additions to the medium.