The utilization of carbohydrate intermediates and amino acids by Vibrio Fetus
by James K Alexander

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

Abstract:

A suitable basal medium was developed to determine the utilization of various energy sources by Vibrio fetus. This basal medium consisted of 0.575 per cent Difco's dehydrated brain liver heart medium, 0.5 per cent sodium chloride, 0.1 per cent disodium phosphate, and 0.05 per cent agar.

Under the conditions of these experiments lactate, pyruvate, acetate, oxaloacetate, alpha-ketoglutarate, succinate, fumarate, malate, aspartate, asparagine, glutamate, and proline were found to be suitable energy sources. Serine supported growth of only one of the two strains used in this study. Glucoae, hexosediphosphate, glycerol, glycerophosphate, citrate, isocitrate, oxalosuccinate, tartrate, glycine, alanine, leucine, lysine, ornithine, arginine, histidine, phenylalanine, threonine, cysteine and methionine all were unable to support appreciable growth.

Resting cell suspensions oxidized lactate, pyruvate, isocitrate, and fumarate. Hexosediphosphate may have been oxidized slightly. Glucose and glycerophosphate were not oxidized.

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INTRODUCTION

Although Vibrio fetus was recognized as the probable causative agent of cases of infectious abortion over 40 years ago, little is known of the cultural characteristics except that it is an obligate microaerophile and difficult to cultivate. Consequently, the cultural methods presently employed are unsatisfactory for there is no way to distinguish V. fetus from similar vibrios. Moreover, it is difficult to isolate this organism.

The inadequacy of the cultural methods has discouraged studies of the natural habitat of the organism and of transmission factors involved in the disease.

In view of this lack of knowledge it appears that a more complete understanding of the metabolic characteristics of this organism might aid in developing more suitable cultural methods. A better understanding of the metabolism might make it possible to distinguish V. fetus from similar vibrios. Moreover, a knowledge of the nutritional requirements could possibly lead to the development of a partially selective medium for the isolation of this organism.

The determination of the utilizable energy sources was undertaken as the first step in studying the metabolism of this organism. Various compounds have been investigated to see if they would serve as sources of energy for the growth of this organism. In addition to this, resting cell suspensions were used to determine the ability of this organism to oxidize several compounds. The growth factor and amino acid requirements have been left for future studies.

HISTORICAL REVIEW

The first report of vibrios being associated with infectious abortion in sheep and cattle was made by McFadyean and Stockman in 1913 (cited by Smith, 1918).

Smith and Taylor (1919) studied the cultural characteristics of an organism which was presumably the same as the vibrio isolated by McFadyean and Stockman and named the organism Vibrio fetus. Although the organism is found both in the form of a spirilium and in the form of a vibrio, the latter form predominates in young cultures, hence, the generic name of Vibrio was chosen. The organism was described as relatively difficult to cultivate immediately after isolation. After repeated transfers the organism sometimes became saprophitized and cultivation became less difficult. Indole was not formed in bouillon, and no growth was attained in milk or nutrient gelatin or on potato medium. The organism produced no gas and the reaction remained neutral or slightly acid in fermented bouillon with 1 per cent dextrose, lactose, or saccharose. A reduced oxygen tension was reported as necessary for growth of the organism.

Stockman (1919) reported that no growth of V. fetus occurred either in a free supply of air or under strictly anaerobic conditions. Barger (1928) grew the organism in an atmosphere in which about 10 per cent of the air was replaced by carbon dioxide.

The concentration of atmospheric carbon dioxide most suitable for growth was determined by Plastridge and Williams (1943). With a semisolid medium of liver infusion and peptone adjusted to pH 7.4 the most abundant growth was obtained when 10 per cent of the air was replaced by carbon dioxide.

No growth was obtained when 30 per cent of the air was replaced by carbon dioxide. These workers also investigated the utilization of carbohydrates by this organism. No evidence for acid production was found with 1 per cent arabinose, dextrose, dextrin, dulcitol, gelactose, inulin, lactose, levulose, maltose, mannitol, raffinose, salicin, sucrose, sorbitol or trehelose. The basal medium used in this study consisted of beef infusion, 1.0 per cent peptone, 0.3 per cent agar, and 1.0 per cent Andrade's indicator.

The nutrition of V. fetus was studied by Batlin and Wilson (1950). A medium consisting of hydrolysed casein, salts, and growth factors was sufficient to maintain growth through serial transfers. Likewise a medium composed of 16 amino acids, salts, and growth factors was also sufficient to maintain growth. These media were prepared with 1 per cent methocel as a thickening agent and incubation was under 10 per cent added carbon dioxide.

According to Plastridge et. al., (1951) increased carbon dioxide tension is required for growth of recently isolated strains of V. fetus and enhances the growth of other strains. May (1953) found that the organism apparently did not require increased carbon dioxide in the atmosphere. The most abundant growth was obtained when about one-half of the air was removed. No surface growth was attained in a free supply of air.

The atmospheric requirements of V. fetus are somewhat puzzling. From a review of the literature it appears that this organism is an obligate aerobe, but requires either a reduced oxygen tension or an increased carbon dioxide tension for growth. Apparently it is more difficult to provide favorable atmospheric conditions for surface growth than for growth in a semisolid medium or in a broth medium. The effect of additional carbon dioxide in the atmosphere does not appear to be understood.

MATERIALS AND METHODS

Two strains of V. fetus, both of ovine origin, were used in this study. These strains, obtained from the Veterinary Research Laboratory, Montana State College, Bozeman, Montana, had been assigned accession numbers 2035 and 4440 by that laboratory.

Stock cultures were maintained in tubes containing about 7 ml of Difco's brain liver heart semisolid medium. This medium contained 46 g of dehydrated brain liver heart per liter. The dehydrated brain liver heart was stored in the cold as a precaution against deterioration for May (1953) found that this medium sometimes underwent progressive deterioration if stored at room temperature.

Preliminary Work. A search was made for a suitable basal medium for the growth experiments. This basal medium was designed to supply an excess amount of the growth factors and amino acids necessary for the growth of this organism but to supply only a limited amount of utilizable energy sources. Since the growth factor and amino acid requirements of this organism are unknown, no attempt was made to develop a chemically defined medium.

Two basal media were tried. One contained yeast extract, peptone, soluble starch, sodium chloride and agar. The other contained six growth factors (thiamine, pyridoxal, riboflavin, nicotinamide, calcium pantothenate, and biotin) casamino acids, additional tryptophane and cysteine, soluble starch, sodium chloride and agar. No appreciable increase in growth resulted when a suitable energy source was added to these media.

In the course of this preliminary work, a variant of <u>V. fetus</u> was encountered that grew well on these basal media. However, since it obviously was less demanding in its cultural requirements than was the parent strain, it was not used for the remainder of the study.

A medium containing about 0.3 per cent dehydrated brain liver heart and additional sodium chloride and agar was found to support a slight amount of growth. No growth was attained unless sodium chloride was added to the medium. Apparently the organism requires a certain, osmotic pressure for growth because the addition of 5 per cent yeast autolysate or horse serum or 0.1 per cent casamino acids would not replace sodium chloride.

The organisms were grown on agar slants at 35 C in Brewer anaerobe jars with about one-half of the air removed. A marked increase in the amount of growth was noted when a suitable energy source was added to this basal medium. However, attempts to measure the amount of growth attained on the agar slants were unsuccessful because it was difficult to obtain uniform cell suspensions. Also, when washing the cells off the agar surface, a small amount of agar was often introduced into the cell suspension. It was therefore necessary to find a method whereby quantitative measurements could be made.

Several unsuccessful attempts were made to develop a broth medium using a small concentration (about 0.3 per cent) of dehydrated brain liver heart with additional sodium chloride. No growth was attained in this medium unless a reducing agent was added. A slight amount of growth was attained when sodium thioglycollate or sodium sulfide were added to the medium. However, no growth resulted when sodium formaldehyde sulfoxylate was added to the medium.

Basal medium. A satisfactory basal medium was developed by increasing the concentration of dehydrated brain liver heart from about 0.3 per cent to about 0.6 per cent and using a small amount of agar, presumably to maintain a reduced oxygen tension. The basal medium used in the growth studies contained 0.57 per cent dehydrated brain liver heart medium, 0.5 per cent sodium chloride, 0.1 per cent disodium phosphate and 0.05 per cent agar. The concentration of the energy sources was 0.2 per cent.

The pH of the medium was adjusted electrometrically to 7.0 after the addition of the energy sources. The phosphate buffer was added to prevent the pH of the medium from changing during autoclaving. The buffer kept the pH of the medium within a range of 6.8 to 7.3, whereas without the buffer the pH changed from 7.0 to about 8.0 when certain energy sources were added. Apparently this change in pH was due to the partial decomposition of some of the added energy sources when heated. The medium was autoclaved at 121 C for 15 minutes.

Glucose, hexosediphosphate, oxaloacetate, oxalosuccinate, and isocitrate solutions were adjusted to neutrality, sterilized by filtration, and then added to the sterile basal medium aseptically. The oxaloacetate was prepared by the method described by Krampitz and Werkman (1941). The remaining energy sources and also glucose were added to the medium and sterilized by autoclaving. A series with no additional energy source in the basal medium and a series with lactate added to the basal medium were included in each experiment. Each compound was run at least in triplicate.

This basal medium supported only minimal growth. However, when a suitable energy source was added a marked increase in growth resulted. This increase was much greater than any difference which occurred in replicates of the same compound.

The small amount of agar added to the basal medium did not increase the viscosity of the medium appreciably. In order to obtain a uniform cell suspension the medium had to be shaken. Although this shaking caused the formation of air bubbles in the medium, these bubbles soon disappeared, and the amount of growth could be estimated turbidimetrically by determining the optical density of the suspension.

The seed cultures for the growth experiments were grown in 150 ml pyrex milk dilution bottles plugged with rubber stoppers. Each bottle contained 20 ml of Difco's brain liver heart semisolid medium and was incubated on its side. One-day-old cultures of strain 2035 were used for inoculum. Strain 4440 appeared to grow slower than did strain 2035, hence, two-day-old cultures were used as inoculum. However, it was later observed that after several rapid transfers strain 4440 apparently could grow at about the same rate as strain 2035. Each bottle received 0.1 ml of inoculum. The medium was inoculated within a few hours after autoclaving as a precaution against oxidation of the medium before growth could be established.

The organisms were grown in 25 ml of the basal medium and the added energy sources contained in milk dilution bottles. These bottles were plugged with rubber stoppers and were incubated at 35 C on their sides. Strain 2035 was incubated two days; strain 4440 was incubated five days.

The amount of growth was measured with a Klett-Summerson photoelectric colorimeter using a blue filter. Each culture was examined microscopically for contamination.

Manometric studies. A few manometric experiments were conducted in order to compare the amount of growth attained with various compounds with the ability of the organism to oxidize these compounds. The activity of resting cell suspensions was determined by conventional manometric methods (Umbreit et al., 1947).

The cells were grown in a broth medium containing 1.0 per cent tryptose, 0.5 per cent sodium chloride, 0.1 per cent yeast extract, 0.1 per cent soluble starch. The cultures were incubated one day at 35 C. It was found that a large surface area in relation to the amount of medium was necessary for the organisms to grow well. Apparently the amount of growth in this medium was limited by the amount of oxygen available. Hence, the depth of the broth was not more than about 0.7 cm. The amount of growth attained in a liter of this medium provided enough cells for one experiment.

The seed cultures were grown in 25 ml of the same medium contained in 125 ml erlenmeyer flasks and were inoculated from one day-old cultures. The contents of one flask served as inoculum for about 350 ml of medium.

The cells were centrifuged and suspended in M/50 dipotassium phosphate buffer, pH 7.0. The cells were not washed because the endogenous respiration of the unwashed cells was not great and washing appeared to render the cells less active. The oxygen uptake with unwashed cells was about 20 per cent greater than with cells washed in 1 per cent solutions of yeast extract or peptone. The concentration of the cell suspensions was adjusted turbidimetrically. Cell suspensions of about the same turbidity were used in each experiment.

One ml of cell suspension was used in each vessel; the total vessel contents were 2.3 ml. The oxygen uptake was measured in an air atmosphere at 37 C. The carbon dioxide was absorbed by alkali in the center well. The concentration of substrate in the vessels was 0.005 M. The substrates were dissolved in M/50 phosphate buffer and the pH was adjusted to 7.0. The side arm of the endogenous vessel contained 0.5 ml of M/50 phosphate buffer which was added to the vessel at the same time the substrates were added. Lactate was used as a positive control in each experiment.

Except with fumarate, the experiments were terminated after the rate of oxygen uptake in the vessels containing substrate became about the same as the rate of oxygen uptake in the endogenous vessel. All determinations were made at least in duplicate.

RESULTS

Growth studies. The amount of growth attained by two strains of *V. fetus* with various non-nitrogen containing compounds and amino acids was determined in the growth studies.

The results showing the amount of growth obtained with the non-nitrogen containing compounds are summarized in Table I. Lactate, pyruvate, alpha-ketoglutarate, succinate, fumarate, and malate all served as excellent energy sources. Acetate supported excellent growth of strain 4440 but only slight growth of strain 2035.

Glucose, whether sterilized by filtering or autoclaving, did not support appreciable growth. Of the carbohydrate intermediates,

Table I

Growth of Vibrio fetus with 0.2 per cent of the various non-nitrogen containing compounds added to the basal medium.

	Average turbidity of at least 3 determinations.	
Compound added	Strain 2035 2 Days	Strain 4440 5 Days
None*.	36	40
Glucose.	31	31
Glucose**.	38	33
Hexosediphosphate**.	40	38
Glycerol	38	36
Glycerophosphate	31	37
Lactate*	155	116
Pyruvate	193	152
Acetate.	68	144
Oxaloacetate**	68	94
Citrate.	31	31
Isocitrate**	46	38
Oxalosuccinate**		58
Alpha-ketoglutarate	183	123
Succinate.	173	189
Fumarate	206	142
Malate	166	184
Tartrate	30	43

* Represents the average of at least 21 determinations.

** Compound sterilized by filtration and added aseptically to the basal medium. Compounds not so marked were sterilized by autoclaving.

hexosediphosphate, glycerol, glycerophosphate, citrate, isocitrate and oxalosuccinate did not support appreciable growth. Likewise, no increased growth was attained with tartrate.

Tartrate was tried as a possible energy source because of the similarity between its molecular structure and the structure of succinate, fumarate, and malate. The inability of the organism to utilize tartrate might be due to the specificity of one or more enzymes involved.

Most of the compounds of the Krebs cycle served as excellent energy sources. It might possibly be that citrate was not utilized because of the lack of permeability of the cell membrane for this compound. Likewise, the poor utilization of oxaloacetate and oxalosuccinate may have been due to the limited permeability of the cell membrane for these compounds.

No increased growth was attained with any of the compounds of the glycolytic scheme which were investigated, except pyruvate. This may indicate that the organism does not have the ability to respire anaerobically. If this is true, then it may possibly be an explanation of the need for oxygen by this organism.

The results showing the amount of growth obtained when different amino acids were added to the basal medium are summarized in Table II. Aspartate, asparagine, fumarate, and proline supported growth of both strains of <u>V. fetus</u>. Serine supported excellent growth of strain 2035 but supported no appreciable growth of strain 4440. Aspartate and asparagine would be expected to be converted into oxaloacetate by deamination. Strain 4440 utilized these latter compounds better than did strain 2035. The greater utilization of these compounds by strain 4440 may be due to a more active

Table II

Growth of Vibrio fetus with 0.2 per cent of the various amino acids added to the basal medium.

Compound added	Average turbidity of at least 3 determinations. Strain 2035 2 Days	 Strain 4440 5 Days
None*	36	40
Glycine	22	33
DL-Alanine	29	38
L-Alanine	30	45
DL-Serine	104	40
L-Serine	178	47
DL-Aspartate	69	118
L-Aspartate	119	129
Asparagine	93	191
L-Glutamate	90	227
L-Proline	101	185
Leucine	58	32
L-Ornithine	52	55
L-Arginine	36	32
Histidine	25	35
DL-Threonine	22	23
L-Lysine	43	39
DL-Phenylalanine	40	2
Cysteine**	13	30
DL-Methionine	25	3

* Represents the average of at least 21 determinations.

** Some of the turbidity probably due to the formation of cystine in the medium.

enzyme or enzyme system in this strain than in strain 2035.

Strain 4440 utilized glutamate and proline better than did strain 2035. Both of these compounds would be expected to be converted into alpha-ketoglutarate. However, strain 2035 utilized alpha-ketoglutarate better than did strain 4440.

With the exception of serine the amino acids which supported appreciable growth were either transaminating amino acids or compounds which might be expected to form transaminating amino acids in the process of dissimilation. Obviously the transaminating amino acids would be expected to be most readily introduced into the Krebs cycle.

L-aspartate supported better growth of both strains than did DL-aspartate. More growth of strain 2035 was attained with L-serine than with DL-serine. However, only slight growth of strain 4440 resulted with either L-serine or DL-serine. The configuration of the alanine molecule made little difference in the amount of growth attained with either strain. With each amino acid with which growth was attained with the DL-configuration an increased amount of growth was attained with the L-configuration. A possible explanation for the more abundant growth with the L-isomer might be that the D-isomer had a blocking effect on the enzyme involved. Another possibility is that only the L-isomer was utilized and that the increased growth was due to a greater supply of the utilizable form.

Glycine, alanine, threonine, phenylalanine, histidine, lysine, arginine, cysteine, and methionine were all unable to support appreciable growth. Slight growth was afforded strain 2035 by leucine but not strain 4440. Ornithine supported only slight growth of both strains. It is possible that phenylalanine and methionine entirely inhibited growth of strain 4440. With the exception of alanine, none of these amino acids would be expected to be easily introduced into the Krebs cycle.

Manometric studies. The results of the manometric studies are summarized in Table III. In general these results are in line with the results obtained from the growth experiments. The active oxidation of isocitrate and the slight oxidation of hexosediphosphate, however, are not in accord with the results of the growth studies.

The large amount of oxygen uptake with isocitrate is difficult to understand in view of the slight amount of growth obtained with this compound.

The slight oxidation of hexosediphosphate may indicate that this compound can be oxidized by V. fetus. On the other hand, the lack of increased growth with this compound and other compounds in the glycolytic scheme, and the inability of the organism to oxidize glucose and glycerophosphate, would seem to be reason not to expect hexosediphosphate to be oxidized. The oxygen uptake with hexosediphosphate might possibly have been due to the oxidation of an impurity in the preparation and not due to the oxidation of hexosediphosphate.

Figure 1 shows the rate of oxygen uptake in one experiment. A short lag at first was evident with lactate, followed by a rapid rate of oxygen uptake. The rate of oxidation then gradually diminished, presumably because of the lack of substrate, until the rate of oxygen uptake became about the same as in the endogenous vessels. It is evident from figure 1 that the rate of oxidation of fumarate was relatively constant up to the time that

Table III

Oxidative dissimilation of energy sources by strain 2035 of *Vibrio fetus*.

Substrate (0.005M)	Oxygen uptake in microliters* (Average of at least 2 determinations)
Glucose	0
Hexosediphosphate	50
Glycerophosphate	-3
Lactate**	262
Pyruvate	280
Isocitrate	238
Fumarate***	162

* Endogenous respiration has been subtracted from the total oxygen uptake.
** Represents the average of 5 determinations.
*** Experiment terminated before oxidation was complete.

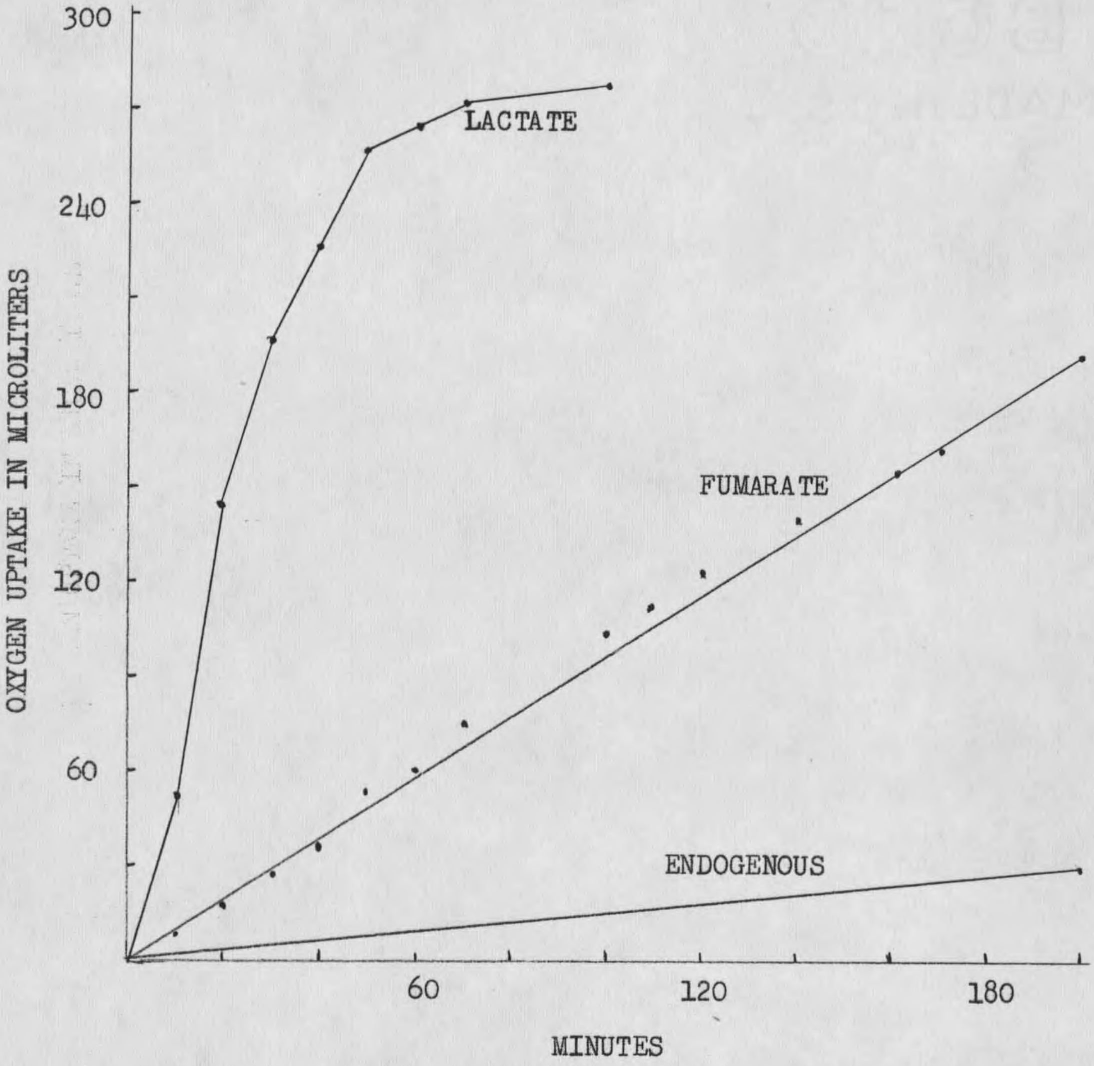

Figure 1. Rate of oxidation of lactate and fumarate by strain 2035 of *Vibrio fetus*.

the experiment was terminated. The oxidation of fumarate may be limited by the permeability of the cell membrane for this compound.

Fumarate was oxidized at a slower rate than were lactate, pyruvate, and isocitrate.

DISCUSSION

Previous to this study the media used for the cultivation of this organism have been extremely complex. As a result, isolation has been difficult because contaminants often overgrew V. fetus. The information obtained in this study might be used in developing special media for the isolation of V. fetus. Lactate, for instance, would probably serve as a suitable energy source in such media for it can be readily utilized and can be autoclaved without decomposing.

In the past, there has not been enough known to identify V. fetus by its cultural characteristics. The inability of V. fetus to either oxidize or ferment glucose would appear to be an excellent criterion for distinguishing this organism from most other vibrios.

The results of this study indicate that this organism cannot utilize any of the compounds in the glycolytic scheme except pyruvate. From what is known only compounds associated with aerobic metabolism can be readily utilized. It appears that this organism can only obtain energy from compounds in the Krebs cycle or compounds which can be easily introduced into the Krebs cycle.

In many respects V. fetus and Brucella abortus appear to resemble each other. The similarity of these organisms appears to extend beyond their pathogenic effect in cattle. Both organisms seem to have strict atmospheric requirements, are relatively difficult to cultivate, and apparently require a certain osmotic pressure for growth. In addition to these similarities there appears to be a marked resemblance between the ability of B. abortus to oxidize compounds in the Krebs cycle and amino acids and the utilization of these compounds by V. fetus. B. abortus has been found to oxidize most of the compounds in the Krebs cycle (Gerhardt, Levine, and Wilson, 1950); V. fetus was afforded excellent growth by most of these compounds. Asparagine, glutamate, and proline were oxidized by B. abortus; these compounds supported considerable growth of V. fetus. This resemblance was not absolute, however, for alanine was oxidized by B. abortus but was not utilized by V. fetus. Moreover, serine was not oxidized at an appreciable extent by B. abortus but supported growth of one strain of V. fetus. Aspartate, arginine, ornithine, histidine, leucine, lysine, phenylalanine, methionine, and threonine were not oxidized at an appreciable rate by B. abortus. With the exception of aspartate, none of these amino acids supported appreciable growth of V. fetus.

SUMMARY

The utilization of energy sources for the growth of Vibrio fetus has been investigated. It appears that only compounds in the Krebs cycle or compounds which can easily be introduced into the Krebs cycle will serve as suitable energy sources for this organism.

Lactate, pyruvate, acetate, oxaloacetate, alpha-ketoglutarate, succinate, fumarate, malate, aspartate, asparagine, glutamate and proline all served as suitable energy sources under the conditions of these

experiments. Glucose, hexosediphosphate, glycerol, glycerophosphate, citrate, isocitrate, oxalosuccinate, tartrate, glycine, alanine, threonine, cysteine, methionine, histidine, phenylalanine, leucine, lysine, ornithine and arginine did not support appreciable growth.

Resting cell suspensions oxidized lactate, pyruvate, isocitrate, and fumarate. Hexosediphosphate may have been oxidized slightly. Glucose and glycerophosphate were not oxidized.

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