METABOLIC PATHWAYS OF *CAMPYLOBACTER FETUS*

by

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to my parents

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ABSTRACT

LIST OF ABBREVIATIONS

Acetyl phosphate	Acetyl P
Acetyl Coenzyme A	Acetyl CoA
Adenosine-5'-diphosphate	ADP
Adenosine-5'-monophosphate	AMP
Adenosine-5'-triphosphate	ATP
Coenzyme A	CoA
Dihydroxyacetone phosphate	DHAP
2,6-Dichlorophenol-indophenol	DPIP
5,5'-Dithiobis-(2-nitrobenzoic acid)	DTNB
Erythrose-4-phosphate	E4P
Ethylenediamine tetraacetate	EDTA
Fructose-1,6-diphosphate	FDP
Fructose-6-phosphate	F6P
Glucose-6-phosphate	G6P
Glucose-6-phosphate dehydrogenase	G6PDH
D-glyceraldehyde-3-phosphate	D-G3P
Guanosine-5 -diphosphate	GDP
α-Glycerophosphate dehydrogenase and triose phosphate isomerase	α-GPH, TPI
2-Keto-3-deoxy-6-phosphogluconate	KDPG
α-Ketoglutarate	α-KG
Lactic dehydrogenase	LDH
Malate dehydrogenase	MDH

LIST OF ABBREVIATIONS (Continued)

Oxidized and reduced nicotinamide adenine dinucleotide	NAD, NADH
Oxidized and reduced nicotinamide adenine dinucleotide phosphate	NADP, NADPH
Oxaloacetate	OAA
Phenazine methosulfate	PMS
Phosphoenolpyruvate	PEP
6-phosphogluconate	6PG
6-phosphogluconate dehydrogenase	6PG-DH
Sedoheptulose-7-phosphate	S7P
Thiamine pyrophosphate	TPP
Triethanolamine-HCl	TEA-HCl
Tris (hydroxymethyl) aminomethane	Tris

INTRODUCTION

Campylobacter fetus is a pathogenic organism which infects cattle and sheep and causes abortion in pregnant animals. It is also the causative organism which induces hepatitis in avian species and severe diarrhea in calves and swine. It infects man and many well documented illnesses have been reported in the past twenty five years. The organism has been isolated from blood, spinal fluid, synovial fluid and stool specimens of patients suffering from abortion (Hood and Todd, 1960), meningitis (Eden, 1962), endocarditis (Lee et al. 1970), sepetic arthritis (Kilo et al.), enteritis (Dekeyser et al. 1972) and others. However, the epidemiology of human infection remains obscure (King, 1957 and Eden, 1962.)

This microaerophilic organism has been found to grow very poorly under aerobic or anaerobic conditions. The optimal gaseous environment for growth contains 5% O_2 and 10% CO_2. The common carbohydrates such as glucose, fructose, mannose, galactose, arabinose, sucrose, lactose, maltose, xylose, ribose, etc. are neither fermented nor oxidized by the organism (Plastridge and Williams, 1943; Lecce, 1958). A few compounds such as pyruvate and lactate, dicarboxylic acid members of the citric acid cycle and acetate can be oxidized by C. fetus. Aspartate, asparagine, glutamate, proline and cysteine are amino acids which can be oxidized (Kiggins and Plastridge, 1958). Presumably, the citric acid cycle is actively functioning in the organism. The metabolism of glucose in C. fetus is unknown.

It was the purpose of this study to establish the existence of the citric acid cycle pathway in C. fetus and to investigate the permeability of the organsim to glucose as well as the presence of any catabolic pathways for glucose in C. fetus.

REVIEW OF LITERATURE

Campylobacter fetus, a gram-negative, non-sporeforming, spirilly curved rod was first isolated by McFadyean and Stockman in England from cases of infectious abortion in sheep in 1909, and in cattle in 1911. Seven years later, Smith (1918) isolated the organism from aborted bovine fetuses in the United States. Smith and Taylor (1919) studied the morphological, and biological characteristics and pathogenesis of the organism and named it Vibrio fetus. But unlike the aerobic cholera group, the organism is micro-aerophilic and does not ferment carbohydrates. Furthermore, the guanine and cytosine (G+C) content of deoxyribonucleic acid (DNA) of C. fetus ranges from 32-35 moles% (Smibert, 1974). Whereas the average G+C content of DNA for members of Vibrio cholerae is 47 moles% (Shewan and Veron, 1974). Sebald and Veron (1963) therefore, proposed to remove this species from the genus Vibrio and created a new genus of Campylobacter. Smibert (1974) described the phenotypic characteristics of Campylobacter fetus in Bergey's Manual of Determinative Bacteriology, eighth edition.

A. Animal Infection by Campylobacter fetus

Campylobacter fetus is now recognized as an important cause of reduced breeding efficiency in dairy and beef cattle by lowering fertility, reducing the conception rate and causing abortion. As a result, both milk and calf production are decreased and farmers suffer serious economic losses. Grant (1955) indicated that the infection of cattle by C. fetus was considered a veneral transmitted disease. The bull acted as a sub-chronic carrier and the organisms might be retained in the testes of the bull for life.

Abortion is also a common sympton of the disease in sheep. Smibert (1965) isolated thirty seven strains of microaerophilic vibrios from the fecal and intestinal content of clinically normal sheep of age ranging from 3 months to 8 years old. Based on their morphological, cultural and biochemical characteristics, the vibrios were identified as C. fetus var. intestinalis. He also indicated that C. fetus var. intestinalis was one of the natural intestinal flora of sheep. Jensen et al. (1957) and Miller et al. (1959) showed that the infection in sheep was orally transmitted through contaminated feeds and water. Firehammer et al. (1956) reported that the ram was not a carrier in the spread of the infection.

Campylobacter fetus was reported to be the causative agent of abortion in antelope (Trueblood and Post, 1959). It was also isolated from the stomach and liver of an aborted goat fetus (Dobbs and McIntyre, 1951).

Vibrios similar to C. fetus were isolated from the intestine of cattle and calves with severe diarrhea and were named Vibrio jejuni by Jones et al. (1931). The disease has been reproduced by feeding the pure cultures of the vibrios or feces from naturally infected cows to normal calves. Doyle (1944) reported that swine dysentery, a bloody diarrhea, was caused by local infection of a vibrio and named it V. coli. Large numbers of the organisms were found in the bowel discharge, and in the cecal and colonic mucosa of infected hogs. The disease could also be reproduced by feeding a portion of the colon or the bowel discharge to a healthy pig.

An unidentified organism similar to C. fetus was isolated by Russel (1955) from the intestine of hoggets afflicted with watery scour. Maciak and Winkenwerder (1964) isolated C. fetus from various sections of the intestines, intestinal lymph nodes and gallbladder of infected pigs.

Winkenwerder (1966) reported that *C. fetus* was an apathogenic intestinal saprophyte of dogs and cats.

Morse and Ristic (1954) reported that the adult guinea pig was resistant to infection following intraperitoneal inoculation with *C. fetus*. However, in pregnant guinea pigs, abortion or premature birth was produced by 31 strains of *C. fetus* isolated from various sources. Ristic and associates (1955) showed that young male hamsters became infected by intraperitoneal inoculation of *C. fetus* isolated from bovine, ovine and human sources. Vibrios were recovered from the testes of animals where the lesions were observed. Winkenwerder and Bisping (1964) also reported that white mice could be infected with a large dose of the organism isolated from poultry.

In addition to venereal transmission and oral transmission of *C. fetus* infection, Frank *et al.* (1965) suggested that American magpies might be the carriers and vectors spreading the disease from one herd or flock of animals to another.

Avian vibrionic hepatitis characterized by degeneration of the liver is the result of a systemic infection by *C. fetus*. The causative agent had been isolated from bile (Winterfield, 1959; Hagan, 1964), liver (Hofstad and associates, 1958; Peckham, 1958; Truscott and Morin, 1964), and the cecum (Barnes and Impey, 1970) of birds. Truscott and Stockdale (1966) showed that the disease was transmitted by fecal contamination.

Truscott and Morin (1964) found that *C. fetus* was the causative agent of transmissible enteritis in turkeys. The disease was reproduced by oral inoculation of pure cultures; and the organisms were reisolated from the infected birds.

According to Waldhalm et al. study (1964), the magpie (Pica pica) could be experimentally infected with ovine strains of C. fetus. Thirty nine days after the culture was orally administered, vibrios were still found in the feces.

Smibert (1969) isolated C. fetus from the intestinal content of sparrows, starlings, pigeons, blackbirds, chickens and turkeys. These birds were commonly found around sheep and cattle barns. Furthermore, these isolates were morphologically and biochemically similar to the vibrios isolated from intestinal tracts of sheep. He concluded that birds might act as both a vector and a reservoir of C. fetus infection.

B. Human Infection by Campylobacter fetus

Campylobacter fetus infects man, causing various symptoms. Since 1947, more than 80 well documented cases of human infections have been recorded in the world literature. However, many investigators believed that human infections of C. fetus might be more prevalent than presently encountered.

The major conditions and their symptoms due to the etiology of C. fetus infection can be briefly presented as follows:

1. Abortion:

Vinzent, et al. (1947) first reported a human case of abortion in France. The patient obtained milk from a neighboring farm and the milk was presumably contaminated with C. fetus. She experienced chills, dizziness, fluctuating temperature and headache. During the 7th month of pregnancy, she had an abortion. Campylobacter fetus was recovered from the patient's blood on several occasions. In 1950, they described

three cases of pregnant women infected with C. fetus. Large areas of necrosis on the placentas were observed.

The first case of human abortion caused by C. fetus in the United States was reported by Hood and Todd (1960). The patient had four consecutive pregnancies that terminated in abortion or premature delivery. C. fetus was isolated from the patient's placenta and from the brain of the aborted fetus. Serum agglutination tests showed C. fetus antibodies in the blood of both the patient and her husband. Her husband recalled that several years ago he had observed cattle abortions; but he did not contact or handle these animals. Nevertheless, it was assumed that he was infected, and transmitted the disease to his wife, who consequently suffered the abortion.

Morbidity and Mortality Weekly Reports (1965) reported a case of premature birth that was confirmed to be caused by C. fetus. At delivery, the lower portion of the body showed extensive petechia; and the body surface was covered by a foul smelling, oily film. The infant died 14 hours later. Campylobacter fetus was cultured from the infant's spinal fluid and blood. Agglutination tests performed with the serum of the mother against the pure culture isolated from the baby showed a very high antibody titer (1:1280 dilution).

2. Meningitis:

The central nervous system of newborn infants is most often attacked by C. fetus. Eden (1962) treated a three-week old premature infant who developed subdural effusions, abscesses, and cystic formations in the left and right hemispheres of the brain. Campylobacter fetus was cultured

from the cerebrospinal fluid. Despite chloramphenicol and penicillin therapy, the infant died at four and a half months of age.

Burgert and Hagstrom (1964) made a clinical and pathological report on a 9-day old infant who died from a meningitis. Campylobacter fetus was cultured from post-mortem samples of cerebrospinal fluid and brain tissue.

Bader and associates (1966) also recovered a spirillum from the cerebrospinal fluid of a 3-week old male infant with purulent meningitis. The organism was morphologically, biochemically and serologically identified as C. fetus.

Campylobacter fetus was also found to be the causative agent of meningitis in adults. Collins and associates (1964) isolated a spirillum-like organism from the blood and spinal fluid of a 55-year old man. The organism was morphologically and biochemically established as C. fetus. The patient suffered from an undulating fever, muscular pain, migratory arthralgias, weight loss, chronical illness, and meningitis. All signs of infection subsided after he was placed on tetracycline therapy.

3. Endocarditis:

One of the important human diseases caused by C. fetus infection is endocarditis. Lee and associates (1970) described 9 cases of C. fetus endocarditis. The symptoms of those cases are summarized in Table I. Four of the patients died; two of them were confirmed as C. fetus endocarditis by post-mortem examination. In one case, the cardiac illness was probably due to infection of Escherichia coli rather than that of C. fetus. Three cases involved extraction of infected teeth. Thus an

Table I. Summary of 9 cases of *C. fetus* endocarditis.

Occupation Sex and Age	Main Symptoms	Cardiac Findings	Outcome
Lathe operator Male 51	Fever and chills	Not described	Cured; cerebral embolism resulting in paresis
Unknown Male ?	Fever and chills	Diastolic murmur (location not described)	Cured; heart murmur persisted
Stockman in warehouse Male 44	Fever and chills, and headache	Murmur of aortic insufficiency; apical systolic murmur	Cured; aortic insufficiency and cardiac decompensation persisted
Painter Male 50	Fever and chills	Subacute bacterial endocarditis (no further description)	Unknown
Janitor Male 47	Fever	Subacute bacterial endocarditis	Died, gastrointestinal hemorrhage; endocarditis possibly due to *E. coli*

Table I. (Continued)

Occupation Sex and Age	Main Symptoms	Cardiac Findings	Outcome
Construction laborer Male 67	Fever, chills, and mental confusion	Normal on admission	Died, 8th hospital day; autopsy confirmed aortic and mitral endocarditis
Cab driver Male 49	Fever, chills and pain and tenderness of left thigh	Murmur of aortic insufficiency and stenosis; aneurysm of left femoral artery	Died, 1 mo. later; acute pulmonary edema; no autopsy
Housewife Female 49	Fever and chills	Murmur of mitral stenosis and insufficiency and aortic insufficiency	Cured; well until 1 yr. later, congestive heart failure secondary to mitral and aortic insufficiency (by catheterization); blood and cervix cultures were negative
Farmer Male 67	Fever and chills; left sural thrombophlebitis	Moderately loud systolic aortic murmur	Died, 12th hospital day, bacterial endocarditis; aortic mitral, and tricuspid valves affected

Ref. Lee *et al.* (1970).

tooth might be one of the possible routes of C. fetus infection.

4. Enteritis and gastroenteritis:

Levy (1945) described an acute outbreak of gastroenteritis among the inmates of two adjacent institutions. The major symptoms were nausea, vomiting, abdominal cramps, diarrhea and fever. One hundred and fifty one patients were hospitalized. Contaminated pasteurized milk and some raw milk shipped by error, were suspected to be the source of infection. The organism cultured from patients' blood and feces was morphologically and biochemically identified as V. jejuni.

Mandel and Ellison (1963) presented a clinical report on a 45-year old retired air force sergeant who suffered from an acute dysentery syndrome. Campylobacter fetus was cultured from blood specimens. The purified culture reacted only with the antisera prepared against animal strains, but not with those against human strains of C. fetus.

Dekeyser and associates (1972) developed a procedure to isolate C. fetus from stool specimens from patients with acute enteritis. They checked 1000 stool specimens, and isolated 35 strains of C. fetus from 28 children, under 6 years old, and 2 adults. Nineteen of the children and both adults suffered from diarrhea.

A meat-packing house worker, Park et al. (1973), was admitted to a hospital suffering from abdominal pain, violent diarrhea, chills, and a high fever. Blood cultures contained C. fetus. Presumably the patient was infected through direct contact with contaminated meat.

King (1957) reported three infections in infants and one in a child due to C. fetus. Wheeler and Barchers (1961) reported on four more cases of infant enteritis. Middlekamp and Wolf (1961) recorded one case. In

all the cases, C. fetus was isolated from the patient's blood. The clinical feature of the infections in children by C. fetus are listed in Table II. Bloody diarrhea appears to be the major feature of the disease. Fever and vomiting were observed in most cases. The children all recovered after antibiotics therapy.

5. Septic arthritis:

The involvement with C. fetus infection of the joints was first reported by King and Bronsky (1961). A positive culture was obtained from synovial fluid of a 74-year old male patient who was hospitalized because of fever associated with a painful swelling in the right knee.

Kilo et al. (1965) also presented a case of acute synovitis in a 62-year old male salesman, who upon hospital admission, was delirious with a high fever and his left knee was red, swollen, and painful. Campylobacter fetus was cultured from the patient's synovial fluid and blood specimens.

The third case of septic arthritis due to C. fetus infection reported by Kutner et al. (1970), occured in a knee joint of a 74-year old lady, who complained of severe pain and swelling on her right knee. An organism isolated from her synovial fluid was identified and characterized as C. fetus.

6. Pustule Formation:

Ward (1948) described the first instance of C. fetus infection as the appearance of a pustule. A laboratory technician who was handling C. fetus, found a facial pustule developed on his cheek. The same organism that he handled was recovered from that pustule. Lawrence, et

Table II. Clinical feature of infant enteritis due to the infection of *C. fetus*.

Age	Symptoms and Duration	Reference
3 mos.	Bloody diarrhea; 2 wks.	King (1957)
5 yrs.	Diarrhea, vomiting; unknown	King (1957)
2 mos.	Fever, diarrhea, vomiting; 3 wks.	King (1957)
2 mos.	Bloody diarrhea, vomiting; 3 wks.	King (1957)
11 mos.	Fever, bloody diarrhea, vomiting; 9 mos. (?)	Wheeler & Borchers (1961)
7 wks.	Bloody diarrhea, vomiting; 3 wks.	Wheeler & Borchers (1961)
8 mos.	Bloody diarrhea, vomiting; 1 wk.	Wheeler & Borchers (1961)
4 yrs.	Fever, bloody diarrhea; 9 mos. (?)	Wheeler & Borchers (1961)
2 mos.	Fever, bloody diarrhea; 2 wks.	Middlekamp & Wolf (1961)

al. (1967) also presented a patient with localized abscess. The patient had sustained an injury on the upper left side of the chest which was later found to be infected by C. fetus.

7. Fever and Septicemia:

Fever and chills of undulant pattern with no signs of localized infection are the major symptoms of septicemia due to C. fetus. Hinton (1959) reported that C. fetus was isolated from blood cultures of an old farmer. He was hospitalized because of fever and chills.

A case was reported in Morbidity and Mortality Weekly Reports (1965) of a two and a half year old child who suffered with fever of inconstant pattern, anorexia, and joint and muscle pain. The organism was also recovered from a blood specimen of a 52-year old teacher who experienced an undulating fever, chills, headache, tiredness, loss of weight and night sweat (Bokkenheuser, 1970).

8. Fever and Phlebitis:

Spink (1957) presented a meat-packing worker suffering from fever, chills, headache, and thrombophlebitis in his right arm vein. Campylobacter fetus was cultured from the patient's blood. The culture was serologically identified as a human strain by King, and as of bovine origin by Plastridge. Three cases of thrombophlebitis with C. fetus septicemia were reported by Kahler and Sheldon (1960). In each of the cases, high fever and shaking chills accompanied by phlebitis were the clinical symptoms.

The epidemiology of C. fetus infection in man is not yet understood. King (1957) suggested several posibilities of C. fetus infection including

direct contact with infected animals, direct venereal transmission between humans, and ingestion of contaminated food or water. She also indicated that patients may acquire the infection after extraction of teeth.

Reyman and Silberberg (1969) considered that aerosols are not the means of spreading the infection; since no infection was ever transmitted from patients to their physicians or nurses. On the other hand, a farmer (Jackson et al., 1960), workers at meat-packing plants (Park et al., 1973, and Spink, 1957), and a laboratory technician (Ward, 1948) who were in close association with animals, in direct contact with animal tissue and blood, and who worked with the microorganism, became infected. Furthermore, Bokkenheuser (1970) postulated that infants or premature infants may obtain the disease by either placental transfer or exposure at the time of delivery.

Direct venereal transmission was also proposed by Hood and Todd (1960), Willis and Austin (1966). If human infection of C. fetus follows the venereally transmitted pattern in cattle, the organism should remain indefinitely in the testes of the male who was infected, and thus the disease might be spread. But such a hypothesis has not been confirmed (Eden, 1962).

Transmission of C. fetus via contaminated food or milk has been considered as a possible means of spreading the disease. Contaminated milk was suspected as the agent associated with first human abortion by Vinzent et al. in 1947. The organism also caused a gastroenteritis outbreak in adults (Levy, 1946). Raw milk was suspected as the means of transmission of the infection to two patients who lived on farms in the same county in Oregon (Wills and Austin, 1966). Raw beef liver was considered the possible source of infection in a septicemia patient (Soonattrakul et al., 1971).

A Korean patient with *C. fetus* subacute endocarditis ingested raw beef (Chung and Lee, 1970). However this possibility can not be documented until a culture is isolated from these foods.

Children and adults who have had no contact with animals have become infected by *C. fetus*. The mode of transmission in these cases is unknown. King (1962) proposed a hypothesis of an endogenous mode of infection. She believed the organism was carried in the human body as part of the gastrointestinal flora. When a person suffers from other diseases such as cancer, diabetes, cirrhosis of the liver, or suffers from a weakened metabolic state such as alcoholism, malnutrition, and pregnancy; the host resistance is lowered so that the organism is able to cause generalized infection.

C. Biological Nature of *Campylobacter fetus*

1. Morphology:

Campylobacter fetus was defined (Smibert, 1974) as a gram-negative, non-spore forming, slender rod with one or two curves. The organism appears S-shaped or gull-winged when two cells are joined in pairs. Spherical forms and spiral shaped filaments may also be seen in older cultures. A single flagellum is attached to one or both poles of the cell. It is very actively motile with a characteristic corkscrew-like motion.

2. Colonies:

Bryner *et al.* (1962) examined the types of colonies formed when strains of *C. fetus* were cultured on thiol agar. Seventy three strains of bovine origin were employed in the study. Four major variant types

of colonies were observed that had the following features:

Smooth colonies (designated S) are circular, about 0.5-1.0 mm in diameter, slightly raised, smooth, translucent, colorless to cream colored, and fluid. Colonies appeared to swarm when covered with a drop of saline solution.

Rough colonies (R) are similar to S colonies in size and shape, and are finely granular, opaque, solid and non-swarming.

Cut glass colonies (GC) are larger than S colonies, circular, raised, granular with a light refracting quality, and are translucent and fluid. Swarming activity was observed.

Mucoid colonies (M) are viscous resulting from prolonged incubation.

When S colonies were successively subcultured, variant types termed soft cut glass I(SCGI), rough cut glass I(RCGI), and soft cut glass IA were found. From R colonies, rough cut glass I, II and III (RCG I, II and III) and soft cut glass II and III (SCG II and III) were derived.

3. Atmospheric Requirements:

Campylobacter fetus is a microaerophilic organism that grows poorly aerobically or anaerobically. Smith and Taylor (1919) observed that the organism failed to grow in fluid media, but did grow in a sealed tube on the slanted agar covered with a small amount of broth. The failure of growth in fluid media was due to convection currents continually disturbing the establishment of concentration gradients of oxygen along the layers of the fluid medium. On the other hand the oxygen gradient was uniformly organized through the layers of slanted agar in the sealed tube. Growth of C. fetus took place at a zone where the oxygen concentration would be proper to cultivate them.

King (1962) inoculated _C. fetus_ in semisolid thioglycollate medium. After incubation, growth was obtained only in the medium a few mm below the surface.

Reich _et al._ (1956) reported _C. fetus_ grew better on agar surfaces or in broth when the percentage of oxygen in the air environment was lowered by replacing a portion of the air with another gas, or by placing the culture in a jar with a partial vacuum. The optimum growth was at an oxygen level of 6 percent. The author stated that carbon dioxide served only as a diluting gas to lower the oxygen concentration in the air environment and was not required. They found that as much growth occured in an atmosphere diluted with nitrogen or helium as in that diluted with carbon dioxide.

Plastridge and Williams (1943) studied six strains of _C. fetus_ of bovine origin and found that they grew in an environment containing 10% CO_2 and 90% air. No growth occured in ordinary atmospheric conditions nor in that containing 30% CO_2.

Kiggins and Plastridge (1956) studied the gaseous requirement for growth of _C. fetus_ on blood agar. No growth occured aerobically or anaerobically. The microaerophilic environment for optimum growth was found to be an air atmosphere containing 5% O_2 and 10% CO_2.

Reich _et al._ (1957) devised a procedure for growing _C. fetus_ in large quantities in broth. The batch culture of _C. fetus_ was aerated by a closed gas circulation system. The gas mixture used in the system was composed of 5% CO_2, 35% air, 10% H_2 and 50% He. The procedure reduced the lag phase time and increased the rate of growth and total cell yield. The percentage of oxygen and hydrogen in the enclosed gas mixture was reduced during

the growth of the culture while the concentration of CO_2 was increased.

Fletcher and Plastridge (1964) reported that on yeast extract agar and in the presence of 10% CO_2, the percentage of oxygen for optimal growth of 49 strains of _C. fetus_ was 2.5% for one group, and 5% for the other. For several strains that were able to grow on a chemically defined solid medium, the optimal gaseous environment contained 1% O_2 and 10% CO_2.

4. Growth of _C. fetus_ on Solid Media:

Smith and Taylor (1919) cultivated _C. fetus_ of bovine origin on plain agar slants with a few drops of condensing water or broth added. Growth took place beneath the water-drop at the surface where the agar slant leaned upon the glass. Defibrinated horse or other mammalian blood added to the condensing water stimulated growth. They also reported that no growth occurred in nutrient gelatin.

Plastridge _et al_. (1949) reported that _C. fetus_ grew well on a thiol agar slant when the culture was incubated at 37 C in an atmosphere having 5-10% CO_2. The medium was prepared by adding 19 g of agar and 0.05 g of glutathione to one liter of thiol medium (Difco).

King (1957) studied the cultural characters of 23 strains of _C. fetus_ isolated from human and animal sources. In a Brewer anaerobic jar or a candle jar, nonhemolytic colonies appeared on rabbit blood agar, and light growth was also seen on MacConkey's agar. There was no growth on SS (Shigella-Salmonella) agar, Simmons' citrate agar, and Christensen's urea agar.

5. Growth of _C. fetus_ in Liquid Media:

Smith and Taylor (1919) successfully grew _C. fetus_ in simple beef

peptone broth in a sealed tube. Multiplication was enhanced by addition of a small quantity of defibrinated blood. No growth of the organism occurred when it was inoculated in milk.

Plastridge and Williams (1943) failed to grow 6 strains of C. fetus of bovine origin aerobically, in liver infusion broth and beef infusion broth. To these bases, serum, ascitic fluid, amniotic fluid and cysteine were individually added; growth of the organism was not observed.

Hansen et al. (1952) used BBL-B5C thioglycollate broth to produce large quantities of the microorganism in 300-ml Erlenmeyer flasks.

Roberstad and Morrison (1957) applied a shaker technique to grow the organism in Brucella broth. Sixty percent of the air in the flask was replaced with nitrogen. They claimed that a good yield of cells could be obtained in 48 hours.

Dennis and Jones (1959) developed a succinate-cysteine (S.C.) medium which has proved satisfactory for the growth of C. fetus. The gas atmosphere was diluted with nitrogen to 6% oxygen. In their 14-liter batch culture study, the logarithmic growth began after 20 hours incubation and lasted for the next 20 hours. The yield of cells recovered was 0.6 g dry wt. per liter of medium.

When catalase-positive strains of C. fetus were grown in a medium containing catalase, Kingscote (1961) found that the size of minimum inoculum could be reduced by 64 fold in broth medium and by 16 fold on agar medium. No significant growth was obtained when catalase was added to blood agar, since blood cells furnished adequate amounts of catalase to increase the initial growth of a small inoculum.

Fung and Winter (1968) cultured two actively growing strains of C.

fetus (C. fetus venerealis and C. fetus intestinalis) in a broth medium simulating Cystine Heart broth (Difco). The cultures were incubated in a gas mixture containing 2.5% O_2, 10% CO_2 and 87.5 % N_2. The lag phase of growth was 2 hours. The log growth occurred for the next 2 hours for the intestinal strain, and 8 hours for the venereal strain.

6. Growth of C. fetus in Semi-solid Media:

Plastridge and Williams (1943) inoculated C. fetus into liver-infusion broth containing 0.1 to 0.5% agar. The cultures were incubated under an air environment containing 10% CO_2. Maximum growth was observed in the medium having 0.3% agar. Initial growth became visible in a thin layer 0.5 cm to 1.0 cm beneath the surface. Growth then gradually developed upward and the zone of cells became dense and might reach from 2 to 4 mm in depth. This medium was found to be suitable for the maintenance of stock cultures. Robertstad and Morrison (1957) also prepared stock cultures in a semisolid Brucella medium containing 0.1% agar. Fung and Winter (1968) maintained stock cultures in a semi-solid medium containing 0.16% agar.

Huddlesen (1948) prepared a thiol semisolid medium for the isolation, cultivation and maintenance of C. fetus. He found that cultures remained alive in thiol semisolid agar for 150 days when stored at room temperature. Hansen et al. (1952) reported excellent growth of C. fetus on BBL-B5C thioglycolate semi-solid agar. Cultures could be kept alive for months at room temperature. Lecce (1958) grew the stock cultures in thiol broth with 0.3% agar. Zemjanis and Hoyt (1960) showed that Brucella broth plus 0.1% agar provided the most favorable environment for growth of C. fetus.

7. Fermentative Ability:

Smith and Taylor (1919) reported no acid or gas formation in broth containing 1% dextrose, lactose or sucrose. Good growth was obtained in all of the fermentation tubes.

Plastridge and Williams (1943) found no acid production in media that contained 1% for the following carbohydrates: arabinose, dextrose, dextrin, dulcitol, galactose, inulin, lactose, levulose, maltose, mannitol, raffinose, salicin, sucrose, sorbitol, or trehalose. The basic medium used in the study was composed of beef infusion, 1.0% peptone, 0.3% agar, and 1.0% Andrade's indicator, the pH was adjusted to 7.4. Good growth was observed in all tubes.

King (1957) also reported that 21 strains of _C. fetus_ (_C. fetus_ and related vibrios) isolated from human and animal sources did not attack the following carbohydrates: glucose, d-xylose, d-mannitol, lactose, sucrose, and maltose. The fermentation tests were studied in a heart infusion broth with brom cresol purple as the indicator. The cultures were incubated in candel jars.

8. Oxidative Ability:

Campylobacter fetus appears to be wholly deficient in fermentative powers. Consequently, the oxidative capabilities of the organism become important for obtaining energy.

Lecce (1958), measuring the reduction of triphenyl tetrazolium chloride in Thunberg tubes tested the oxidative capabilities of 27 strains of _C. fetus_. Among 30 potential electron donors, lactate, formate, pyruvate, α-ketoglutarate and succinate were the only five compounds able to reduce triphenyl

tetrazolium chloride. Acetate, glutamate, malate, citrate, fumarate, aspartate, glycerol, rhamnose, arabinose, fructose, lactose, sucrose, mannose, galactose, glucose, gluconate, xylose, ribose, maltose, ethyl alcohol, trehalose, sorbitol, mannitol, alanine, and glycine were negative after a 2-hour reaction. Formate was the most active electron donor, but it completely inhibited the growth of C. fetus when it was added to growth medium (heart infusion broth) at a concentration of 0.5%; at 0.2% it delayed the growth.

Alexander (1957) studied the energy sources utilized for the growth by C. fetus. 0.2% of various substrates was added to a minimal peptone medium and the increase in growth of microorganisms was measured. Among the non-nitrogenous compounds, acetate, lactate, pyruvate, α-ketoglutarate, succinate, fumarate, and malate all served as energy sources. But glucose, hexose-diphosphate, citrate, isocitrate, glycerolphosphate, oxaloacetate, oxalosuccinate, and tartrate did not support significant growth. With various amino acids, aspartate, asparagine, glutamate, and proline were utilized for growth. Glycine, alanine, threonine, phenylalanine, histidine, lysine, arginine, cysteine, methionine, leucine, or ornithine did not serve as energy sources. Furthermore, lactate, pyruvate and fumarate were studied manometrically and proved to be excellent energy sources and were readily oxidized by a resting cell suspension. There was no oxygen uptake when glucose, glycerophosphate or hexose-diphosphate were tested.

Kiggins and Plastridge (1958), employing the Warburg manometric technique, investigated the oxidative activities of two strains of C.

fetus. Whole cells and crude cell free extracts were tested. There was no oxygen uptake with any of the 16 common carbohydrates tested. Of the glycolytic intermediates, pyruvate was the only compound that was oxidized. All members of the citric acid cycle were rapidly oxidized when a crude cellular extract was employed, but whole cells showed no reaction with citrate, cis-aconitate, isocitrate, and α-ketoglutarate. Of 23 amino acids tested, glutamate, glutamine, aspartate, asparagine, proline, and cysteine were oxidized. Ten fatty acids were tested, and acetate was the only one oxidized. They concluded that an Embden-Meyerhof glycolytic pathway was not operative in C. fetus and the organism could only obtain energy from citric acid cycle intermediates or compounds that might rapidly enter the cycle.

9. Nutritional Requirements:

Batlin and Wilson (1950) successfully grew 5 strains of C. fetus in a chemically defined semi-solid medium. The medium contained 16 amino acids which were comparable with the composition of casein, plus salts, growth factors and 1% methocel as a thickening agent.

The studies of Kuzdas and Morse (1956b) first indicated that to obtain the best possible growth condition for C. fetus, nicotinic acid, thiamine, calcium, pantothenate, pyridoxal, and biotin were needed.

Zemjanis and Hoyt (1960) studied the effect of a number of substances on the growth of 22 catalase-positive bovine strains of C. fetus. The substances included reducing agents, metal ions, intermediates of glycolytic and citrate acid cycles, purines, pyrimidines and vitamins. Glutathione was unable to stimulate the growth of all strains. Cysteine supported growth

at low concentrations; but at higher levels, it was inhibitory. Marked visible growth was shown by the addition of magnesium ($MgCl_2$), manganese ($MnCl_2$) or iron (ferrous sulfate). Molybdenum (MoO_3) slightly stimulated the growth when cultures were incubated aerobically; but growth was inhibited in a gaseous environment containing 15% CO_2. Cobalt acetate showed no effect on growth at 0.05% level, but was bactericidal with higher concentrations. At lower levels of concentration, lactate, α-ketoglutarate, glutamine, uracil, thymine, p-aminobenzoic acid, and 17-β-estradiol enhanced the growth of C. fetus; some of these were inhibitory at higher concentrations. One percent of pyruvate completely inhibited the growth of C. fetus, while lower levels of pyruvate did not affect the growth. The growth of all strains of C. fetus was not affected by aspartate, adenine or urea. 0.015 to 1.0 % fumarate depressed their growth.

Fletcher and Plastridge (1963) developed a defined medium which supported the growth of 22 out of 26 strains of C. fetus. The organisms were catalase-positive, H_2S-negative and were isolated from cattle, sheep, swine, chickens and man. The composition of the medium consisted of aspartate, cysteine, glutamate, sodium acetate, $MgCl_2 \cdot 6H_2O$, NaCl, K_2HPO_4, KH_2PO_4, and niacin. The pH was adjusted to 8.0. When biotin, pyridoxine, riboflavin, and thiamine were added, individually or in combination, to the defined medium, no stimulation of growth was obtained.

Smibert (1963) prepared a chemically defined medium for C. fetus. The liquid or semi-solid medium that supported growth of 87 strains of C. fetus from various sources contained 18 amino acids, B-vitamins and minerals. The pH was adjusted to 7.0-7.1. Spermine, cadavarine, putrescine, betaine and reducing agents including thioglycolate, thiosulfate,

cysteine, and ascorbic acid did not affect the growth of slower growing strains in liquid medium. Sodium carbonate and sodium thiosulfate slightly stimulated the growth of some strains. Sodium bicarbonate showed no effect. Magnesium and ferrous salts were the only major minerals required. Purines and pyrimidines were not needed. The only essential vitamin required by most strains was nicotinic acid; however, addition of other B-vitamins caused better growth of C. fetus. The amino acid requirements of 87 strains of C. fetus were diverse. Some strains needed only a few amino acids, others required a very complex number of amino acids. Eighteen amino acids were used in the complete medium that supported growth of all of the strains tested.

10. Miscellaneous Biochemical Properties:

Price et al. (1955) reported that C. fetus did not produce indole, reduce nitrate to nitrite, failed to grow in nutrient gelatin medium (BBL) and was not found to acidify media containing 1% of lactose, glucose, sucrose, maltose, xylose, mannitol, inositol, or sorbitol. The study also indicated that several strains grew in litmus milk and reduced litmus. Some produced hydrogen sulfide.

The studies of Kuzdas and Morse (1956a) indicated that 59 strains of C. fetus from various sources were indole negative and urease negative. Growth was not observed in nutrient gelatin medium (Difco), but the addition of 0.1% agar to the same medium supported good growth of all strains of C. fetus. Gelatin was not liquified. There was no growth in litmus milk. However, when 0.1% agar was added to the litmus milk, growth of all C. fetus strains were seen after 48 hours of incubation; and litmus was reduced. Nitrate was reduced by most strains, but was not reduced by

two strains of C. jejuni. All cultures were catalase positive except for 8 isolated from bovine vaginal mucus and semen, and one of human origin.

Smibert (1965) investigated the biochemical characteristics of 17 representative strains of C. fetus var. intestinalis isolated from feces and the intestinal content of clinically normal sheep. The cultures were all catalase-positive and oxidase-positive, reduced nitrate and selenite, grew in medium containing 1.0% glycine, but did not grow in a medium containing 3.5% NaCl. They also grew in a chemically defined medium (Smibert, 1963) and grew slightly on MacConkey's agar. They grew at 37, 42, and 45 C, but not at 25 C. Growth and reduction of litmus by all vibrios were observed in litmus milk when it was fortified with 1% peptone. There was no phosphatase and arylsulfatase activity. Hippurate and esculin were not hydrolyzed, and gelatin was not liquified. About 80% of the strains tested were able to hydrolyze casein. Hydrogen sulfide was not detected by any strains when they were grown on triple sugar iron agar. A small amount of H_2S was revealed when isolates were cultured in semisolid medium and lead acetate-impregnated paper strips were used as the indicator. A large quantity of H_2S was produced when they were grown in the semi-solid medium with 0.02% cysteine. They were unable to ferment or oxidize sugars.

D. Classification and Taxonomy of Campylobacter fetus

Since the first description of V. fetus by Smith and Taylor (1919), a similar organism was isolated by Jones et al. (1931) from inflamed intestinal tracts and livers of cows or calves suffering from winter scours, and was named V. jejuni. Doyle (1948) named V. coli as a causative

organism of swine dysentery. On the basis of catalase and H_2S tests, Bryner and Frank (1955) divided _V. fetus_ of genital tract origin into two distinct types:

(1) Catalase positive and H_2S negative type: Strains included in this type were considered the typical _V. fetus_ and associated with animal infection.

(2) Catalase negative and H_2S positive type: Strains of this type were considered normal genital flora in cattle and were not a causative agent of abortion and infertility in cattle and man. The latter was named _V. bubulus_ by Thouvenot and Florent (1954). King (1957) studied isolates of _V. fetus_ from 4 cases of children with fever and diarrhea, and referred to the organism as a "Related vibrio". Peckham (1958) called the organism associated with avian vibrionic hepatitis _V. hepaticus_. Two subspecies of _V. fetus_ were proposed by Florant (1959). They were named _V. fetus_ subsp. _venerialis_ (type I) and _V. fetus_ subsp. _intestinalis_ (type II). The former was unable to survive in the intestinal tract, and was associated with enzootic infertility and abortion in cattle. The latter occurred in the intestinal tracts of sheep, cattle, swine, birds, and humans, and caused sporadic abortion in sheep and cattle. It also caused human infection. Bryans _et al_. (1960) described a new variety of _V. fetus_ that produced both catalase and hydrogen sulfide. The culture was obtained from an aborted ovine fetus. King (1962) differentiated _V. fetus_ and "Related vibrios" by their temperature tolerance. The former grew at 25 C and 37 C but not at 42 C while the latter showed growth at 37 C and 42 C but not at 25 C.

Sebald and Veron (1963) reclassified the vibrios which had a G + C

content of deoxyribonucleic acid (DNA) between 29 and 35 moles% into the new genus Campylobacter.

Bryner et al. (1962), studying the colony dissociation pattern on Albimi agar and the correlated biochemical reaction of cultures, divided 87 strains of bovine vibrios into two major types and a subtype of type I:

Type I included the strains that grew poorly on plain Albimi agar, but growth was improved by the addition of glutathione and sodium thioglycollate. They were H_2S-negative at five days incubation. One smooth and 4 variant colonial forms were found.

Type II included the strains that grew well on plain Albimi agar and were H_2S-negative at 24-hour incubation, but H_2S-positive at 5-day incubation. One smooth and 8 variant colonial forms were found.

Subtype I included the strains that grew moderately on supplemental Albimi agar and were H_2S-negative at 24-hour incubation but H_2S-positive at 5-day incubation. Only one stable smooth cut glass colony was found.

Berg and associates (1971) proposed 5 groups of V. fetus listed as follows:

(1) Sero-type A - bio-type I: glycine negative, H_2S-negative.

(2) Sero-type A - bio-subtype I: glycine negative, H_2S-positive.

(3) Sero-type A - bio-type II: glycine positive, H_2S-negative.

(4) Sero-type B: glycine positive, H_2S-positive.

(5) Sero-type C: glycine variable, H_2S-positive.

Truscott and Morin (1964) also reported a catalase-positive, H_2S-positive strain of V. fetus which was isolated from turkey with infectious enteritis.

Vibrio meleagridis, named by Mathey and Rissberger (1964), was isolated from sinus exudate of turkeys. Firehammer and Berg (1965) used temperature tolerance to identify V. fetus subsp. venerealis and V. fetus subsp. intestinalis.

On the basis of phenotypic characteristics, Smibert (1970) divided V. fetus into three biotypes. The carbohydrate composition of cell wall of biotypes I and II strains contained galactose and mannose, and usually did not have glucose. Most strains of biotype III contained galactose and glucose in their cell wall. Some also had only galactose.

Veron and Chatelain (1973) examined the biochemical and serological properties of 40 Campylobacter strains. They divided the species of C. fetus into two subspecies of C. fetus subsp. fetus and C. fetus subsp. venerealis.

Smibert (1974) in Bergy's Manual of Determinative Bacteriology, 8th ed. classified the genus Campylobacter into three major species: C. fetus, C. sputorum and C. fecalis. In this classification, C. fetus was divided into three subspecies, C. fetus subsp. fetus, C. fetus, subsp. intestinalis and C. fetus subsp. jejuni. Table III shows the characteristics of C. fetus while Table IV shows several differential characteristics of the subspecies and the major illness caused by them. C. fetus subsp. fetus was the V. fetus by Smith and Taylor (1919), the V. fetus var. venerealis of Florent (1959) and Veron and Chatelain (1973). C. fetus subsp. intestinalis was the V. fetus var. intestinalis of Florent (1959), the C. fetus subsp. fetus of Veron and Chatelain (1973). C. fetus subsp. jejuni was the V. jejuni of Jones et al. (1931), the V. meleagridis of Mathey and Rissberger (1964), the V. hepaticus of Peckham (1958) and the "Related vibrio" of King (1957).

Table III. Biological characteristics of <u>Campylobacter</u> <u>fetus</u>.

Test	Result
Gram Stain	Negative
Spores	None
Morphology	Spirally curved rod
Flagellum	Single polar flagellum at one or both ends of the cell
Motility	Corkscrew-like motion
Oxygen Requirement	Macroaerophilic
CO_2 Requirement	10% (candle jar)
Optimum Temerature	37 C
Optimum pH	7.0
Microaerophilic Growth:	
Blood Agar	+
Thiol Agar	+
Chocolate Agar	+
MacConkey Agar	+
SS Agar (Shigella-Salmonella)	-
Simmons' Citrate Agar	-
Christensens' Urea Agar	-
0.16% Semisolid Agar (Aerobically)	+
+ 1% Bile	+
+ 3.5% NaCl	-
+ 1% Glycine	v
Carbohydrates fermentation	-
Pigment Production	-
Hemolysis	-
Indol Production	-

Table III. (Continued)

Test	Result
Methyl Red	-
Vosges-Proskauer	-
H_2S Production	-
TSI Slant	-
Semi-solid with Cysteine (Lead Acetate Strips)	v
Nitrate Reductase	+
Nitrite Reductase	-
Catalase	+
Peroxidase	+
Urease	-
Deamination:	
Glutamic Acid	+
Glutamine	+
Aspartic Acid	+
Asparagine	+
Phenylalamine	-
Tyrosine	-
Tryptophan	-
Decarboxylation:	
Lysine	-
Ornithine	-
Arginine	-
Hydrolysis:	
Casein	v
Hippurate	-

Table III. (Continued)

Test	Result
Esculin	-
Ribonucleic Acid	-
Deoxyribonucleic Acid	-
Gelatin	-

+ : Most (90%) strains positive.
- : Most (90%) strains negative.
v : Some strains positive, some negative.

Ref. Smibert (1974).
King (1957).

Table IV. The differential characteristics of *C. fetus*.

	C. fetus ss. *fetus*	*C. fetus* ss. *intestinalis*	*C. fetus* ss. *jejuni**
Catalase	+	+	+
Nitrite Reductase	-	-	-
H_2S on TSI	-	-	-
H_2S by Lead Acetate Paper	-	+	+
1% Glycine	-	+	+
3.5% NaCl	-	-	-
25 C	+	+	-
42 C	-	-	+
Mode of Transmission	Venereally	Orally	Orally
Disease Induced	Infectious Abortion & Infertility in Cattle	Abortion in Sheep Sporadic Abortion in Cattle Human Infections: Fever, Septicemia, Menigitis, Arthritis, Endocarditis, Placentitis & Abortion, Phlebitis, etc.	Abortion in Sheep Human Infections: Gastroenteritis with Fever

+ : most (90%) strains positive. - : most (90%) strains negative.

* : "Related vibrios" King (1957) are included in this subsp. *C. fetus* ss. *jejuni* is the normal flora present in the intestines of cattle, sheep, swine, poultry and wild birds (pigeons and Sparrows,etc.).

Ref: Smibert (1975).

MATERIALS AND METHODS

A. Test Organisms:

Campylobacter fetus subsp. fetus 482, Campylobacter fetus subsp. intestinalis PB1 and Campylobacter fetus subsp. jejuni H840 kindly supplied by Dr. R. M. Smibert, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, were used in this study. Stock cultures were maintained in Brucella semi-solid medium containing 0.16% Bacto-agar, incubated aerobically at 37 C, and transferred weekly.

B. Chemicals and Reagents:

All chemicals and reagents were obtained commercially at the highest purity available.

Acetic anhydride, ammonium chloride, ammonium sulfate, cysteine-HCl dextrose, disodium ethylenediamine tetraacetate (EDTA), ferrous sulfate, fumaric acid, glycerin, lactic acid, magnesium acetate, magnesium sulfate, manganese sulfate, phenol reagent (2N solution), phenylhydrazing-HCl, potassium chloride, potassium hydroxide, potassium phosphate (dibasic anhydrous), potassium phosphate (monobasic), scintanalyzed toluene, sodium acetate, sodium bicarbonate, sodium bisulfite, sodium carbonate, sodium chloride, sodium cyanide, sodium hydroxide, sodium sulfate, and soluble starch were obtained from Fisher Scientific Co. (Fairlawn, N.J.).

Cobalt chloride, cupric sulfate, magnesium chloride, pyridine, sodium citrate, succinic acid (disodium salt), trichloroacetic acid and tris (hydroxymethyl) amino methane-HCl were obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.).

L-aspartic acid, L-malic acid, and D-ribose were obtained from Eastman Organic Chemicals (Rochester, N.Y.).

Brucella broth and trypticase were obtained from BBL (Cockeysville, Maryland).

Bacto-agar and yeast extract were obtained from Difco Laboratories (Detroit, Mich.).

PPO (2,5-diphenyloxazole) and POPOP (1,4-Bis-(2-(5-phenyloxazolyl)) benzene) were obtained from ICN Pharmaceuticals Inc. (Irvine, Calif.).

UV glucose reagent set was obtained from Worthington Biochemical Corp. (Freehold, N.J.).

Methyl-α-D-glucopyranoside (glucose-U-C^{14}) sp. act. 52.2 mci/mM was obtained from Cal-atomic (Los Angeles, Calif.).

Adenosine-5'-monophosphate monohydrate (A grade), acetyl coenzyme A trilithium salt 0.3 H_2O, Bovine albumin (crystalline), malate dehydrogenase (porcine heart), methyl-α-D-glucopyranoside (A grade), nicotinamide adenine dinucleotide (reduced) disodium 0.3 H_2O, phosphoglucose isomerase (yeast) were obtained from Calbiochem (San Diego, Calif.).

Acetyl coenzyme A sodium salt (grade 1), acetyl phosphate lithium potassium salt, adenosine-5'-diphosphate barium salt (equine muscle, grade 1), adenosine 5'-triphosphate sodium salt (grade 1), aldolase (rabbit muscle, grade 1), coenzyme A lithium salt (yeast, grade 1), 2,6-dichloro-phenol-indophenol sodium salt (grade 1), 5,5'- dithiobis-(2-nitrobenzoic acid), erythrose-4-phosphate sodium salt, fructose-1,6-diphosphate tri-sodium salt (grade 1), D-fructose-6-phosphate disodium salt (grade 1), D-gluconate sodium salt, β-D-glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase (baker's yeast, type XV), α-glycerophosphate dehydrogenase-triose phosphate isomerase (rabbit muscle type III), gly-cylglycine (free base), glyoxylic acid sodium salt monohydrate, guanosine-

5'-diphosphate sodium salt (yeast type 1), imidazol (grade 1), glutathione, DL-isocitrate trisodium salt (reduced), α-ketoglutaric acid mono potassium salt, lactic dehydrogenase (rabbit muscle, type III), β-nicotinamide adenine dinucleotide, β-nicotinamide adenine dinucleotide phosphate, β-nicotinamide adenine dinucleotide reduced form disodium salt, β-nicotinamide adenine dinucleotide phosphate reduced form disodium salt, cis-oxaloacetic acid, phenazine methosulfate, phosphoenolpyruvic acid trisodium salt hydrate, 6-phosphogluconic acid trisodium salt, 6-phosphogluconic dehydrogenase (yeast, type IV), pyruvic acid sodium salt pyruvate kinase (rabbit skeletal muscle type II), thiamine pyrophosphate chloride, triethanolamine-HCl, xylulose-5-phosphate sodium salt (grade II), were obtained from Sigma Co. (St. Louis, Mo.).

C. Media:

1. Brucella Semi-solid Medium:

7 ml of Brucella semi-solid medium per culture tube (screw capped; 150mm X 16mm) were used for maintenance of stock cultures. The composition of medium is as follows:

Components	Amt./liter
Brucella broth	28 g
Bacto-agar	1.6g
0.05% Neutral red	25 ml
Distilled water	1000 ml

Brucella broth contains 2% of polypeptone™ peptone, 0.1% of dextrose, 0.2% of yeast autolysate, 0.5% of sodium chloride and 0.01% of sodium bisulfite.

2. Growth Medium of Biphase Culture System:

The biphase culture system is a mass culture technique adapted from Dr. Smibert's Laboratory. The organism is cultured in a one-liter filtering flask containing a layer of 200 ml of solid medium, above that is 300 ml of liquid medium. Cells multiply best along the surface of the solid medium; with mild shaking, the mass of cells are washed away from solid phase and are dispersed in the liquid medium. The following two sets of media were used for growth experiments and for culture preparations during this study.

(1) Basal Medium: Basal medium was used to study the growth curves of strains of C. fetus. The effects of 0.5% glucose, citrate, acetate, or lactate on the growth of cultures were tested when 5 g per liter of the corresponding carbon source were added to the broth phases of basal medium. The pathways of glucose degradation were examined in the crude cellular extract of cultures that were grown in basal medium. The components of the basal medium are listed as follows:

Components	Liquid phase g/liter	Solid phase g/liter
Trypticase	20	20
Yeast extract	2	2
Sodium bisulfite	0.1	0.1
Sodium chloride	5	5
Bacto-agar	-	15

(2) Enriched Medium: Cultures grown in enriched medium were tested for enzymes of the citric acid cycle pathway and glyoxylate bypass; also some major ancillary enzymes of the citric acid cycle were tested. The medium was initially formulated by Smibert (1975). Its composition is listed as follows:

Components	Liquid phase g/liter	Solid phase g/liter
Brucella broth	28	28
Sodium succinate	2	2
Soluble starch	5	5
Ferrous sulfate	0.1	0.1
Bacto-agar	-	15

D. Growth Curve Procedure:

1. Inoculum Preparation:

About 6 ml of stock culture were transferred into a 500 ml Erlenmeyer flask which contained 100 ml of solid medium and 150 ml of liquid medium. The flask was set in an anaerobic jar and incubated at 37 C for 48 hours. The percentage of oxygen in the jar was reduced to 5% by partial vacuum of the system.

2. Growth Study:

Growth was initiated by transferring 30 ml of the inoculum into a one-liter filtering flask containing 300 ml of liquid phase and 200 ml of solid phase. The culture was aerated by a mixture of gas composed of 5% O_2, 10% CO_2 and 85% N_2 and incubated in a water bath shaker (Fermentation Design Inc., Allentown, Pa.) at 37 C and 100 RPM. The growth of the organism was followed by measuring the optical density of the culture at 600 nm with a Bausch and Lomb Spectronic 20 photometer; the liquid medium was used as the blank.

E. Culture Preparation and Harvesting Conditions:

Approximately 3 ml of one-week old stock culture was directly inoculated into a one-liter filtering flask containing a total of 500 ml of biphasic culture medium. The culture was incubated in the water bath

shaker at 37 C and 100 RPM. 5% O_2 was maintained in the flask by either partial vacuum of the system or by passing a gas mixture consisting of 5% O_2, 10% CO_2 and 85% N_2 through the system. After 48 hours incubation for the culture grown in the enriched medium, or 108 hours incubation for the culture grown in either the basal medium or basal medium plus 0.5% carbon sources, the organisms were harvested at the middle stage of logarithmic growth. Cells were collected by centrifugation in a Sorvall RC2-B refrigerated centrifuge at 8,000 x g at 4 C for 10 minutes; the cell paste was washed twice with 25 ml of 0.1 M phosphate buffer (pH 7.0), and resuspended in a few ml of the same buffer. The cell suspension was used in the following studies.

F. Standardization of Cell Concentration:

The number of cells per ml of cell suspension was estimated by a direct microscopic counting method. The cell suspension was diluted with 0.1 M phosphate buffer (pH 7.0) to various levels of absorbancies. Each preparation was then rediluted with glycerol, as described by Pierson (1970) to a final percentage of 20% glycerol; thus the motion of the microorganisms was restricted. The Petroff-Hausser counting chamber was filled with the glycerol diluted suspension; the number of cells in 20 squares were counted. The average cell number for one square was obtained. Then, the number of cells per ml of suspension could be calculated by the following equation:

The number of cells per ml = average cell number per square x 20 x 20 x 50 x 1000 x dilution factor.

G. Uptake of α-methyl-D-glucoside:

The possible existence of a glucose permease in C. fetus was investigated through its uptake of α-methyl-D-glucoside (α-MG). The rate of accumulation of α-MG in the organism is the function of rate of uptake of α-MG. Therefore, the accumulations of ^{14}C-labelled α-MG by C. fetus at various time intervals of incubation were measured. The method described by Pierson (1970) was used in this experiment.

108-hour old cultures of C. fetus 482 grown both in basal medium and in basal medium + 0.5% glucose were separately harvested, washed and resuspended in sterilized 0.1 M phosphate buffer (pH 7.0). The final cell concentrations of these suspensions were adjusted to about 2×10^9 cells/ml. These preparations were used to study α-MG uptake.

A 14 ml volume of such a cell preparation was placed in a 50 ml Erlenmeyer flask and incubated in a 37 C water bath. A submersible magnetic stirrer (Cole-Parmer Instrument Co., Chicago, Ill.) was placed in the water bath to gently mix the cell suspension with a stirring bar. The system was aerated with a mixture gas of 5% O_2, 10% CO_2 and 85% N_2. To the suspension, one ml of 0.6 mM α-methyl-D-glucoside (glucose-U-C^{14}) having a specific activity of 0.83 μCi per μmole, was added. One ml suspension was immediately removed from the reaction mixture and vacuum filtered onto a 0.45 μm pore size Millipore membrane filter (Millipore Filter Corp., Bedford, Mass.). The reaction mixture was then sampled every 3 minutes, up to 30 minutes. The cells were mounted on the filter as fast as possible. They were washed twice with 1 ml of cold phosphate buffer (pH 7.0), and dried under a heat lamp (General Electric). The dried cells on the filter were transferred into a vail containing 15 ml of

scintillation fluid which was composed of 0.5% PPO (2,5-diphenyloxazole) and 0.05% POPOP (2,2'-p-phenylene-bis(5-phenyloxazole)) per liter of liquid scintillation counting grade toluene. The radioactivity of samples was measured in a Beckman LS-100 Liquid Scintillation System with a C^{14} narrow window. The filter mounted with one ml of cell suspension but without substrate was used as the background. All samples were counted for 20 minutes or until a 20,000 counts (1.5% error) had been obtained.

H. Assimilation of Glucose and Citrate:

Glucose and citrate assimilation during the growth of C. fetus 482 was studied. A 30 ml volume of the 48-hour old inoculum grown in basal medium was transferred into each of the 3 flasks that contained the following growth media of biphase culture system:

1. A total 500 ml of basal medium.
2. A total 500 ml of basal medium + 0.5% glucose.
3. A total 500 ml of basal medium + 0.5% citrate.

A 15-day growth study was performed. Every 24 hours, 6 ml of culture were removed from each flask. After the optical density of the culture was read at 600 nm, the cells were removed by centrifugation with a Sorvall RC2-B refrigerated centrifuge at 8000 x g, at 4 C for 10 minutes. The pH of the supernatant was measured with a Corning Model 12 Research pH Meter (Corning Scientific Instruments, Corning Glass Works, Medfield, Mass.). The supernatant was then frozen until used for the glucose and citrate determinations.

I. Preparation of Crude Cellular Extract:

4 ml of cell suspension were mixed with 3 ml of glasperlen (0.10-

0.11 mm in dia., Kat Nr. 54140, B. Braun, Melsungier Apparatebau). The mixture was filled in a 10 ml stainless steel beaker mounted on the beaker holder of Vibrogen cell mill (RHO Scientific, Inc., Commack, New York) and cooled with ice water. The cells were disintegrated with the mill at the vibration speed of 4500 strokes/minute for 5 minutes. The fine glass beads were removed from the homogenate by low speed centrifugation in the Sorvall RC2-B refrigerated centrifuge at 121 x g, at 4 C for 5 minutes. The homogenate was subjected to a high speed centrifugation in the same centrifuge at 27,000 x g, at 4 C for 20 minutes. The precipitant that included some intact cells and cell debris was discarded. The supernatant was collected and used as crude cellular extract in which various enzymatic reactions were studied. An overall preparation procedure is presented in the following chart (Figure 1).

J. Enzyme Assays:

All enzyme assays were performed at 25 C in a Perkin-Elmer double beam Coleman model 124 spectrophotometer equipped with Perkin-Elmer model 56 recorder (Coleman Instruments Division, Maywood, Ill.). The temperature of reactions was maintained at 25 $\pm$ 0.05 C with a Lauda Constant-Temperature Circulator model WB-20/R (Brinkmann Instruments, Westbury, New York). The total volume of reaction mixture was 3 ml which contained the appropriate substances and cofactors. The reactions were initiated by the addition of the properly diluted crude cellular extracts. The rates of reactions were measured as the initial change in absorbance (1-cm light path) at a fixed wavelength and expressed as micromoles of substrate transformed per minute (international units). The specific

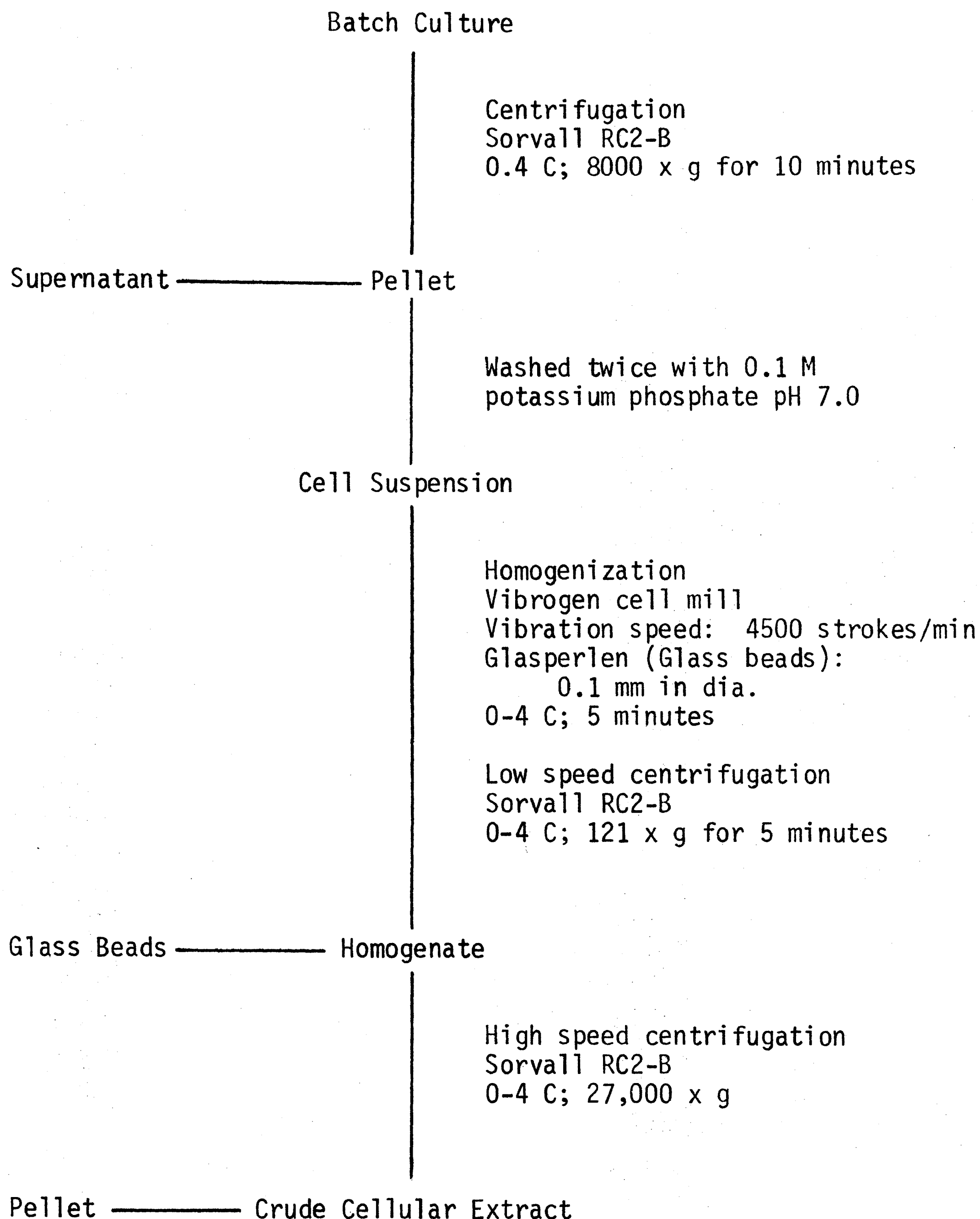


Figure 1. The preparation procedure of crude cellular extract of *C. fetus*.

activity of an enzyme was then defined as the rate of catalytic reaction by 1 mg of protein.

NAD and NADP and their reduced forms have been used as cofactors in many enzymatic reactions. Their reaction rates, therefore, can simply be followed by measuring the reduction (or oxidation) of the dinucleotides at 340 nm. The reactions listed in Table V were assayed by this method.

Many enzymatic activities can be spectrophotometrically assayed by determining the rate of product formation through coupling with a second enzyme reaction. When the substrate and coupling enzyme are present in excess, the step of product formation (the major reaction) is consequently a slow reaction step and is a rate-determining step. Therefore, the rate of the overall coupling reactions is essentially the same as that of the slow step reaction. The activities of the enzymes listed in Table VI were measured by the rate of overall coupling reactions.

Compounds with ethylene (C=C) linkage or with a thio ester have characteristic light absorption bands in the ultraviolet region. These optical properties can be used to assay several enzyme reactions by simply measuring the appearance or disappearance of these functional groups. The enzymatic reactions listed in the next Table (Table VII) were assayed in this manner.

A few artificial dyes were used in the following enzymatic reactions (Table VIII) to either accept electrons or react with the product. The reduced dyes or the derivatives of the product are chromogenic. Consequently, the rate of reaction can be spectrophotometrically determined by measuring the rate of formation of such a compound at its specific wavelength.

Table V. The enzymes assayed by the formation of NAD, NADP, or their reduced forms at 340 mμ

Enzyme and Reference	Assay Mixture[1,2]
Isocitrate dehydrogenase NAD - specific (EC 1.1.1.41) NADP - specific (EC 1.1.1.42) Hatchaway & Atkinson (1963)	Tris-HCl (pH 7.3), 200; Isocitrate, 10; $MgCl_2$, 10; NADP (or NAD) 1.
α-ketoglutarate dehydrogenase (EC 1.2.4.2) Reed & Mukherjee (1969)	Phosphate (pH 8.0), 150; $MgCl_2$, 3; NAD, 6; Cysteine, 9; TPP, 0.6; CoA, 0.18; α-KG, 3.
Malate dehydrogenase (EC 1.1.1.37) Yoshida (1965b)	Tris-HCl (pH 8.8), 250; OAA, 3.7; NADH, 0.8.
Pyruvate dehydrogenase (EC 1.2.4.1) Reed & Mukerjee (1969)	Phosphate (pH 8.0), 150; $MgCl_2$, 3; NAD, 6; Cysteine, 9; TPP, 0.6; CoA, 0.18; Pyruvate, 3.
Glutamate dehydrogenase (EC 1.4.1.4) Grisolia *et al*. (1964)	Phosphate (pH 8.0), 150; α-KG, 60; NH_4Cl, 330; NADH, 0.22; (NADPH, 0.6).
Malic enzyme (EC 1.1.1.40) Hsu & Lardy (1967)	Tris-HCl (pH 7.5), 200; Malate, 1.5; $MgCl_2$, 18; NADP, 0.1.
Lactic dehydrogenase (EC 1.1.1.27) Yoshida (1965a)	Phosphate (pH 6.0), 100; Pyruvate, 2.4; NADH, 0.2.

Table V. (Continued)

Enzyme and Reference	Assay Mixture[1,2]
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) Noltmann *et al*. (1961)	Glycylglycine (pH 8.0), 260; G-6-P, 3; NADP, 1; $MgCl_2$, 30.
6-phosphogluconate dehydrogenase (EC 1.1.1.44) Pontremoli and Grazi (1966)	Glycylglycine (pH 7.6), 100; 6-PG, 4.5; $MgCl_2$, 60; NADP, 1.
Glucose dehydrogenase (EC 1.1.1.47) Sadoff (1966)	Tris-HCl (pH 8.0), 150; Glucose, 60; NAD (or NADP), 0.5; $MnSO_4$, 0.3.
Reduced NAD(P) dehydrogenase (EC 1.6.99.2) Bragg (1965)	Tris-HCl (pH 8.0), 100; NADH (or NADPH), 0.2; $MgCl_2$, 2.

1. Numerals without unit refer to the micromoles of compound used in the 3.0 ml of assay mixture.

2. All assays were performed at 25 C.

Table VI. The enzymes assayed by coupling reactions.

Enzyme and Reference	Assay Mixture[1,2]
Phosphoenolpyruvate carboxylase (EC 4.1.1.31) Quayle (1969)	Tris-HCl, (pH 8.5), 100; $MgCl_2$, 4; $NaHCO_3$, 40; NADH, 0.2; 10.1 μg of MDH (12 IU).
Pyruvate caboxylase (EC 6.4.1.1) Seubert & Weicker (1969)	Tris-HCl, (pH 7.2), 110; $MgCl_2$, 15; $NaHCO_3$, 20; Pyruvate, 2; NADH, 0.2; ATP, 2; 2 μg of MDH (30 IU).
Pyruvate kinase (EC 2.7.1.40) McQuate & Utter (1959)	Tris-HCl, (pH 7.4); KCl, 75; NADH, 0.2; $MgCl_2$, 4; ADP, 0.5; PEP, 3.12; 1.25 μg of LDH (1.3 IU).
Phosphoenolpyruvate snthetase (EC 2.7.1.dd) Cooper & Kornberg (1969)	Phosphate, (pH 6.8), 300; $MgCl_2$, 15; AMP, 3; PEP, 3; NADH, 0.4; 1.25 μg of LDH (1.3 IU).
Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) Chang and Lane	Tris-HCl, (pH 7.5), 200; PEP, 2; GDP (or ADP), 0.6; $NAHCO_3$, 50; $MnCl_2$, 1; GSH, 2; NADH, 0.16; 5 μg of MDH (6 IU).
Phosphofructokinase (EC 2.7.1.11) Ling *et al*. (1966)	Tris-HCl, (pH 8.0), 100; CH_3CH_2SH, 10; ATP, 2; $MgCl_2$, 10; NADH, 0.2; F6P, 3.75; 100 μg of Aldolase (1.4 IU); 16 μg of α-GPDH and TPI (1.9 IU and 15.7 IU).

Table VI. (Continued)

Enzyme and Reference	Assay Mixture[1,2]
Fructose-1,6-diphosphatase (EC 3.1.3.11) Rosen *et al*. (1965)	Glycine, (pH 9.5), 200; FDP, 0.6; $MgCl_2$, 3; NADP, 1.5; 6.6 μg of G6PDH (2 IU); 10 μg of phosphoglucose isomerase (3.75 IU).
Fructose diphosphate aldolase (EC 4.1.2.13) Groves *et al*. (1966)	Tris-Acetate, (pH 7.5), 150; $CoCl_2$, 2.1; FDP, 6; NADH, 0.6; 8 μg of α-GPDH and TPI (0.95 IU and 7.85 IU).
Transaldolase (EC 2.2.1.2) Tchola and Horecker (1966)	TEA-HCl (pH 7.6), 100; EDTA, 25; F6P, 8.4; E4P, 0.6; NADH, 0.2; 16 μg of α-GPDH and TPI (1.9 IU and 15.7 IU).
6-phosphogluconate dehydrase (EC 4.2.1.12) and KDPG aldolase (EC 4.1.2.C) Laughan and Krieg (1974)	Imidazol (Ph 8.0), 100; CH_3CH_2SH, 10; $MnCl_2$, 0.3; NADH, 0.6; 6PG, 8; 10 μg of LDH (10.5 IU). in 2 ml reaction mixture measured at 366 nm.
Glucokinase (EC 2.7.1.2) Kamel *et al*. (1966)	Glycylglycine, (pH 7.5), 200; $MgCl_2$, 20; ATP, 10; NADP, 2; D-glucose, 40; 3.3 μg of G6PDH (1 IU).
Phosphoglucose isomerase (EC 5.3.1.9) Noltmann (1964)	Tris-HCl (pH 8.0), 200; F6P, 5; NADP, 1.5; 3.3 μg of G6PDH (1 IU).

Table VI. (Continued)

Enzyme and Reference	Assay Mixture[1,2]
D-Ribokinase (EC 2.7.1.15) Anderson & Wood (1962)	Tris-HCl (pH 7.5), 160; GSH, 30; $MgCl_2$, 20; ATP, 10; D-ribose, 30; PEP, 5; NADH, 0.6; 12.4 μg of LDH (13 IU); 10 μg of pyruvatekinase (3.9 IU).
Acyl phosphate : Hexose phosphotransferase (EC 2.7.1.g) Kamel & Anderson (1964)	Glycylglycine (pH 7.5), 100; D-glucose, 10; Acetyl-p, 10; NADP, 5; EDTA, 10; 6.6 μg of G6PDH (2 IU).
Gluconokinase (EC 2.7.1.12) Laughon & Krieg (1974)	Tris-HCl, (pH 8.0), 100; CH_3CH_2SH, 10; D-gluconate, 50; ATP, 10; $MgCl_2$, 20; NADP, 1; 6.25 μg of 6PGDH (0.25 IU).
Phosphoketolase[3] (EC 4.1.2.9) Goldberg *et al.* (1966)	

1. Numerals without unit refer to the micromoles of compound used in the 3.0 ml of assay mixture.

2. All assays were performed at 25 C.

3. Phosphoketolase (EC 4.1.2.9): The complete system (0.5 ml) contained 10 μmoles of potassium phosphate, pH 6.0, 2 μmoles of D-xylulose-5-phosphate, 3 μmoles of glutathione, pH 6.0, 1 μmole of $MgCl_2.6H_2O$, 0.3 μmoles of thiamine pyrophosphate, pH 7.5, 16 μg of the mixture of Triose phosphate isomerase (15.7 IU) and α-glycerophosphate dehydrogenase (1.9 IU), 50 μg of aldolase (0.7 IU) and crude cellular extract. The mixture was incubated at 37 C for 15 minutes and then placed in a boiling water bath for 1 minute to stop the enzyme reaction. The denatured protein was centrifuged down and discarded. The supernatant was assayed for the formation of fructose-1,6-diphosphate. The assay procedure is outlined as follows: 3 ml reaction mixture contained 150 μmoles of Triethanol amine, pH 7.5, 0.2 μmoles of NADH, 16 g of the mixture of Trisose phosphate isomerase (15.7 IU) and α-glycerophosphate dehydrogenase (1.9 IU), 50 μg of aldolase (0.7 IU) and 0.1 ml of supernatant. NADH oxidation was followed at 340 mμ until no further change of absorbancy was seen.

Table VII. The enzymes assayed by measuring the appearance or disappearance of a functional group.

Enzyme and Reference	Assay Mixture and Wavelength[1,2]
Aconitase (EC 4.2.1.3) Racker (1950)	Phosphate, (pH 7.5), 150; DL-isocitrate, 30; 240 nm.
Fumarase (EC 4.2.1.2) Massey (1955)	Phosphate, (pH 7.3), 100; fumarate, 3.36; 250 nm.
Succinyl Co A synthetase (EC 6.2.1.5) Ramaley (1967)	Tris-HCl, (pH 7.2) $MgCl_2$, 30; ATP, 1.2; Succinate, 30; CoA, 0.3; 230 nm.
Malate synthase (EC 4.1.3.2) Dixon *et al*. (1960)	Tris-HCl, (pH 8.0), 240; $MgCl_2$, 10; Acetyl CoA, 0.5; glyoxylate, 2; 232 nm.
Aspartase (EC 4.3.1.1) Emery (1963)	Tris-HCl, (pH 7.0), 150; L-aspartic acid, 150; $MgSO_4$, 3; EDTA, 0.3; 240 nm.
Phosphotransacetylase (EC 2.3.1.8) Klotzsch (1969)	Tris-HCl, (pH 7.4), 200; GSH, 3.25; CoA, 0.29; Acetyl-P, 21.7; $(NH_4)_2SO_4$, 16.5; 233 nm.

1. Numerals without unit refer to the micromoles of compound used in the 3.0 ml of assay mixture.

2. All assays were performed at 25 C.

Table VIII. The enzymes assayed by measuring the formation of chromogens.

Enzyme and Reference	Assay Mixture and Wavelength[1,2]
Citrate synthase (EC 4.1.3.7) Srere *et al.* (1963)	Tris-HCl, (pH 8.0), 200; DTNB, 0.3; Acetyl CoA, 0.4; OAA, 0.3; 412 nm.
Isocitrate lyase (EC 4.1.3.1) Dixon & Kornberg (1959)	Phosphate, (pH 6.85), 200; $MgCl_2$, 15; Phenyl hydrazine, 10; Cysteine-HCl, 6; DL-isocitrate, 10; 324 nm.
Succinate dehydrogenase (EC 1.3.99.1) Ells (1959)	Phosphate, (pH 7.5), 150; succinate, 50; KCN, 10; DPIP, 0.04; PMS, 0.5; 600 nm.
Glucose dehydrogenase (EC 1.1.99.a) Hauge (1966)	Phosphate, (pH 6.0), 150; glucose, 60; DPIP, 0.14; 600 nm.

1. Numerals without unit refer to the micromoles of compound used in the 3.0 ml of assay mixture.

2. All assays were performed at 25 C.

K. Chemical Analyses:

The method of Lowry et al. (1951) was used to measure the protein concentration in the crude cellular extracts with crystalline bovine serum albumin as the colorimetric standard.

Glucose was enzymatically determined in the supernatants of the culture by the Worthington UV glucose procedure (Worthington Biochemical Corp.): 0.1 ml of a 1 to 5 diluted sample were mixed with 3 ml of reconstituted reagent. The reaction was spectrophotometrically followed at 340 nm for 5 minutes. The results were calculated as mg of glucose per ml of supernatant of culture.

A rapid procedure described by Saffran and Deustedt (1948) was followed to determine the concentration of citric acid in the supernatants of the cultures: 0.2 ml of sample were diluted with 1.8 ml of 5% TCA and then 0.4 ml of the mixture were dehydrated with 5 ml of acetic anhydride followed by the addition of 0.5 ml of pyridine. A yellowish color was developed by keeping the reaction tube in a 40 C water bath for 40 minutes. The intensity of the color was measured at 400 nm.

RESULTS

A. Growth Curves:

The growth curves of biphasic culture systems of strains of C. fetus were studied primarily to determine the culture age for the preparation of crude cellular extracts. Meanwhile, several growth characteristics of the organisms were observed.

The growth of C. fetus was extremely slow in both basal medium and basal medium plus 1.0% or 0.5% of four tested carbon sources, i.e.; glucose, citrate, lactate and acetate respectively (Figures 2 to 10). Cultures also grew very slowly in a total of 500 ml of plain Brucella medium in a biphase system. A slight improvement on the growth of this organism was observed when Brucella biphasic medium was enriched with succinate, ferrous sulfate and soluble starch.

The addition of 1% of a carbon source to the basal medium (Figure 3) showed no stimulatory effect on the growth of C. fetus 482. Glucose (1%) did not stimulate nor inhibit the growth of the culture. However, 1% of organic acid salts did inhibit growth (Figure 3).

At a level of 0.5%, the growth of C. fetus 482 was stimulated by glucose, lactate and acetate (Figures 3 and 4). Citrate neither stimulated nor inhibited growth. The growth of PB1 (Figures 5, 6, 7 and 8) was stimulated slightly by lactate and acetate, while glucose had no effect and citrate inhibited the growth slightly. The growth of H840 (Figures 9, 10 and 11) was stimulated slightly by citrate, but acetate had no effect on growth. Glucose stimulated growth during most of the log phase of the growth curve but growth was slowed during later stages of the curve.

The change in the acidity of the culture was measured with a pH

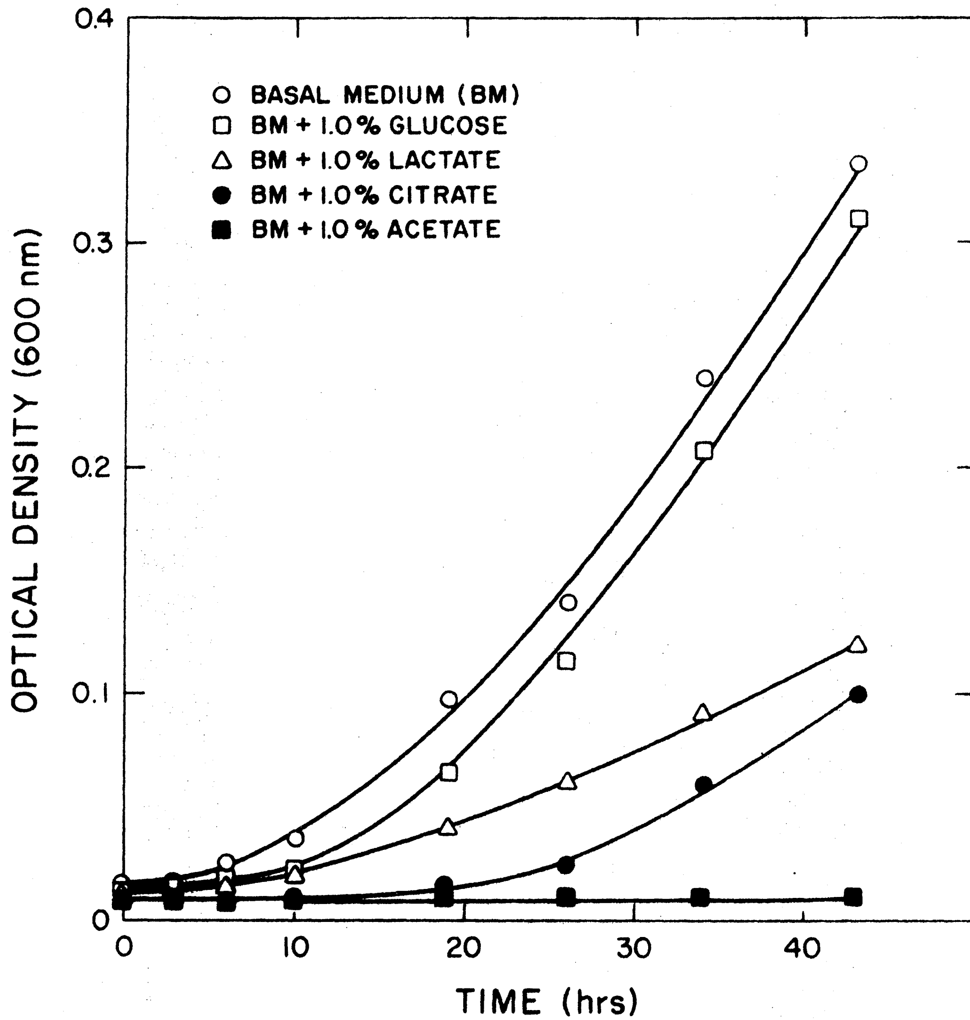


Figure 2. The effect of 1% glucose, citrate, lactate and acetate on the growth of C. fetus strain 482. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2, and 85% N_2.

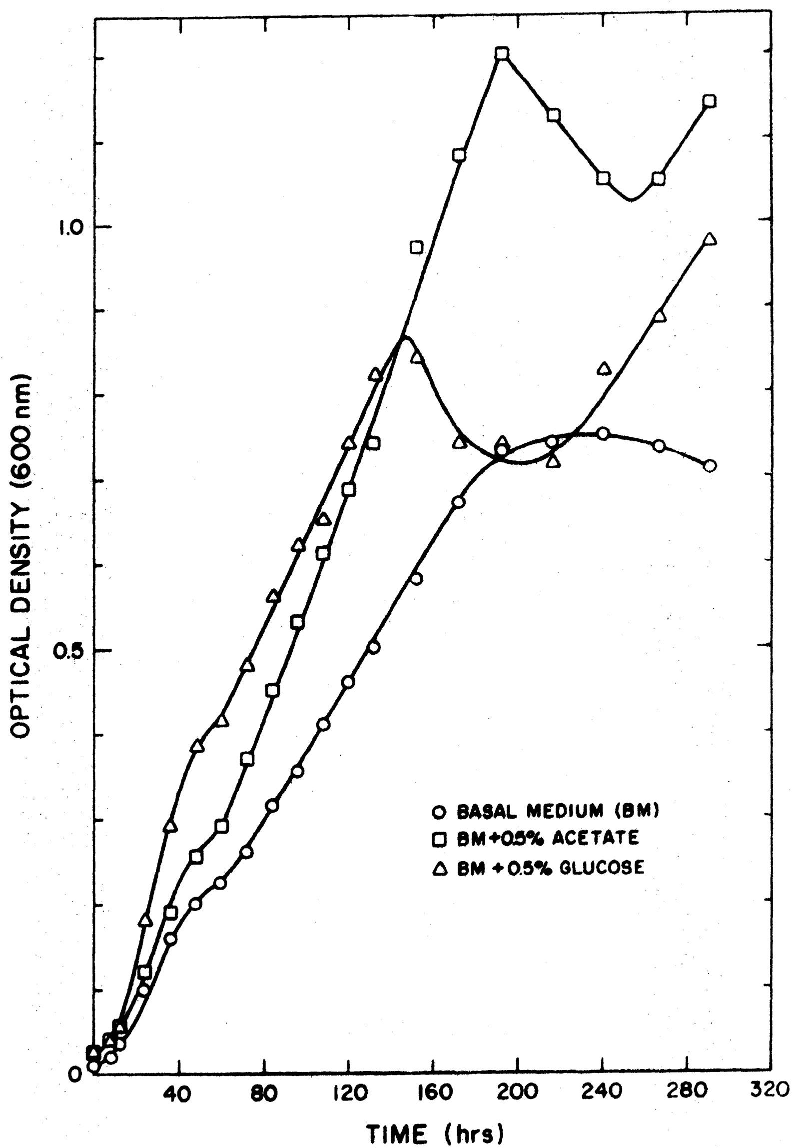


Figure 3. The growth curves for C. fetus 482 grown in basal medium (BM), BM + 0.5% acetate and BM + 0.5% glucose. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2, and 85% N_2.

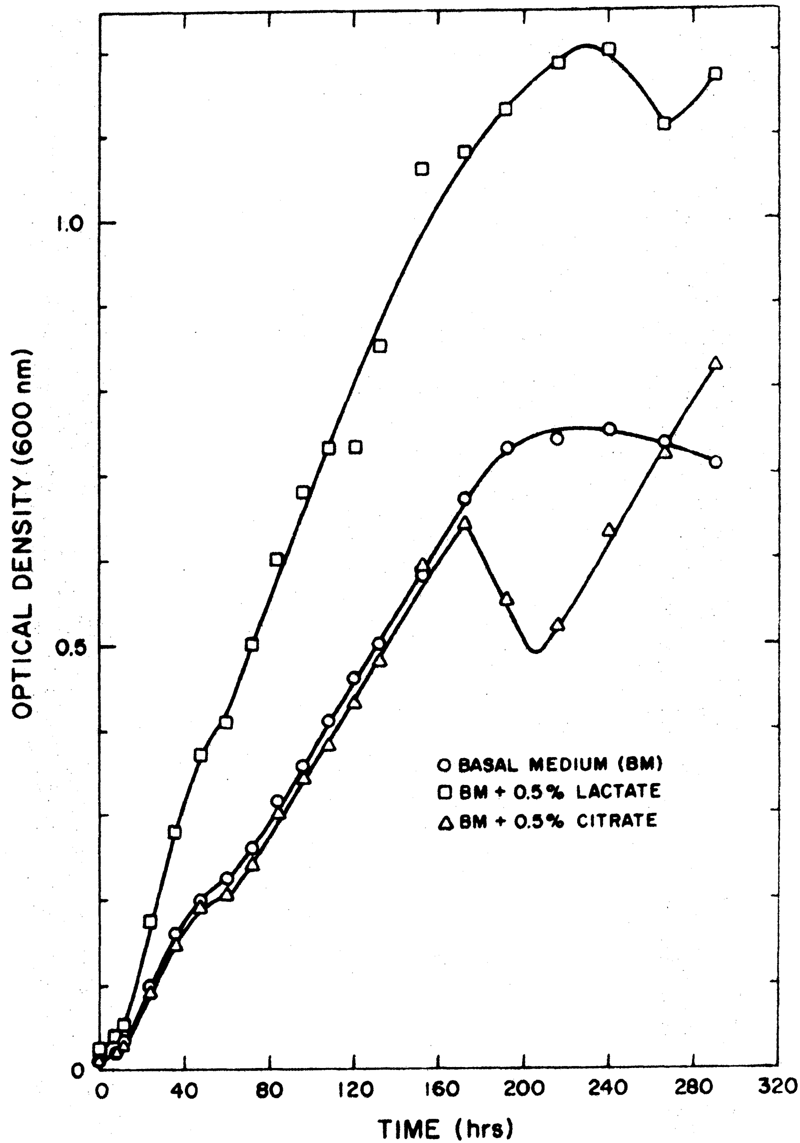


Figure 4. The growth curves for C. fetus 482 grown in basal medium (BM), BM + 0.5% lactate and BM + 0.5% citrate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2, and 85% N_2.

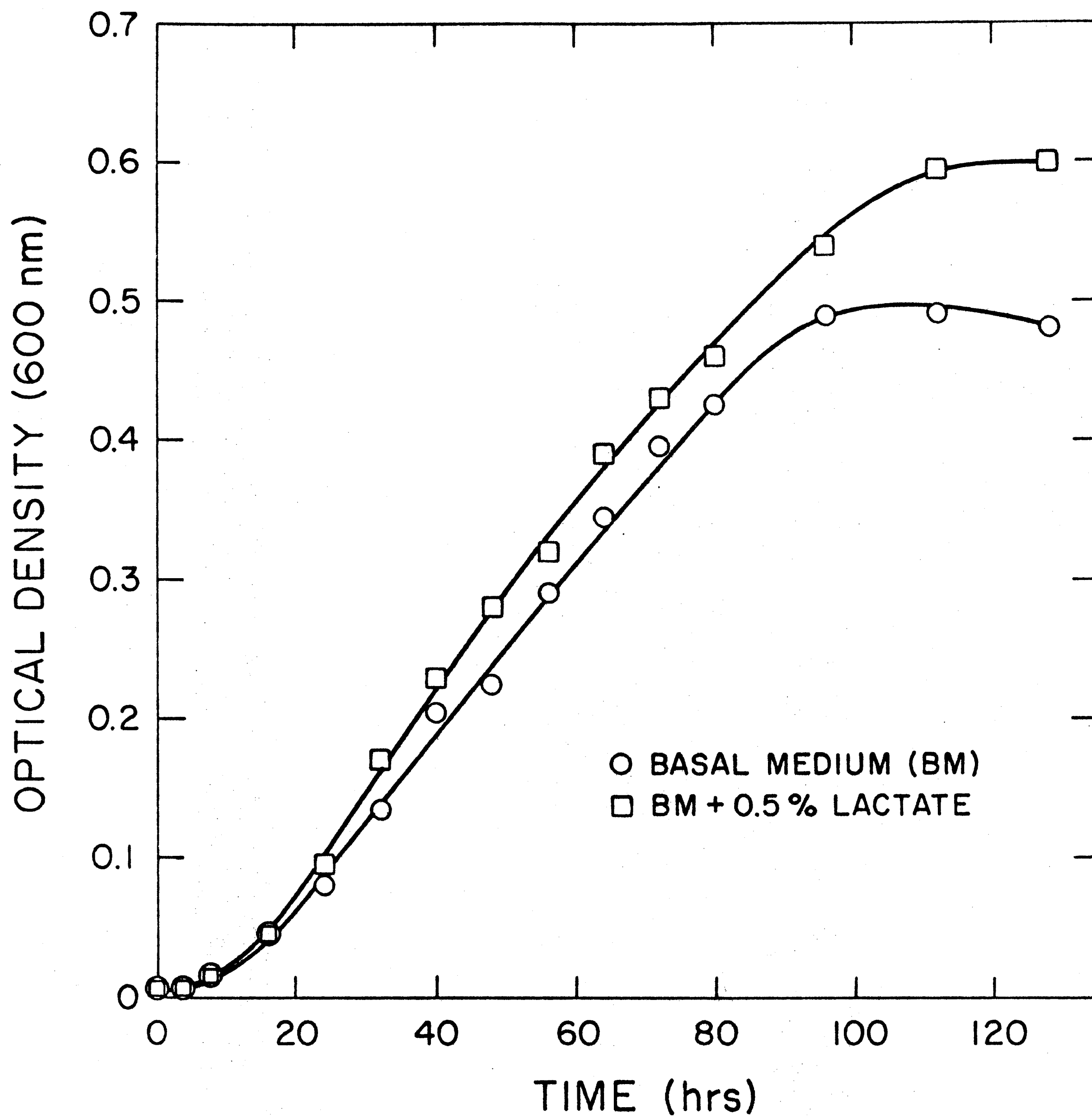


Figure 5. The growth curves for C. fetus PB1 grown in basal medium (BM) and BM + 0.5% lactate. Cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

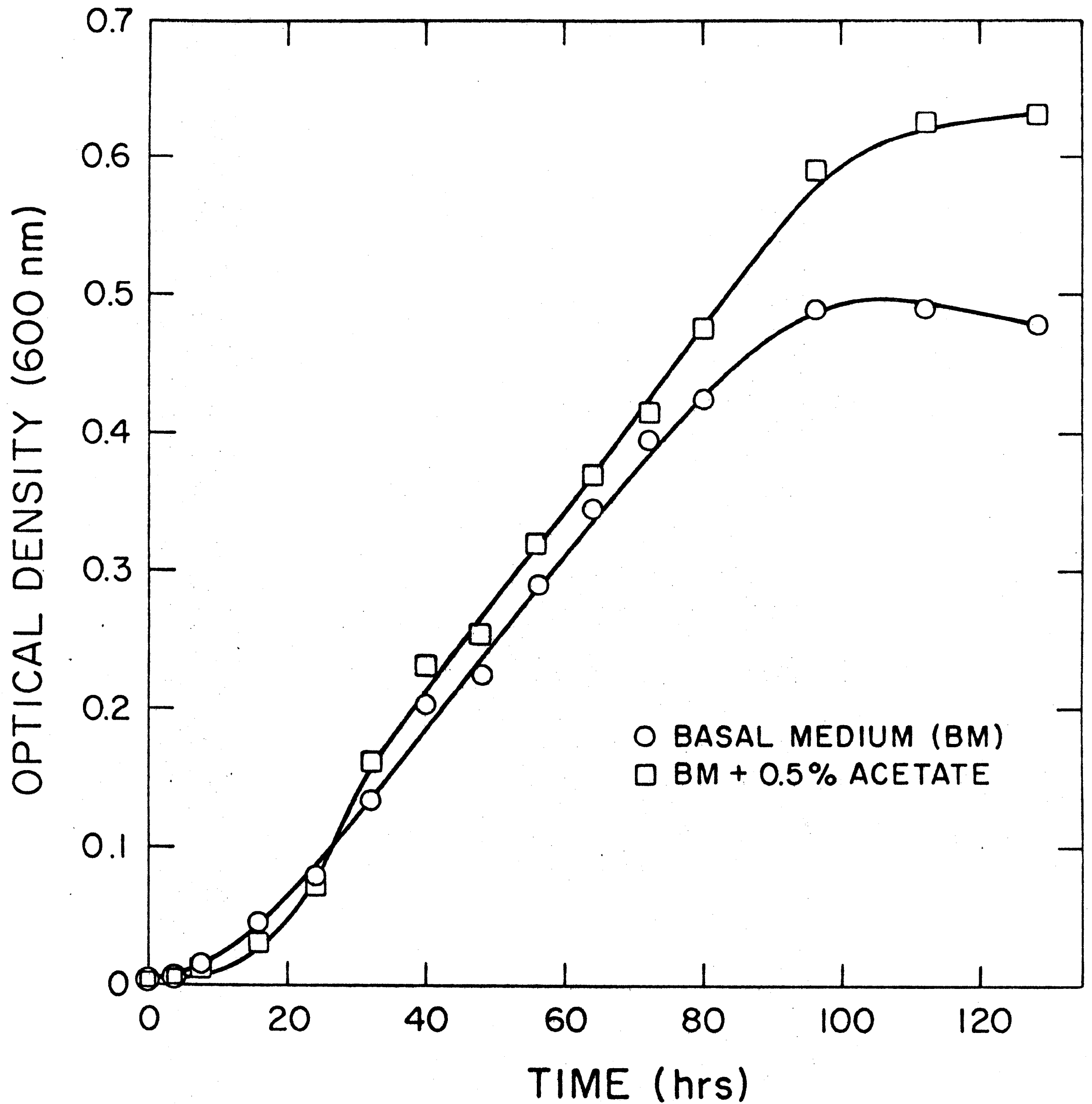


Figure 6. The growth curves for C. fetus PB1 grown in basal medium (BM) and BM + 0.5% acetate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

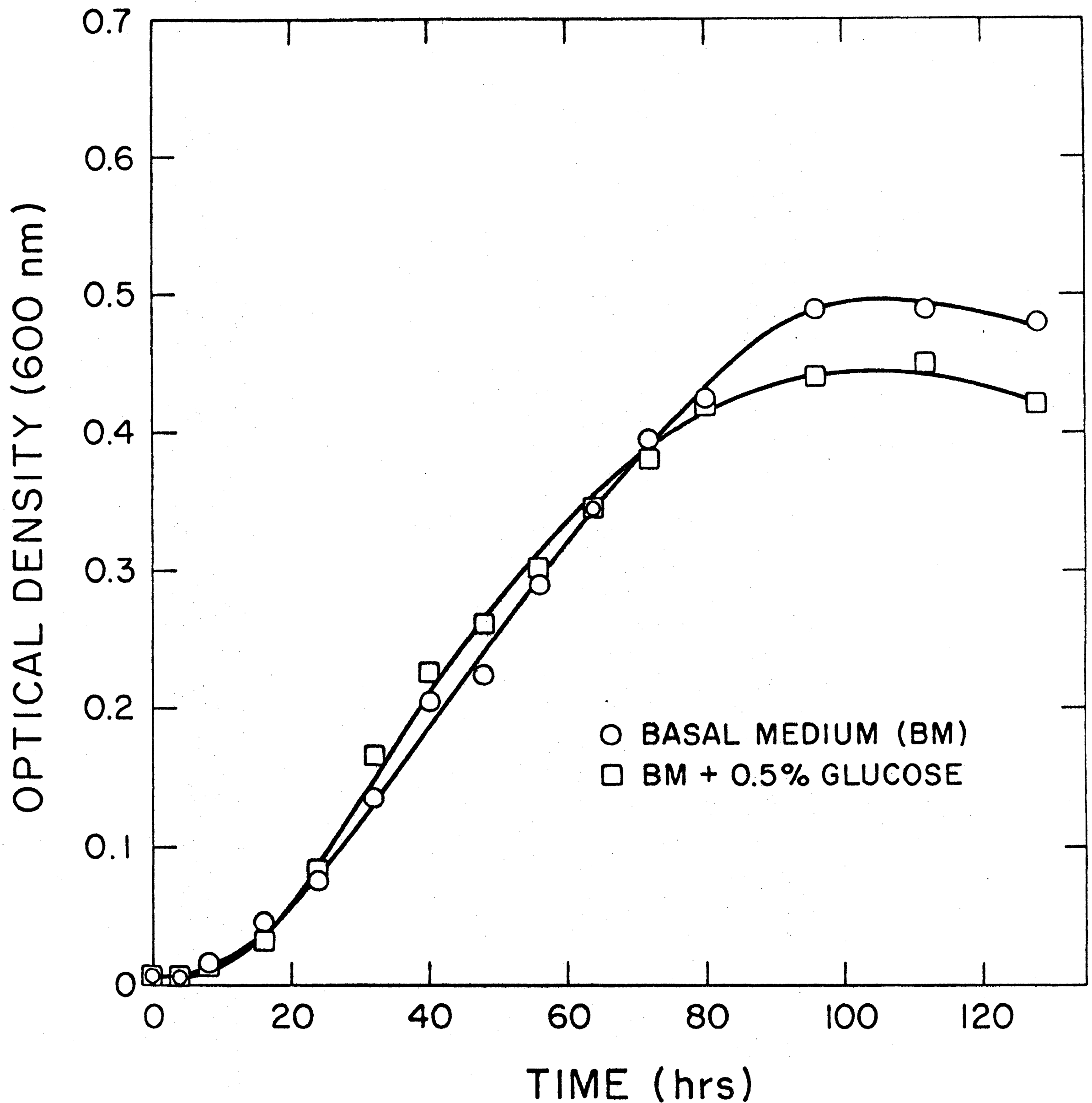


Figure 7. The growth curves for C. fetus PB1 grown in basal medium (BM) and BM + 0.5% glucose. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

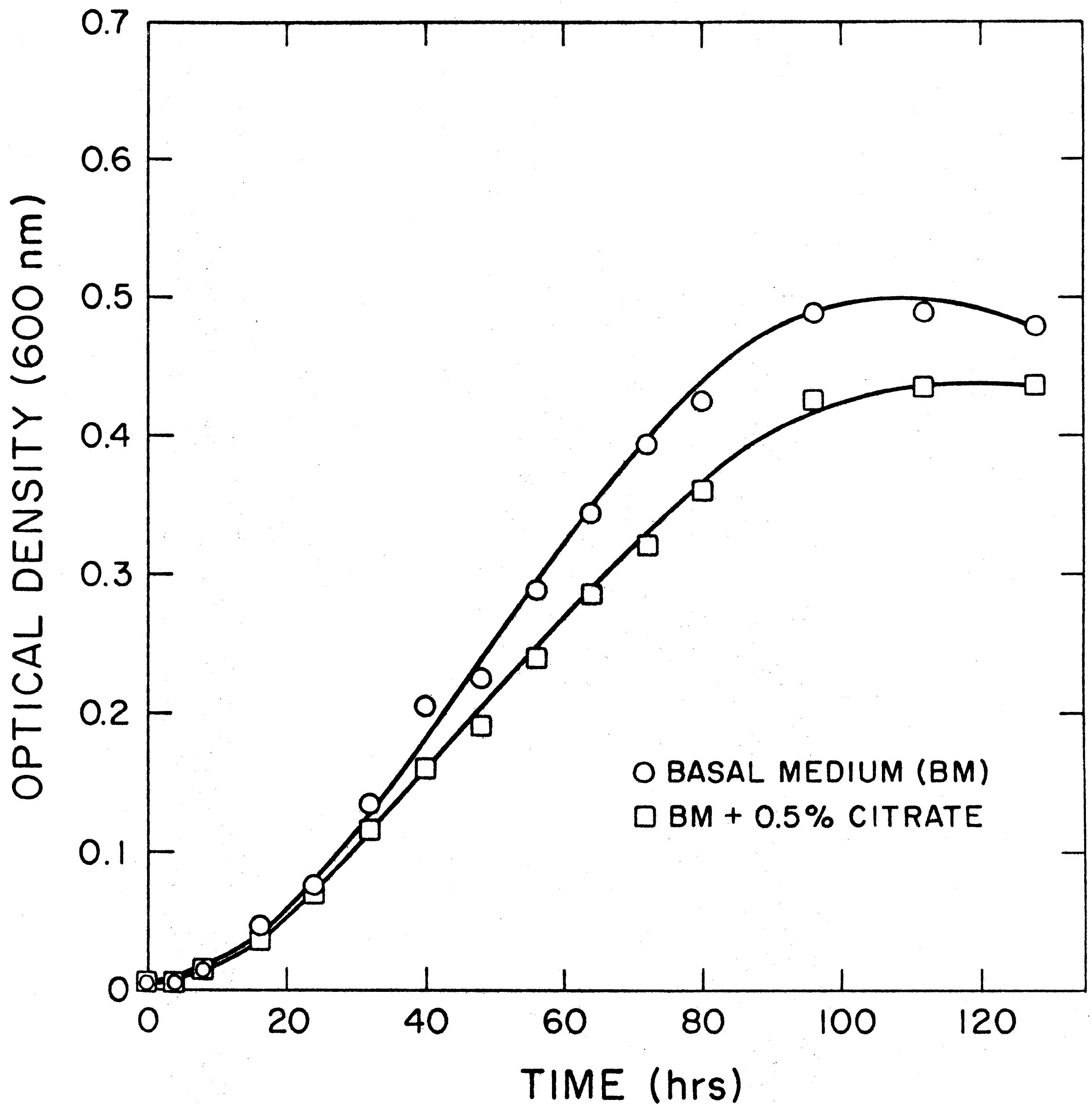


Figure 8. The growth curves for C. fetus PB1 grown in basal medium (BM) and BM + 0.5% citrate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

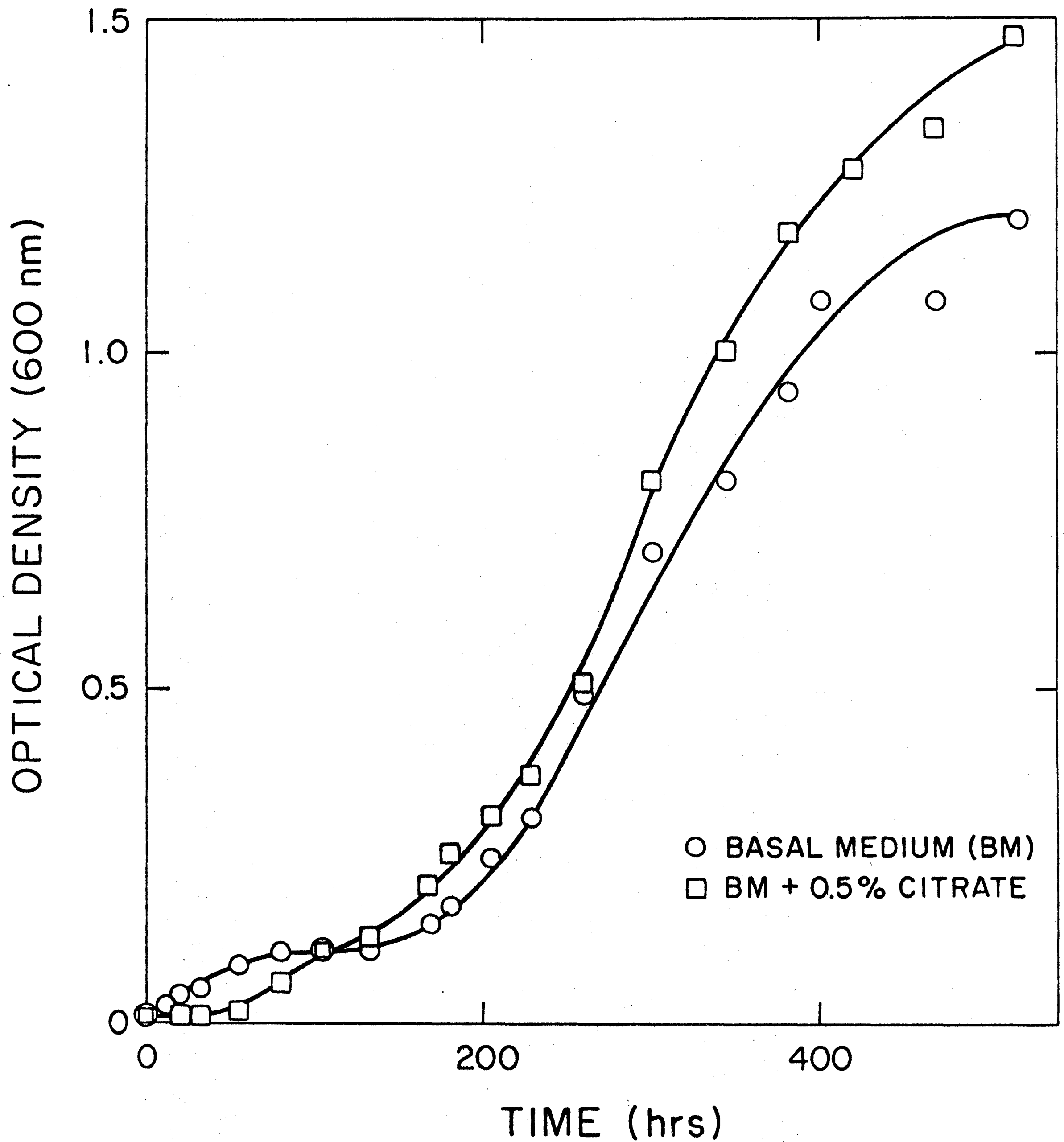


Figure 9. The growth curves for C. fetus H840 grown in basal medium (BM) and BM + 0.5% citrate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2, and 85% N_2.

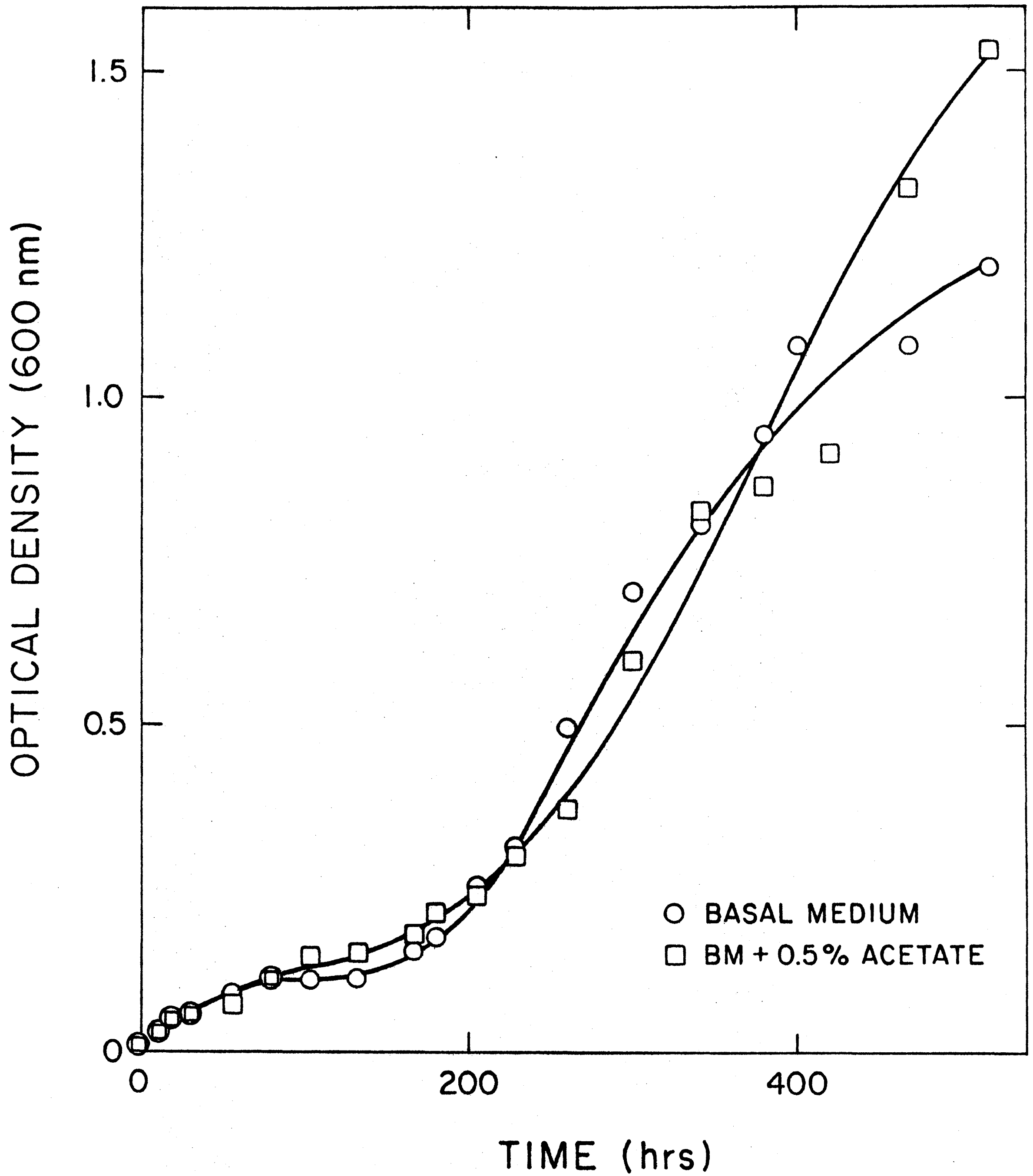


Figure 10. The growth curves for C. fetus H840 grown in basal medium (BM) and BM + 0.5% acetate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

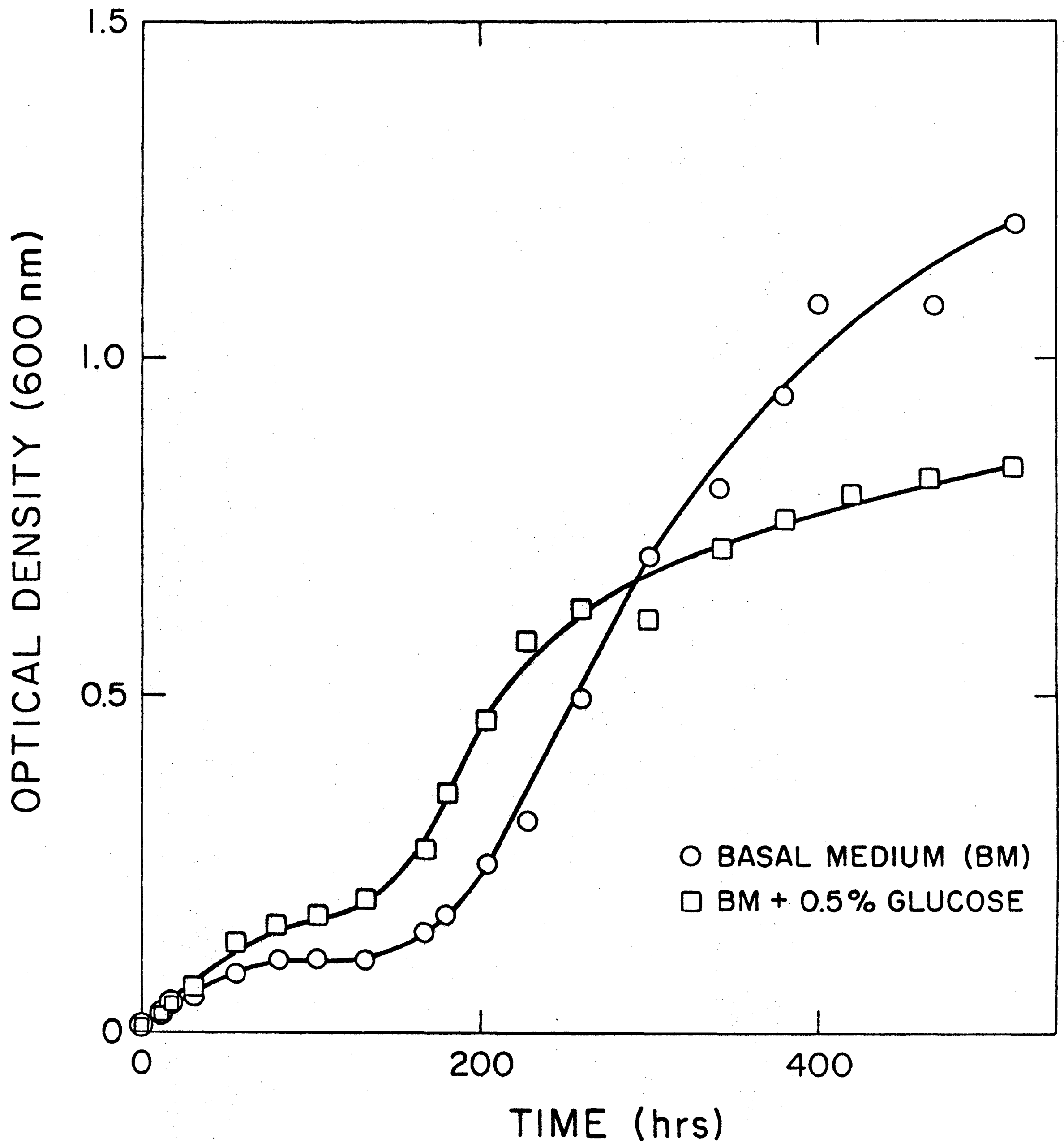


Figure 11. The growth curves for C. fetus H840 grown in basal medium (BM) and BM + 0.5% glucose. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

meter when C. fetus 482 was grown in biphase culture system. The pH of the culture dropped during the first day of growth in basal medium (Figure 12). Since the system was aerated with a gas mixture containing 5% O_2, 10% CO_2 and 85% N_2, the drop in pH was probably caused by the solution of CO_2 in the medium. Shortly after the lag phase, the pH rose very fast with the increased rate of growth. About 4.5 days after inoculation, the culture passed the point of maximum rate of growth and so the climb in pH of the culture also leveled off. Until the 11th day of growth, the pH as well as the growth of the culture increased and ended at pH 7.4 after 15 days. The same pattern of pH-curves for C. fetus grown in basal medium + 0.5% glucose and basal medium + 0.5% citrate is shown in Figures 13 and 14.

The optimal pH for the growth of C. fetus in the chemically defined liquid medium of Smibert (1963) was 6.8 to 7.2. Robertstad and Morrison (1957) reported the optimal pH was 7.1 for their batch culture of C. fetus. Dennis and Jones (1959) reported that optimal pH for the growth of C. fetus in succinate-cysteine medium was between 6.6 and 7.2. Based on the previous experiments of this study, the optimal pH for C. fetus 482 grown in basal medium with or without the addition of carbon sources probably ranged from 6.6 to 7.0.

An unusual feature of the growth of C. fetus 482 was that the optical density of the culture grown in the basal medium plus any one of the 0.5% carbon sources decreased significantly after 140-220 hours (Figures 3 and 4) but the density increased again 25-65 hours later. The cause for this phenomenon is unknown and no such observation has been reported in the literature. Presumably, one of the growth factors in the media supporting

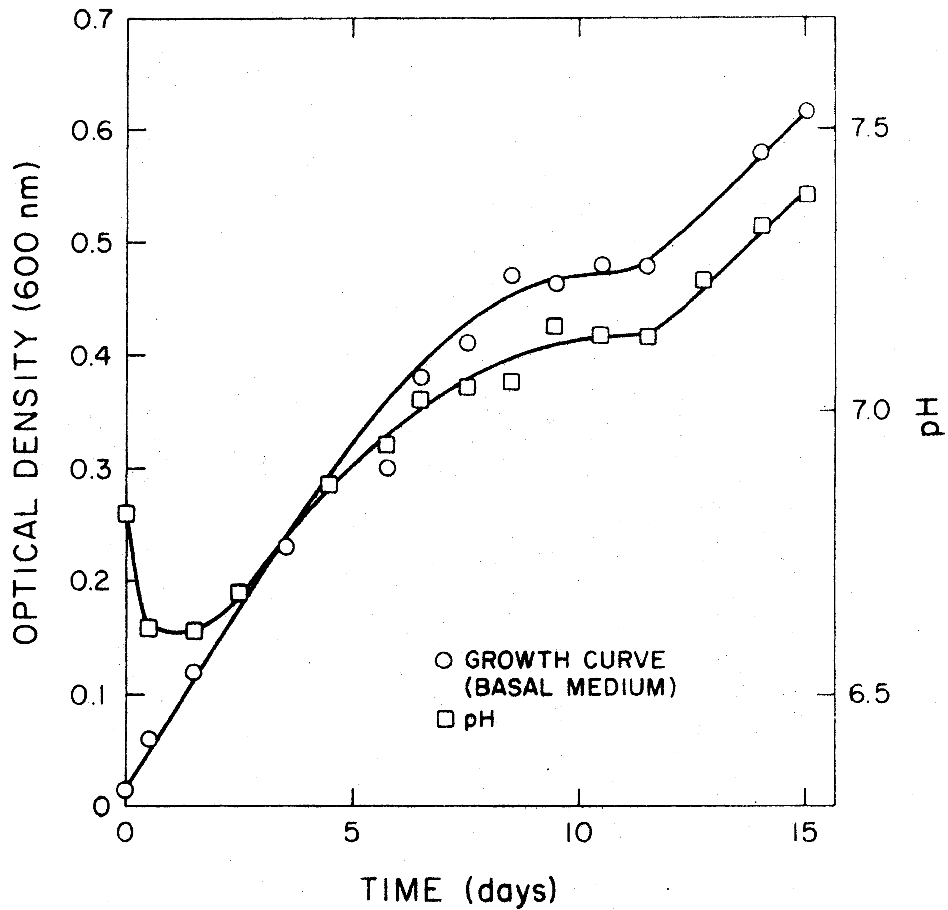


Figure 12. pH and the growth curves of C. fetus 482 grown in basal medium. The culture was incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

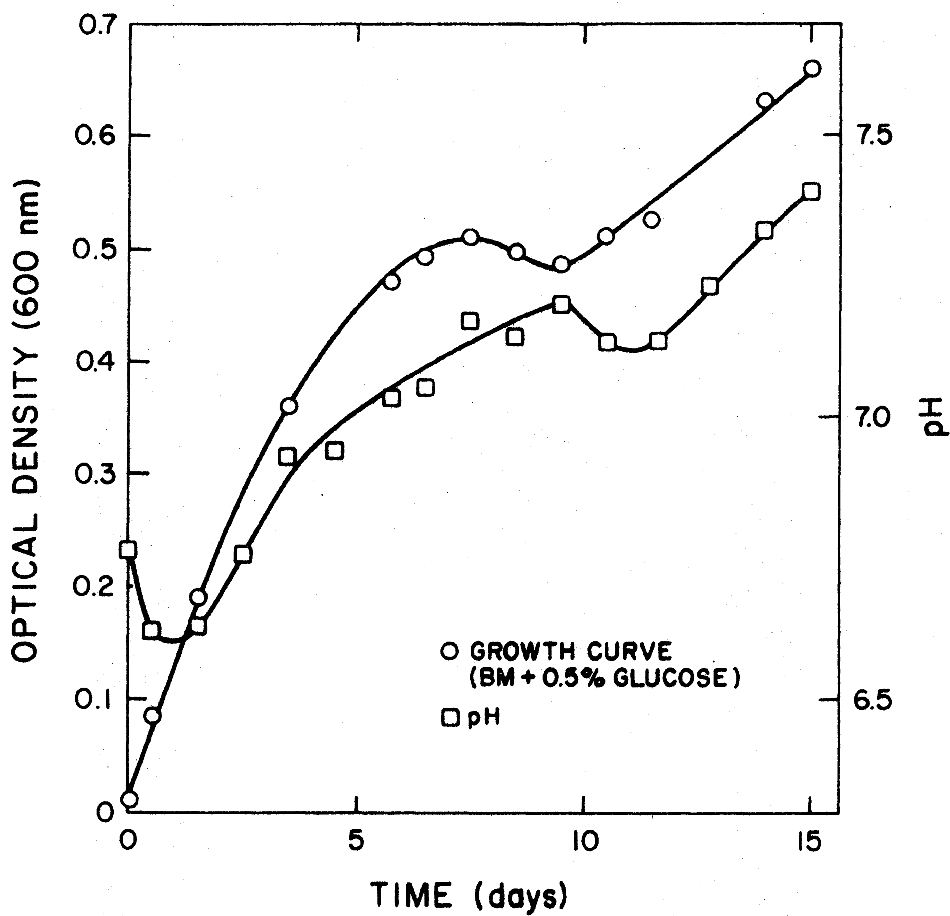


Figure 13. pH and the growth curves of C. fetus 482 grown in BM + 0.5% glucose. The culture was incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

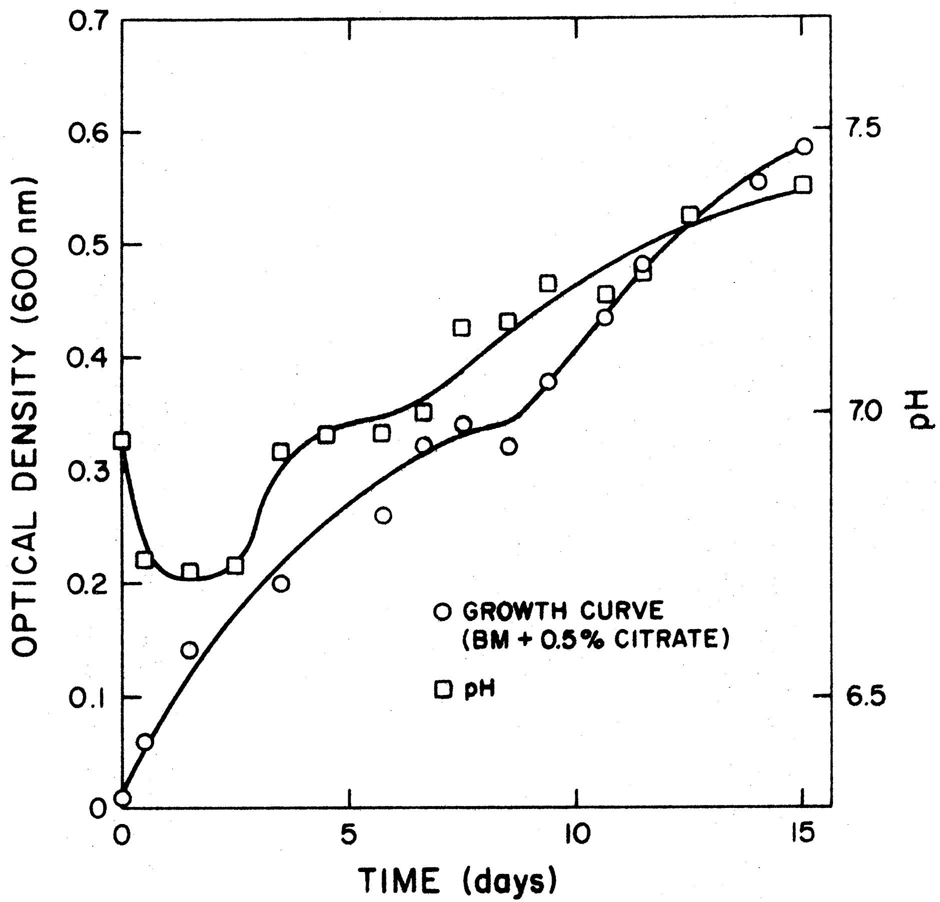


Figure 14. pH and the growth curves of C. fetus 482 grown in BM + 0.5% citrate. The culture was incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

more rapid growth became depleted and the cells died and autolyzed. Consequently, the density of the culture decreased. Twenty-five to sixty-five hours later, the growth of the culture could have resumed by a newly developed mutant which favoured those environmental conditions. On the other hand, the second growth phase could again initiate, because the concentration of the growth factor believed to be limited during the declining phase of growth, was released to the medium from the many lysed cells. To the growth of C. fetus in basal medium + 0.5% citrate, the same phenomenon was probably due to the formation of a complex of citrate and the growth factor. As a result, the effective concentration of the factor in that medium was decreased, and the growth of the organism was retarded. Finally, the cell population declined. This phenomenon may also be a case of diauxie growth. A better illustration of diauxie growth of C. fetus 482 can be seen in Figure 12 (growth in basal medium), Figure 13 (growth in BM + 0.5% glucose) and Figure 14 (growth in BM + 0.5% citrate). During the first cycle of growth, the organism utilized an essential carbon source. When this carbon source was completely consumed, a lag phase occurred which represents the time for inducing a new enzyme system for the metabolism of a second major carbon source. Then, the second growth ensued.

B. Assimilation of Glucose:

It has been reported that glucose is not fermented (Smith and Taylor, 1919; Plastridge and Williams, 1943; King, 1957) nor oxidized (Alexander, 1957; Lecce, 1958; Kiggins and Plastridge, 1958) by C. fetus. However, previous experiments indicated that 0.5% of glucose enhanced the growth of strains 482 and H840 but had no effect on that of PB1. Glucose

assimilation by _C. fetus_ was therefore re-examined.

Campylobacter fetus 482 was grown in basal medium and basal medium + 0.5% glucose. The growth of the culture was followed by measuring the optical density of the culture at 600 nm. The concentration of glucose in the broth was measured by coupling the hexokinase reaction with glucose-6-phosphate dehydrogenase. The results presented in Figure 15, indicate that glucose was not utilized by the organism. In the same Figure, the growth data also indicates that the basal medium containing 0.5% glucose supported better growth of _C. fetus_ 482 than that of a culture grown in basal medium alone. The presence of 0.5% glucose in the medium enhanced the growth of _C. fetus_ but glucose was not assimilated by the organism.

C. Assimilation of Citrate:

Lecce (1958) reported that citrate was not oxidized by _C. fetus_. Kiggins and Plastridge (1958) also indicated that citrate did not permeate the intact cell. The assimilation of citrate by strain 482 was re-examined in this study. Strain 482 was grown both in basal medium and in basal medium + 0.5% citrate. The growth of the culture was followed by measuring its optical density at 600 nm and the citrate concentration of the broth was assayed by Saffran and Deustedt's rapid procedure. The results shown in Figure 16 indicate that growth of the culture was depressed by the addition of 0.5% citrate to the basal medium and that citrate in the medium was not utilized.

D. Uptake of α-methyl-D-glucoside (α-MG):

There are two possible reasons why _C. fetus_ is unable to assimilate glucose. Firstly, it is possible that _C. fetus_ is unable to absorb glucose,

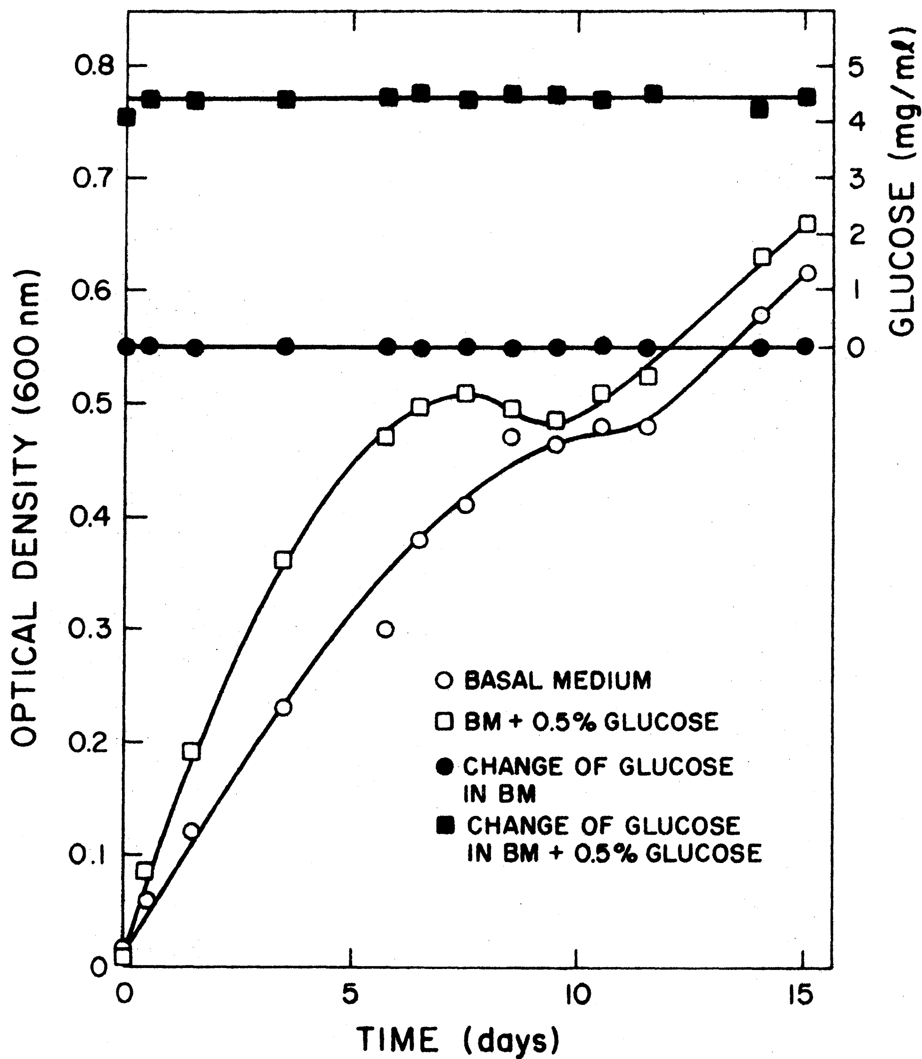


Figure 15. Assimilation of glucose by C. fetus 480 grown in basal medium (BM) and BM + 0.5% glucose. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

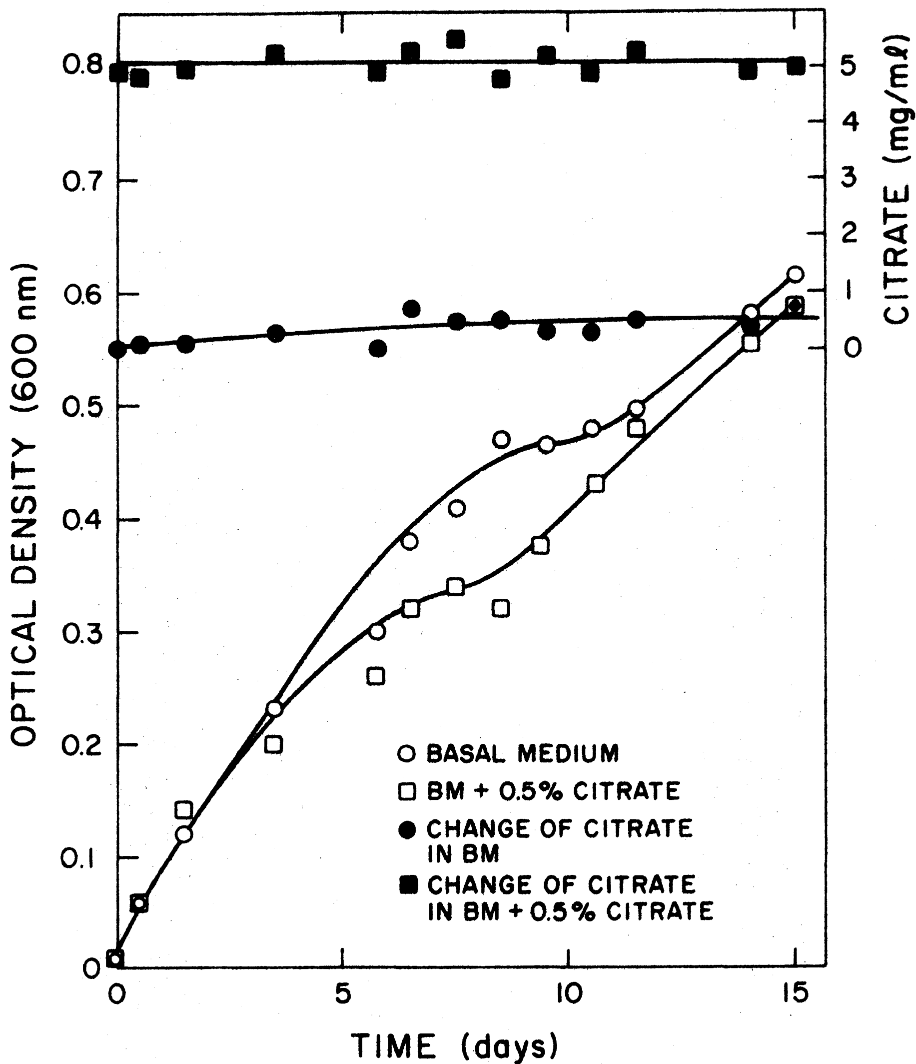


Figure 16. Assimilation of citrate by C. fetus 482 grown in basal medium (BM) and BM + 0.5% citrate. The cultures were incubated at 37 C and in a gaseous mixture containing 5% O_2, 10% CO_2 and 85% N_2.

because the cell membrane lacks a specific glucose permease that prevents glucose from being transferred. Secondly, a missing enzyme or an incomplete metabolic pathway in the cell prevents the glucose from being utilized. This experiment was designed to examine whether or not glucose can be absorbed by C. fetus. α-methyl-D-glucoside, a compound which shares the same permease system with glucose and can not be metabolized in the cell (Cohen and Monod, 1957), took the place of glucose in the study. The accumulation of α-MG can be a simple index that C. fetus is able to absorb glucose. Two batches of C. fetus 482, one grown in basal medium and the other in basal medium + 0.5% glucose, were respectively tested. The radioactivity of the ^{14}C-labelled-α-MG retained in the cells collected from one ml of cell suspension containing 0.04 μmole of ^{14}C-labelled-α-MG (specific activity: 0.83 μCi/μmole) and mounted on a Millipore filter, was measured in a scintillation counter. The results are summarized in Table IX.

There was only a very small amount of ^{14}C-radioactivity retained in the cells grown in the basal medium with or without 0.5% glucose. In both sets of experiments, incubation was continued up to 30 minutes and no accumulation of radioactivity was observed. It may be concluded that α-MG was not absorbed by the organisms which were either grown in basal medium with or without glucose. It seems that C. fetus does not possess a glucose permease system or that such a system can not be induced by growing the organism in a medium containing glucose.

E. Enzyme Assays:

1. Citric Acid Cycle

Alexander (1957) first reported that 0.2% of α-ketoglutarate,

Table IX. The accumulation rate of α-methyl-D-glucoside (^{14}C) by the resting cell of C. fetus 482.

Incubation time min.	^{14}C-Radioactivity, CPM	
	Cells[1] grown in basal medium	Cells[1] grown in basal medium + 0.5% glucose
0	19.8	36.8
3	100.1	55.2
6	25.4	69.9
9	17.5	43.2
12	26.1	47.5
15	70.6	76.8
30	18.1	20.0

1. There was a total of 1.2×10^6 CPM of ^{14}C-radioactivity in the 15 ml of incubated cell suspension.

succinate, fumarate and malate, served as energy sources for the growth of C. fetus. Kiggins and Plastridge (1958) reported that all memebers of the citric acid cycle intermediates were rapidly oxidized by a crude cellular extract of C. fetus. The presence of citric acid cycle enzymes were, therefore, the first to be investigated in this portion of the study. The specific activities of citric acid cycle enzymes in the crude cellular extracts of C. fetus strains 482, H840 and PB1 were studied. The results are presented in Table X.

In the three strains of C. fetus, oxaloacetate was condensed with acetyl CoA by citrate synthase, and the product citrate was interconverted to cis-aconitate and to isocitrate by the action of aconitase. Isocitrate was oxidized and decarboxylated by isocitrate dehydrogenase to α-ketoglutaric acid; both NADP and NAD can be employed as cofactors. The results indicate that NADP served as a better electron acceptor than did NAD.

Next was the oxidative decarboxylation of α-ketoglutaric acid carried out by α-ketoglutarate dehydrogenase, a complex enzyme composed of three individual enzymes and five cofactors. The activity of α-ketoglutarate dehydrogenase could not be detected in the crude cellular extracts of strains 482 and PB1 but was detectable in that of strain H840. The activity of α-ketoglutarate dehydrogenase in crude cellular extract of C. fetus 482 was re-examined. The cells were grown in basal medium, basal medium + 0.5% glucose, basal medium + 0.5% citrate and basal medium + 0.5% lactate, respectively. The results presented in Table XI show that enzyme activity was undetectable in C. fetus 482 grown in any of the four media. The lack of activity of α-ketoglutarate dehydrogenase in strain 482 and PB1 could be due to the absence of any one of the constitutent enzymes of the complex,

Table X. The specific activities of citric acid cycle enzymes in the crude cellular extracts of C. fetus strains 482, H840, and PB1.[1]

Enzymes	Specific Activity[2]		
	482	H840	PB1
Citrate synthase	0.275	0.091	0.128
Aconitase	0.039	0.363	0.072
Isocitrate dehydrogenase			
NADP-specific	0.275	0.849	0.271
NAD-specific	0.008	0.009	0.009
α-Ketoglutarate dehydrogenase	ND[3]	0.004	ND
Succinyl-CoA synthetase	0.297	0.674	0.195
Succinate dehydrogenase	0.007	0.003	0.001
Fumarase	0.691	0.470	1.605
Malate dehydrogenase	0.010	0.151	0.025

1. The cultures were grown in enriched medium.

2. Expressed as micromoles of substrate transformed per minute per milligram of protein.

3. ND = not detectable, specific activity less than 0.0005.

Table XI. The effect of carbon source on the specific activities of α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and malate synthase in the crude cellular extract of *C. fetus* 482.

Medium	Specific Activity[1]		
	α-Ketoglutarate Dehydrogenase	Pyruvate Dehydrogenase	Malate Synthase
Enriched Medium	ND[2]	ND	ND
Basal Medium (BM)	ND	ND	ND
BM + 0.5% Glucose	ND	ND	0.034
BM + 0.5% Acetate	-[3]	-	0.015
BM + 0.5% Citrate	ND	-	-
BM + 0.5% Lactate	ND	ND	-

1. Expressed as micromoles of substrate transformed per minute per milligram of protein.
2. ND = not detectable, specific activity less than 0.0005.
3. - = not analyzed.

or because the whole complex was missing. Until the activities of the participating enzymes in the complex are examined, the reason why strains 482 and PB1 are lacking in α-ketoglutarate dehydrogenase activity will remain an interesting question.

The next reaction on the cycle was catalyzed by succinyl CoA synthetase. Succinyl CoA was cleaved by this enzyme in all three strains of C. fetus. Then succinate was oxidized by the iron flavoprotein, succinate dehydrogenase. The product fumarate was rehydrated by fumarase, and oxidized again by malate dehydrogenase; the final product, oxaloacetate, was regenerated for the initiation of the next cycle.

In strain H840, enzymes of the citric acid cycle were all present and the cycle was complete. In strains 482 and PB1, α-ketoglutarate dehydrogenase was undetectable, so the cycle was probably interrupted at this oxidative decarboxylation step.

2. Glyoxylate Bypass:

In order that the citric acid cycle may continue to provide energy and intermediates for the growth of organisms, the sequential reactions of the pathway should not be interrupted at any point. The incomplete citric acid cycle in strains 482 and PB1 might be supported by an auxillary shunt so that the multifunctions of the cycle could be realized. A glyoxylate bypass in C. fetus was therefore studied. Strains of C. fetus were grown in enriched medium for two days and the key enzymes of the glyoxylate bypass in the crude cellular extracts were examined. The results are shown in Table XII.

The first key enzyme of the bypass is isocitrate lyase which converts isocitrate into glyoxylate and succinate. This reaction could successfully

Table XII. The specific activity of the key enzymes of the glyoxylate bypass in the crude cellular extracts of _C. fetus_ strains 482, H840 and PB1.[1]

Enzymes	Specific Activity[2]		
	482	H840	PB1
Isocitrate lyase	0.002	0.001	0.004
Malate synthase	ND[3]	ND	ND

1. The cultures were grown in enriched medium.
2. Expressed as micromoles of substrate transformed per minute per milligram of protein.
3. ND = not detectable, specific activity less than 0.0005.

make up for the disruption of the citric acid cycle in the absence of α-keto-glutarate dehydrogenase. Isocitrate lyase activity was detectable in the three strains of C. fetus.

The second enzyme in the glyoxylate bypass, malate synthase, catalyzes the following reaction:

$$\text{Acetyl CoA} + \text{glyoxylate} + H_2O \longrightarrow \text{Malate} + \text{CoA} \quad (1)$$

In all three strains of C. fetus, malate synthase activity could not be detected. The activity of malate synthase in crude cellular extract of C. fetus 482 was re-examined. The organism was cultivated in basal medium, basal medium + 0.5% glucose, and basal medium + 0.5% acetate. The specific activities of malate synthase in strain 482 grown in various media are also presented in Table XI. The results indicate that the enzyme was not present when the organism was grown in either basal medium or enriched medium. However, malate synthase was an inducable enzyme which was induced by 0.5% glucose, or 0.5% acetate in basal medium. As C. fetus does not assimilate glucose, it is not understood how 0.5% glucose in the medium induces the formation of malate synthase.

It seems reasonable to conclude that citric acid cycle enzymes were all present in C. fetus H840 and all but α-ketoglutarate dehydrogenase are present in strains 482 and PB1. Isocitrate lyase, a key enzyme of the glyoxylate bypass, was present to circumvent the deficiency of the citric acid cycle in strains 482 and PB1. In strain H840, α-ketoglutarate dehydrogenase was present and the presence of isocitrate lyase may be more significant than just to support the operation of citric acid cycle. Any additional biological functions of this enzyme in H840 or in the other two strains are worthy of further study. No malate synthase activity was

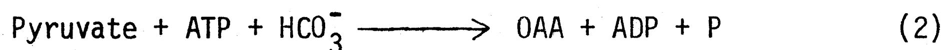
found in any of the strains of C. fetus when they were grown in the medium without the inducer, acetate or glucose. Then, glyoxylate, a product of isocitrate lyase reaction, cannot condense with acetyl CoA to produce malate. The metabolic role of glyoxylate, therefore, should be very significant.

3. Some Major Ancillary Enzymes of Citric Acid Cycle:

Several other enzymes in C. fetus catalyzing the reactions which lead to or leave the citric acid cycle were examined. The specific activities are presented in Table XIII.

No pyruvate dehydrogenase activity was detected in strains of C. fetus. Repeat examinations were made of the crude cellular extracts of C. fetus 482 grown in basal medium, basal medium + 0.5% glucose and basal medium + 0.5% lactate. The results presented in Table XI confirmed the absence of pyruvate dehydrogenase activity in C. fetus. Furthermore, pyruvate dehydrogenase was not induced by growing the organism in the medium containing 0.5% glucose or lactate.

Since pyruvate could not be oxidized by conventional reactions, several anoplerotic enzymes in C. fetus were examined. The first enzyme tested was pyruvate carboxylase which catalyzes the following reaction:



$$\text{Pyruvate} + \text{ATP} + HCO_3^- \longrightarrow \text{OAA} + \text{ADP} + \text{P} \qquad (2)$$

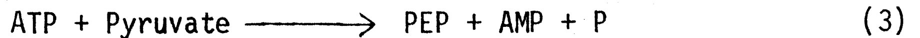
This direct carboxylation of pyruvate was only found in strain H840 but not in 482 and PB1. Oxaloacetate, the product of the reaction, was then oxidized by the citric acid cycle.

Pyruvate may also be converted to phosphoenolpyruvate by the action of phosphoenolpyruvate synthetase (reaction 3).

Table XIII. The specific activity of some major ancillary enzymes of citric acid cycle in the crude cellular extracts of *C. fetus* strains 482, H840 and PB1.[1]

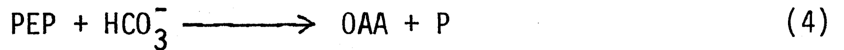
Enzymes	Specific Activity[2]		
	482	H840	PB1
Pyruvate dehydrogenase	ND[3]	ND	ND
Pyruvate carboxylase	ND	0.008	ND
Phosphoenolpyruvate synthetase	0.006	ND	0.004
Phosphoenolpyruvate carboxylase	ND	ND	ND
Phosphoenolpyruvate carboxykinase			
GDP - specific	ND	ND	ND
ADP - specific	ND	-[4]	ND
Malic enzyme (NADP)	0.005	ND	0.002
Glutamate dehydrogenase			
NADH - specific	0.008	0.001	0.007
NADPH - specific	0.338	0.004	0.224
Aspartase	3.565	2.886	4.159

1. The cultures were grown in enriched medium.
2. Expressed as micromoles of substrate transformed per minute per milligram of protein.
3. ND = not detectable, specific activity less than 0.0005.
4. - = not analyzed.

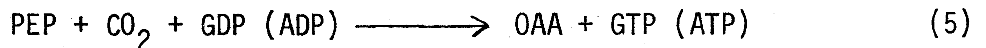


$$ATP + Pyruvate \longrightarrow PEP + AMP + P \quad (3)$$

Phosphoenolpyruvate is then carboxylated by the carboxykinase or phosphoenolpyruvate carboxylase to form oxaloacetate. The reactions are shown as follows:



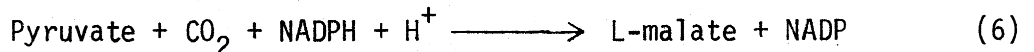
$$PEP + HCO_3^- \longrightarrow OAA + P \quad (4)$$



$$PEP + CO_2 + GDP\ (ADP) \longrightarrow OAA + GTP\ (ATP) \quad (5)$$

PEP synthetase was indeed found in strain 482 and PB1 but was not detected in H840. But neither PEP carboxylase nor PEP carboxykinase were found in three strains of C. fetus.

Pyruvate may enter the citric acid cycle by a pyridine nucleotide-linked decarboxylation, namely; the NADP-dependent malic enzyme reaction which is shown as follows:



$$Pyruvate + CO_2 + NADPH + H^+ \longrightarrow L\text{-malate} + NADP \quad (6)$$

The results indicated that strains 482 and PB1, but not H840, possessed malic enzyme activity.

It may be concluded that pyruvate was carboxylated by pyruvate carboxylase in strain H840 and by malic enzyme in strains 482 and PB1. The product, oxaloacetate or malate, was then oxidized by the citric acid cycle.

Kiggins and Plastridge (1958) reported that glutamate and aspartate were both oxidized by C. fetus. Both amino acids had been selected as the major components in a chemically defined medium by Smibert (1963) and in that of Fletcher and Plastridge (1963). Strains of C. fetus in this study were able to assimilate both amino acids by the actions of glutamate dehydrogenase and aspartase. In addition, experimental data shows that NADPH served as a better cofactor of glutamate dehydrogenase than did NADH.

4. Pathways of Glucose Metabolism:

Glucose, a highly reduced and energy rich compound is utilized as the major source of carbon and energy by most bacteria. However, *C. fetus* is unable to ferment or oxidize glucose. Early experiments in this study also found that glucose was not utilized during growth of the organism. This was also confirmed by the inability of *C. fetus* to absorb α-MG (Table IX). Kiggins and Plastridge (1958) suspected that the enzymes of the Embden-Meyerhof glycolytic scheme were not in operation in *C. fetus*. The deficiency of this metabolic pathway in *C. fetus* provides an explanation for the inability of this organism to utilize glucose.

Accordingly, the key enzymes of four pathways of glucose metabolism were examined in the crude cellular extract of *C. fetus* 482. These enzymatic activities are presented in Table XIV and summarized as follows:

(1) Embden-Meyerhof-Parnas Pathway:

Glucose metabolism is initiated by a phosphorylation reaction, the action of glucokinase, but the enzymatic activity was not found in *C. fetus* 482. Glucose-6-phosphate was degraded to dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate in *C. fetus* 482 by the sequential reactions of phosphoglucose isomerase, phosphofructokinase, and fructose diphosphate aldolase. Pyruvate was derived from phosphoenolpyruvate by pyruvate kinase in the organism.

There are three reactions in the glycolytic scheme which were actually irreversible by their highly exergonic nature. These are the reactions of glucokinase, phosphofructokinase, and pyruvate kinase. Two of these steps can be circumvented or by-passed by fructose-1,6-diphosphatase and phosphoenolpyruvate synthetase, both of which were present in *C. fetus*

Table XIV. The specific activity of key enzymes on various pathways of glucose metabolism in the crude cellular extracts of _C. fetus_ strain 482.[1]

Enzymes	Specific Activity[2]
A. Embden-Meyerhof-Parnas Pathway:	
Glucokinase	ND[3]
Phosphoglucose isomerase	0.010
Phosphofructokinase	0.002
Fructose-1,6-diphosphatase	0.038
Fructose diphosphate aldolase	0.050
Pyruvate kinase	0.089
B. Hexose Monophosphate Pathway:	
Glucose-6-phosphate dehydrogenase	
NADP -specific	ND
NAD - specific	ND
6-phosphogluconate dehydrogenase	ND
Transaldolase	0.012
C. Entner-Doudoroff Pathway:	
6-Phosphogluconate dehydrase and KDPG aldolase	ND
D. Phosphoketolase Pathway:	
Phosphoketolase	0.010

1. The culture was grown in basal medium.
2. Expressed as micromoles of substrate transformed per minute per milligram of protein.
3. ND = not detectable, specific activity less than 0.0005.

482 (Table XIV). The glycolytic scheme that started from glucose-6-phosphate and ended at pyruvate should be complete and well functioning in C. fetus 482.

(2) Hexose Monophosphate Pathway:

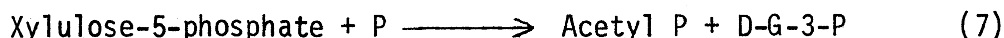
Enzymes on the oxidative portion of the pathway, namely, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were not found in C. fetus 482. The transaldolase reaction was measurable. Presumably, C. fetus 482 also contained transketolase, phosphoribose isomerase, and ribulose-5-phosphate epimerase, so that pentose-5-phosphate, the non-nitrogenous component of nucleotides, might be derived from triose phosphate of the glycolytic pathway.

(3) Entner-Doudoroff Pathway:

The key enzymes of the pathway, namely glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrase, and KDPG aldolase, were all undetectable; presumably, the entire pathway does not exist in C. fetus.

(4) Phosphoketolase Pathway:

The major reaction of the pathway catalyzed by phosphoketolase is shown as follows:



$$\text{Xylulose-5-phosphate} + \text{P} \longrightarrow \text{Acetyl P} + \text{D-G-3-P} \qquad (7)$$

The phosphoketolase activity was found in C. fetus 482.

(5) Miscellaneous Enzymes:

Several enzymes do not belong to any of the pathways described previously. Their specific activities are presented in Table XV.

No lactic dehydrogenase activity was found in either strain of C. fetus grown in enriched medium. When C. fetus strain 482 was grown in basal medium or basal medium + 0.5% lactate, there was low lactic dehydro-

Table XV. The specific activity of miscellaneous enzymes in crude cellular extracts of *C. fetus* strains 482, H840 and PB1.[1]

Enzymes	Specific Activity[2]		
	482	H840	PB1
Lactic dehydrogenase			
NADH - specific	ND[3]	ND	ND
NADPH - specific	ND	ND	ND
Reduced NAD dehydrogenase	0.002	0.002	0.002
Reduced NADP dehydrogenase	0.008	0.007	0.009
Glucose dehydrogenase			
DPIP - specific	ND	-[4]	-
NAD - specific	ND	-	-
NADP - specific	ND	-	-
Gluconokinase	ND	-	-
D-Ribokinase	ND	-	-
Phosphotransacetylase	3.232	-	-
Acylphosphate: Hexophosphotransferase	ND	-	-

1. The cultures were grown in enriched medium.
2. Expressed as micromoles of substrate transformed per minute per milligram of protein.
3. ND = not detectable, specific activity less than 0.0005.
4. - = not analyzed.

genase activity in the crude cellular extract (Table XVI). It may be that excess succinate in the enriched medium depressed the activity of the lactic dehydrogenase. Yoshida (1965a) reported that L-malate and oxaloacetate correspondingly inhibited the oxidation of lactate and the reduction of pyruvate by the lactic dehydrogenase of Bacillus subtilis; the inhibition was competitive. Succinate is the metabolic precursor of both compounds; consequently, excess succinate in the medium could also inhibit the enzymatic activity in C. fetus. It is not understood why NADPH served effectively as an electron donor for lactic dehydrogenase when the organism was grown in basal medium, but not when the organism was grown in basal medium with 0.5% lactate.

Reduced NAD dehydrogenase and reduced NADP dehydrogenase were all found in the three strains of C. fetus. Furthermore, the specific activity of reduced NADP dehydrogenase was about 4-fold that of reduced NAD dehydrogenase in the three strains.

The possibility that glucose may be oxidized to gluconate in C. fetus was examined by measuring the activity of glucose dehydrogenase in the crude cellular extract of 482. Using DPIP, NAP or NADP as electron acceptors, no such activity was found.

No gluconokinase activity or D-ribokinase activity was detected in a crude cellular extract of 482. Therefore, neither gluconate nor D-ribose could be phosphorylated and further metabolized by the Hexose Monophosphate pathway in C. fetus.

Acetyl phosphate could be rapidly converted to acetyl CoA by the action of phosphotransacetylase in C. fetus 482. Acetyl phosphate could be derived from xylulose-5-phosphate by the action of phosphoketolase or

Table XVI. The specific activity of lactic dehydrogenase of *C. fetus* 482 grown in enriched medium, basal medium (BM) and BM + 0.5% glucose.

Medium	Specific Activity[1]	
	NADH	NADPH
Enriched Medium	ND[2]	ND
Basal Medium (BM)	0.002	0.001
BM + 0.5% Glucose	0.001	ND

1. Expressed as micromoles of substrate transformed per minute per milligram of protein.

2. ND = not detectable, specific activity less than 0.0005.

could presumably result from acetate and ATP by the action of acetyl-kinase (Reaction 8):

$$\text{Acetate} + \text{ATP} \longrightarrow \text{Acetyl-P} + \text{ADP} \qquad (8)$$

Previous experiments indicated that *C. fetus* lacks glucokinase and glucose could not be phosphorylated. Kamel and Anderson (1964) reported that glucose was phosphorylated in *Aerobacter aerogenes* catalyzed by acetylphosphate: hexophosphotransferase. Acetyl phosphate served as the phosphate donor, and glucose-6-phosphate was the product of the reaction. The activity of this enzyme in *C. fetus* 482 was examined, but no such alternative was found.

DISCUSSION

A. Glucose and the Growth of *Campylobacter fetus*:

Five tenths percent of glucose in the basal medium was found to enhance the growth of *C. fetus* 482 and slightly increase that of H840 and not affect the growth of PB1 (Figures 3, 7, and 11). The growth of 482 in the basal medium + 0.5% glucose was re-examined. It was found that the growth rate of 482 was increased by the addition of 0.5% glucose. However, the concentration of glucose in the broth of the culture remained constant until the end of the growth (Figure 15). Apparently, glucose was never assimilated by the organism.

Because glucose was sterilized with the basal medium in these experiments, a somewhat different color was visible between the basal medium and basal medium containing glucose. The pH and optical density of the basal medium containing different amounts of glucose, were measured before and after the media were sterilized at 121 C, 15 lbs of pressure for 15 minutes. The results are shown in Table XVII. There were essentially no changes in both pH and optical density of the media in which different amounts of glucose were added before sterilization. After being autoclaved, the basal medium became slightly more alkaline. However, the presence of glucose in the medium had a profound effect on the pH of the medium after autoclaving. The pH decreased from 6.78 to 6.62 as the glucose concentration was increased from 0 to 1.0%. The basal medium also became darker after sterilization as glucose enhanced the Browning reaction. The degree of caramelization of the medium was also proportionally increased with the amount of glucose added. The effect of sterilization on the glucose concentration in the medium was subsequently determined. Media containing 0 to 1% glucose were prepared,

Table XVII. The pH and optical density (400 nm) of the basal medium containing various levels of glucose, before and after sterilization at 121 C, 15 lbs of pressure for 15 minutes.

	pH		O.D. (400 nm)	
Medium	Before	After	Before	After
Basal Medium (BM)	6.720	6.780	0.194	0.230
BM + 0.25% Glucose	6.720	6.750	0.190	0.243
BM + 0.5 % Glucose	6.720	6.710	0.190	0.257
BM + 0.75% Glucose	6.715	6.675	0.190	0.262
BM + 1.0 % Glucose	6.715	6.625	0.194	0.295

and their concentrations were analyzed before and after sterilization. The findings are presented in Figure 17. They show that a certain percentage of the glucose in the medium could not be recovered after sterilization.

Presumably, there was a small amount of glucose in the medium which was decomposed or formed complex derivatives by the Browning reaction, therefore, the percentage of glucose recovered was less after sterilization. It may be these decomposed compounds or derivatives of glucose in the medium that enhanced the growth of C. fetus.

The enhancement of the growth of microorganisms by heated sugar in the medium has been reported several times. Fulmer et al. (1931) reported that the growth of Saccharomyces cerevisiae was enhanced in a heated medium containing sucrose, NH_4Cl and K_2HPO_4. They reported that the stimulatory effect was directly proportional to the degree of caramelization of the medium; however, decolorization of the medium with charcoal failed to reduce the stimulatory activity. Lankford and Ramsey (1956) also found that a compound (or compounds) derived from the sterilization of glucose with phosphate or with a basal medium, markedly promoted the initial growth of Lactobacillus fermenti but it had no effect on the exponential phase of the growth of the organism. The same effect was also seen in the growth of the following organisms: Micrococcus pyrogenes var. aureus, Lactobacillus delbruckeii strain LD3, Bacillus subtilis var. niger, Leuconostoc mesenteroides strain P60, Aerobacillus polymyxa and Streptococcus lactis. An unknown factor isolated from the autoclaved glucose-phosphate solution by Sergeant et al. (1957) was able to stimulate the initial growth of Bacillus globigii. The factor could be absorbed by charcoal and solublized

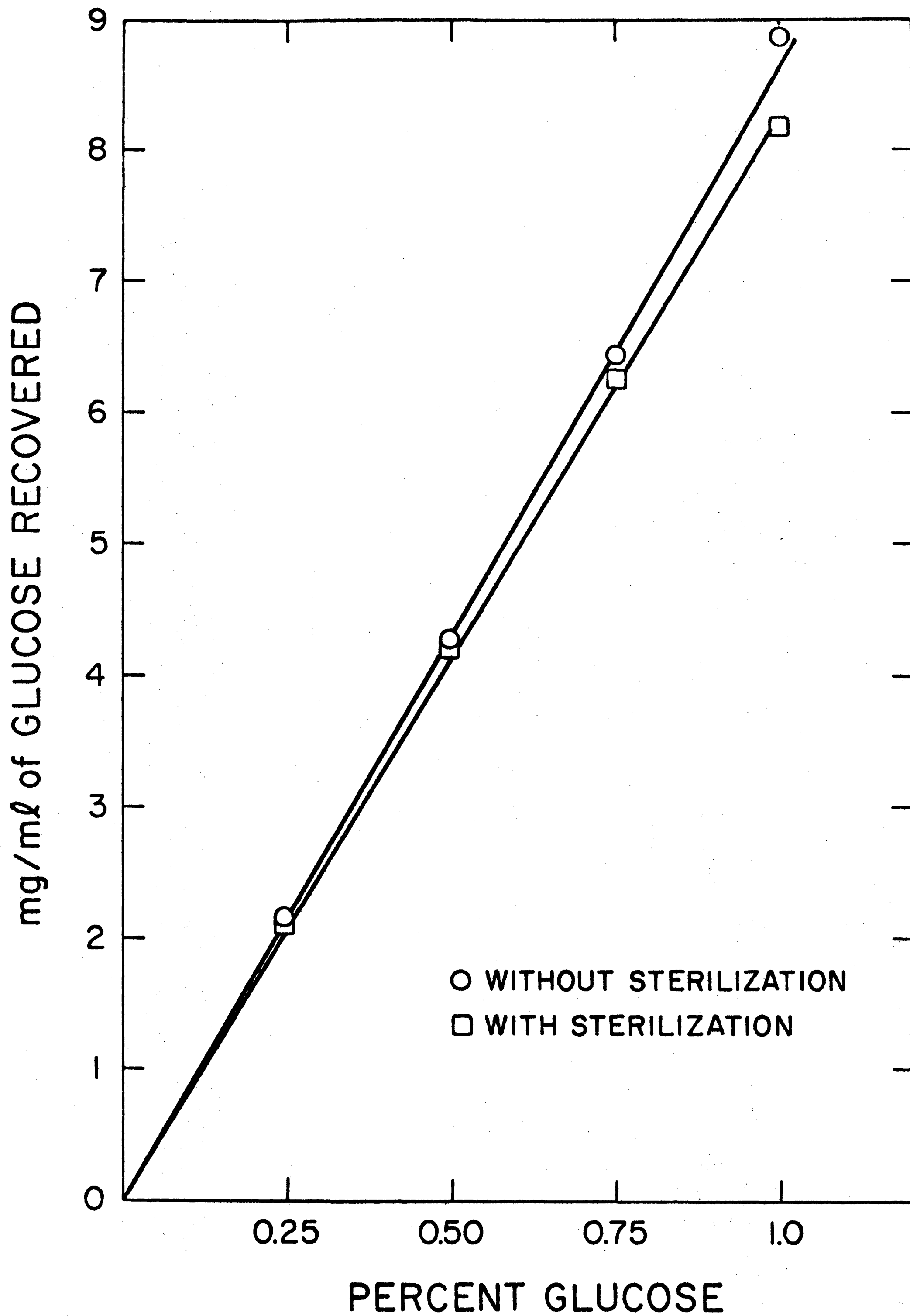
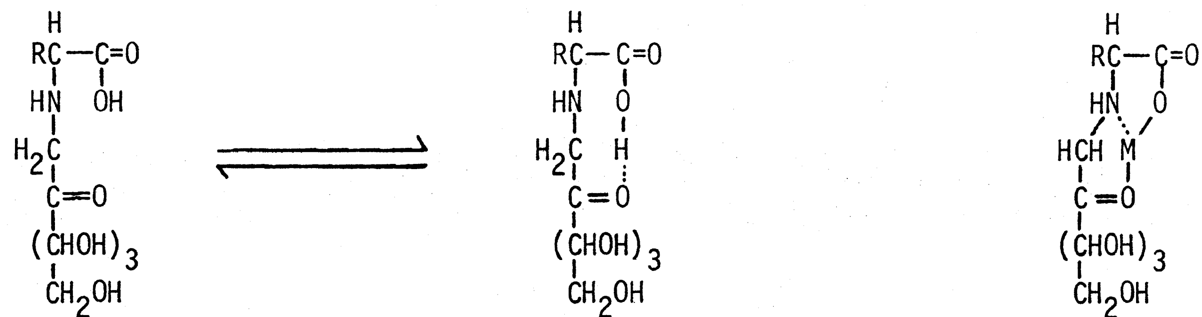


Figure 17. mg of glucose recovered from one ml of basal medium with and without sterilization at 121 C, 15 lbs of pressure for 15 minutes.

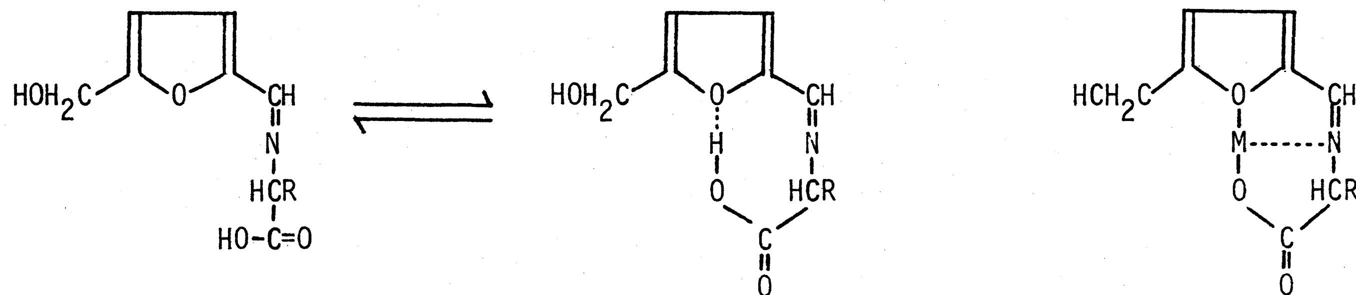
in ether. It was thermostable and an acidic, nonvolatile compound containing a carboxyl group, a hydroxyl group, and unsaturated bonds. This unknown compound was found to have metal chelating potential by Lankford _et al_. (1957).

Krieg (1975) studied the ferrous requirement on the aero-tolerance of _C. fetus_ and found that the addition of very small amounts of several chelating agents such as protocatechuric acid, pyrogallol, epinephrine and dopa or 0.05% of ferrous sulfate increased the aero-tolerance capability of the organism. He assumed that ferrous ion is effectively transferred into the cell by adding a small amount of chelating agent or by an increase in the ion concentration in the medium.

Most likely, a non-enzymatic Browning reaction involving glucose was also taking place when the medium used in this study was sterilized. Some of the products may have chelating ability and serve as carrier agents for the transport of trace metal ions which are essential to the growth of _C. fetus_. In reviewing the mechanism of the Browning reaction of glucose (Braverman, 1963), two of the many reaction products are highly suspected to have chelating qualities. These compounds are 1-amino-1-deoxy-2-ketose and a Schiff's base of hydroxymethyl furfural (HMF). Both of the compounds may possibly be stablized by a hydrogen bonding as the proposed forms of I and III respectively (Figure 18). By the same token, they may become more stable by chelate formation as the proposed forms of II and IV. Furthermore, these compounds fit well with the description for the unknown compounds isolated from autoclaved glucose-phosphate solution by Sergeant _et al_. (1957). Possibly, the Schiff's base of HMF and 1-amino-1-deoxy-2-ketose were derived from glucose during sterilization, which served as chelating agents to



1-Amino-1-Deoxy-2-Ketose I III



Schiff's Base of HMF II IV

Figure 18. The proposed structures of 1-amino-1-deoxy-2-ketose and Schiff's base of hydroxymethyl furfural (HMF), stabilized by hydrogen bonding and by chelate formation.

effectively transport an essential growth factor into the cell of C. fetus. The growth factor by Krieg's observation is possibly the ferrous ion. As a result, C. fetus grows better in a medium in which 0.5% glucose is added before sterilization.

B. The Metabolism of Glucose in Campylobacter fetus:

Several investigators have reported that glucose was neither fermented nor oxidized by C. fetus (Smith and Taylor, 1919; Plastridge and Williams, 1943; King, 1957; Lecce, 1958; Alexander, 1957; Kiggins and Plastridge, 1958 and Smibert, 1974). The results of this study also showed that C. fetus was incapable of assimilating glucose. Because it does not possess a glucose permease system, glucose can not be absorbed by the organism. Furthermore, glucose was unable to be phosphorylated in the crude cellular extract of C. fetus, for there was no glucokinase activity and acyl phosphate: hexophosphotransferase activity ever found in the crude cellular extract.

Glucose, galactose, and mannose were found to be the carbohydrate components of the cell wall of C. fetus (Smibert, 1970). Apparently, glucose was indeed synthesized in the organism. Since glucose is not the carbon or energy source for the growth of this organism, the building blocks, namely, glucose-6-phosphate or UDP-glucose should be synthesized from other sources. The best carbon or energy sources were found to be acetate, lactate and pyruvate, several dicarboxylic acids on the citric acid cycle; and the amino acids: aspartate, glutamate, cysteine and proline (Alexander, 1957 and Kiggins and Plastridge, 1958). It is clear that the Embden-Meyerhof pathway is the major channel for the synthesis of phosphohexoses.

A scheme of glucose-6-phosphate metabolism is proposed and presented in Figure 19. Glucose-6-phosphate is synthesized from short chain carbon sources by the Embden-Meyerhof pathway. Since no glucose-6-phosphate dehydrogenase activity was ever found in C. fetus, glucose-6-phosphate is not oxidized by either the Hexose Monophosphate pathway or by the Entner Doudoroff pathway. The activities of the non-oxidative portion of the Hexose Monophosphate pathway and Phosphoketolase pathway were found in C. fetus. Their metabolic roles will be discussed in the next section. UDP-glucose is indirectly derived from glucose-6-phosphate. Since the enzymatic activities of phosphoglucomutase and uridine diphosphoglucose pyrophosphorylase in this organism were not examined, the reactions are assumed to exist and are shown by the dotted lines in the scheme. It may be concluded that the Embden-Meyerhof scheme is functioning as a synthetic pathway in C. fetus.

C. Pentose Metabolism by Campylobacter fetus:

Several pentoses were known of as not being metabolized by C. fetus (King, 1957 and Lecce, 1958). There was no ribokinase activity ever found in C. fetus in this study (Table XV). Perhaps ribose phosphate, the sugar component of nucleotides, could only by synthesized endogenously. The activities of the non-oxidative portions of both the Hexose Monophosphate pathway and the Phosphoketolase pathway were found in C. fetus (Table XIV). Ribose-5-phosphate may be synthesized by the sections of these two pathways.

A proposed scheme of pentose-5-phosphate metabolism is presented in Figure 20. Solid lines indicate that the enzymatic reaction was detectable in C. fetus, while dotted lines represent the reactions that were not tested but are assumed to be operating in the cell. Pentose-5-phosphate

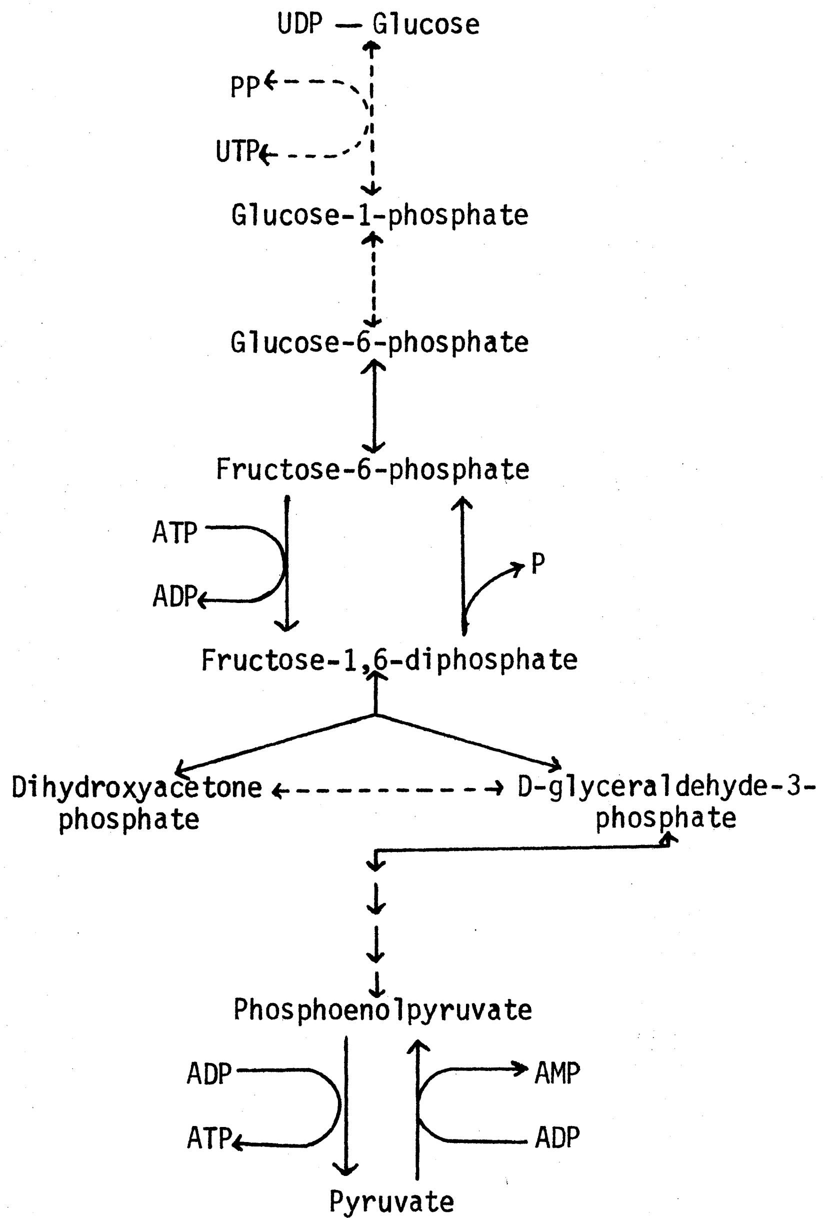


Figure 19. The proposed scheme of glucose-6-phosphate metabolism in *Campylobacter fetus*.

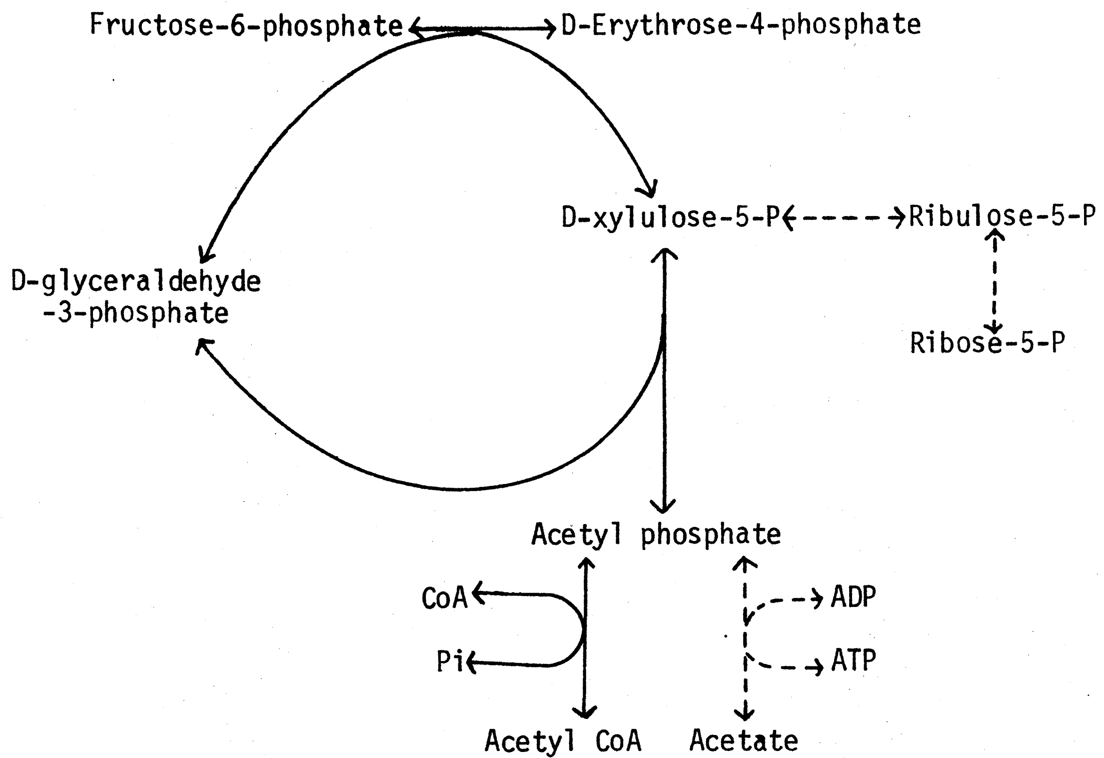


Figure 20. The proposed scheme for pentose-5-phosphate metabolism in <u>Campylobacter fetus</u>.

can be synthesized by the action of phosphoketolase from acetyl phosphate and D-glyceraldehyde-3-phosphate. Acetyl phosphate may be made from acetyl CoA by the phosphotransacetylase reaction or from acetate and ATP by acetylkinase reaction, while D-glyceraldehyde-3-phosphate is derived from the Embden-Meyerhof scheme. The, xylulose-5-phosphate may join the Embden-Meyerhof scheme by the transaldolase reaction. It is believed that ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase are also actively operating to catalyze the interconversion of the three pentose phosphates.

D. The Oxidation of Pyruvate by *Campylobacter fetus*:

The first reaction for the oxidation of pyruvate is the oxidative decarboxylation by a complex enzyme system: pyruvate dehydrogenase. Acetyl CoA is produced from the reaction and then oxidized in the citric acid cycle. However, pyruvate dehydrogenase activity was not found in *C. fetus* (Table XI). Instead, pyruvate came into the citric acid cycle by pyruvate carboxylase in strain H840 and by malic enzyme in strains PB1 and 482. In both reactions, one molecule of CO_2 is needed and pyruvate is carboxylated to become oxaloacetate and malate respectively. These reactions apparently confirmed the observations by Plastridge and Williams (1943), Kiggins and Plastridge (1956), Fletcher and Plastridge (1964), and Smibert (1974), that 10% CO_2 was essential for the growth of *C. fetus*.

A proposed scheme for the oxidation of pyruvate in *C. fetus* 482 and PB1 is presented in Figure 21 and for H840 in Figure 22. In both systems, lactate is dehydrogenated to form pyruvate, which is carboxylated by pyruvate carboxylase and malic enzyme, respectively. Aspartate and gluta-

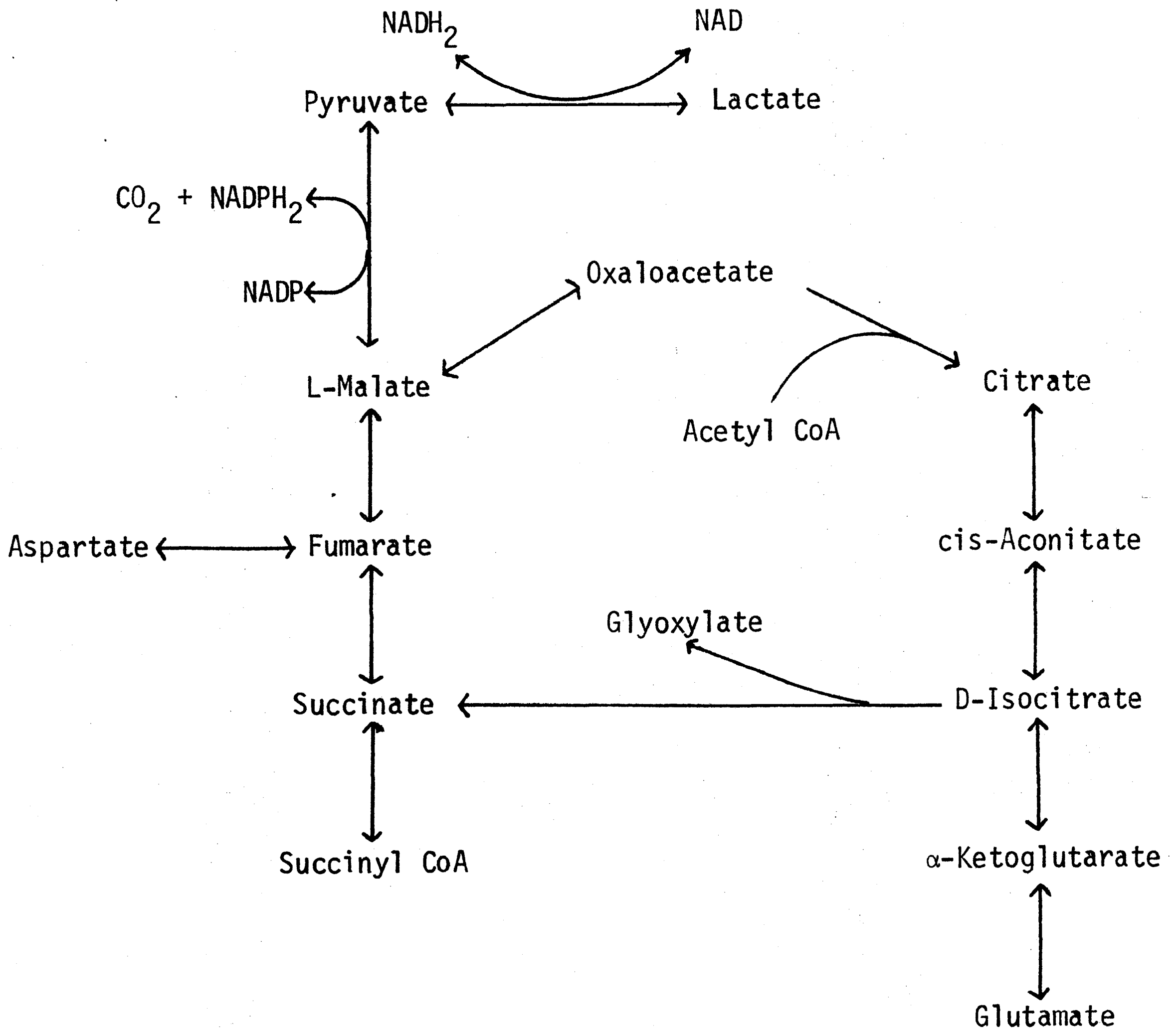


Figure 21. The proposed pathway for pyruvate oxidation in *Campylobacter fetus* strains 482 and PB1.

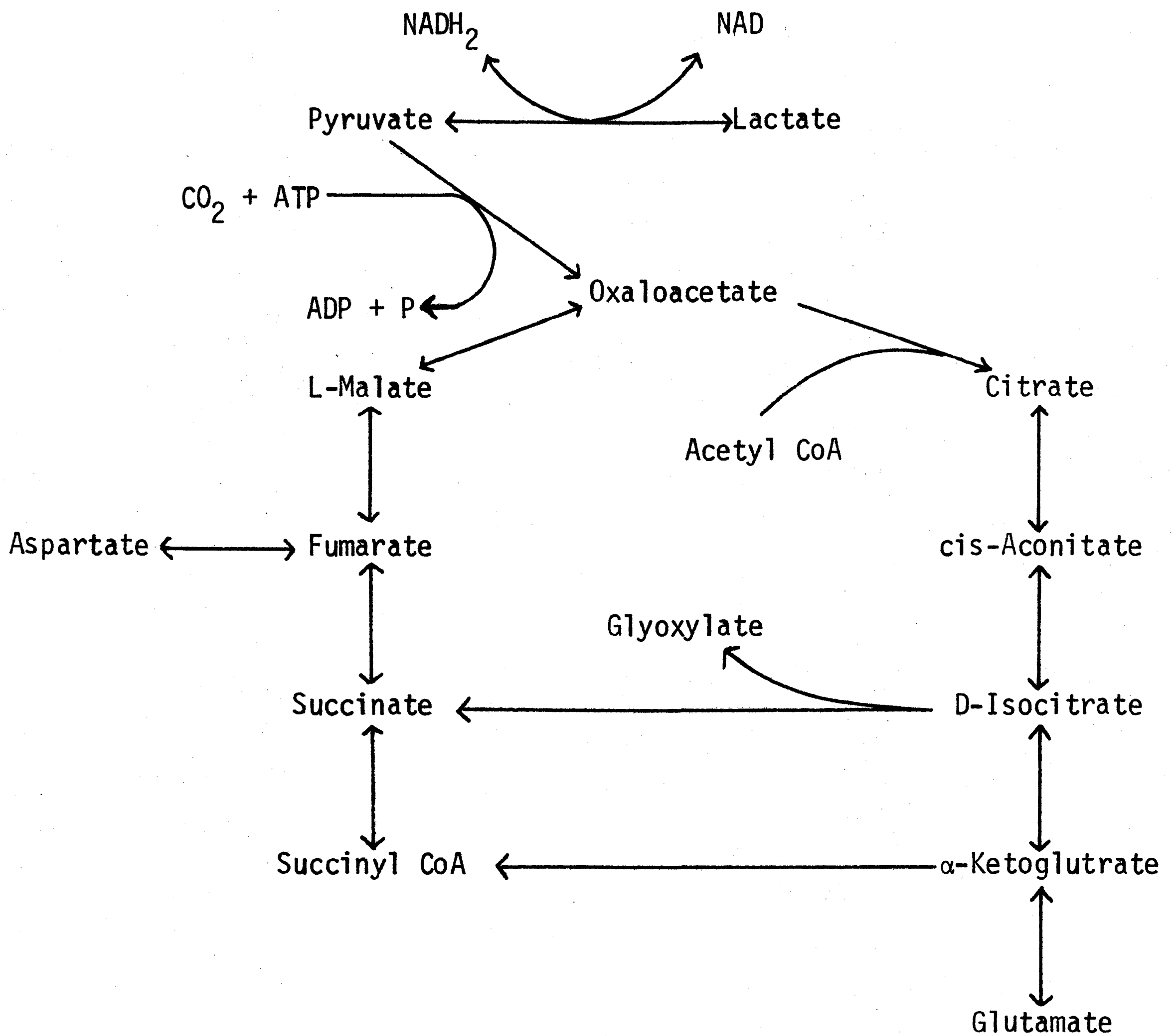


Figure 22. The proposed pathway for pyruvate oxidation in Campylobacter fetus strain H840.

mate may join the two schemes by aspartase and glutamate dehydrogenase reactions.

Both schemes are supported by the action of isocitrate lyase which actively splits isocitrate into succinate and glyoxylate. But malate synthase was absent in both schemes when the organism was grown in a medium containing no inducer, namely, glucose or acetate. Therefore, a question as to how the glyoxylate is metabolized in C. fetus is raised. Acetyl CoA, one of the essential compounds for the initiation of citric acid cycle is ordinarily derived from pyruvate by the pyruvate dehydrogenase reaction. Since the enzyme was not found in C. fetus, the origin of acetyl CoA is another important question which remains to be determined.

α-Ketoglutarate dehydrogenase is another complex enzyme system on the citric acid cycle. Its activity was not found in C. fetus 482 and PB1. But a very weak enzymatic activity was detectable in H840. The lack of pyruvate dehydrogenase activity and α-ketoglutarate dehydrogenase activity (weak activity in H840), provides an explanation for the microaerophilic properties of C. fetus. As there are 5 electron donors on the citric acid cycle, namely, pyruvate, α-ketoglutarate, malate, isocitrate and succinate which generate NADH; the NADH, in turn, is oxidized via the electron transport chain by oxygen. Since pyruvate and α-ketoglutarate are not electron donors in C. fetus, the demand for oxygen as the electron acceptor is accordingly decreased.

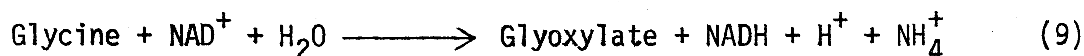
E. The Metabolic Role of Glyoxylate:

According to both proposed schemes for pyruvate oxidation (Figures 21 and 22), glyoxylate is produced in the isocitrate lyase reaction, but it

was unable to condense with acetyl CoA, as no malate synthase was found in <u>C. fetus</u> grown in basal medium or enriched medium. The metabolism of glyoxylate was not studied in this project, but several possible reactions in which glyoxylate may be metabolized are speculated.

Firstly, glyoxylate can induce the synthesis of malate synthase which then catalyzes the condensation of glyoxylate with acetyl CoA to form malate. This is supported by the finding of Reeves and Ajl (1960) that the formation of isocitrate lyase proceeded that of malate synthase in <u>E. coli</u> grown in the chemically defined medium containing acetate.

Secondly, glyoxylate may be converted into glycine by reductive amination. Goldman and Wagner (1962) purified the enzyme glycine dehydrogenase from crude cell-free extract of <u>Mycobacterium tuberculosis</u>, which catalyzes the following reaction:



$$\text{Glycine} + NAD^+ + H_2O \longrightarrow \text{Glyoxylate} + NADH + H^+ + NH_4^+ \qquad (9)$$

The interconversion of glyoxylate and glycine can also be catalyzed by a transaminase reaction which is pyridoxal phosphate dependent. The reaction can be visualized as follows:

$$\text{Glyoxylate} + \text{L-Amino Acid} \longrightarrow \text{Glycine} + \alpha\text{-Keto Acid} \qquad (10)$$

Campbell (1956) found a transamination reaction between glyoxylate and alanine, aspartic acid, glutamic acid, asparagine and glutamine in an unidentified strain of <u>Pseudomonas</u>. The interconversion of glycine and glyoxylate by this transamination was also found in <u>Pseudomonas aeruginosa</u> by Bachrach (1957) and in rat liver by Nakada (1964).

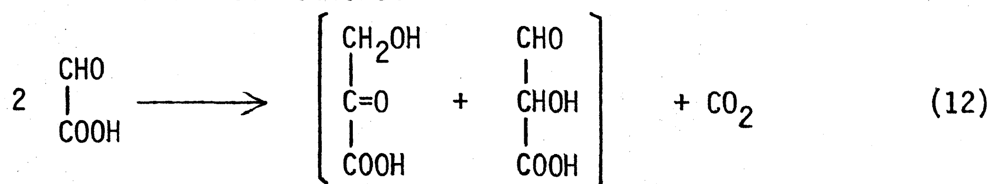
Glyoxylate was also found actively to catalyze the deamidation of asparagine and glutamine in rat liver preparation by Meister <u>et al</u>. (1952).

The reaction is shown as follows:

$$\text{Glyoxylate} + \text{Glutamine} \longrightarrow \alpha\text{-KG} + \text{Glycine} + NH_4^- \quad (11)$$

It is known that C. fetus actively utilizes cysteine, aspartic acid, asparagine, glutamic acid, glutamine and proline as carbon and energy sources (Alexander, 1957 and Kiggens and Plastridge, 1958). One possible role of glyoxylate, therefore, is to catalyze the deamination reactions of these amino acids. Glycine synthesized in these reactions may be used for the synthesis of purine and peptidoglucan.

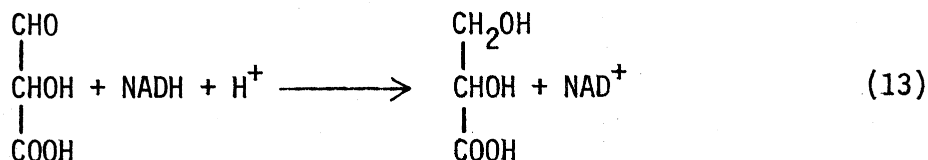
Thirdly, glyoxylate may be used to synthesize D-glycerate. The formation of D-glycerate from glyoxylate in microorganisms has been studied by several scientists. Krakow and Barkulis (1956) reported that glyoxylate was rapidly utilized by a strain of E. coli grown in a chemically defined medium containing glycollate. Incubating the glyoxylate with crude cellular extracts of E. coli revealed the formation of hydroxypyruvate or its isomer tartonic semialdehyde. A reaction of condensive decarboxylation was proposed and is shown as follows:



$$2\ \begin{matrix} CHO \\ | \\ COOH \end{matrix} \longrightarrow \left[\begin{matrix} CH_2OH \\ | \\ C=O \\ | \\ COOH \end{matrix} + \begin{matrix} CHO \\ | \\ CHOH \\ | \\ COOH \end{matrix} \right] + CO_2 \quad (12)$$

The reaction was confirmed by Kornberg and Gotto (1960) by incubating (1-^{14}C) glyoxylate with the cell-free crude extract of Pseudomonas (B_2aba) grown in a medium containing glycollate as the sole carbon source. One molecule of CO_2, which was derived from the carboxyl froup of one of the two glyoxylate, was evolved. The enzyme glyoxylate carboligase which catalyzes the condensive decarboxylation reaction, was purified from crude cellular

extract of E. coli and identified as a flavoprotein containing flavin adenine dinucleotide (Gupta and Vennesland, 1964). Tartronic semialdehyde, the product of condensive decarboxylation was further reduced to glycerate, which was isolated as (^{14}C)-glycerate by Kornberg and Gotto. The reaction is NADH dependent and is shown as follows:



$$\begin{array}{l} CHO \\ | \\ CHOH + NADH + H^{+} \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2OH \\ | \\ CHOH + NAD^{+} \\ | \\ COOH \end{array} \qquad (13)$$

The enzyme which catalyzes the reaction is called tartronic semialdehyde reductase which was purified and crystallized from crude cellular extract of Pseudomonas ovalis chester by Gotto and Kornberg (1961).

Since carbohydrates are not assimilated by C. fetus, structural hexoses have to be synthesized from compounds with short carbon chains. The Embden-Meyerhof pathway is probably the major route for the synthetic operation, reactions from glyoxylate to glycerate may provide the organism a fast alternative pathway.

An overall scheme for the synthesis of glucose-6-phosphate from acetyl CoA, aspartate and glutamate is proposed and shown in Figure 23.

F. The Origin of Acetyl CoA:

Pyruvate is probably not the major source of acetyl CoA in C. fetus, since pyruvate dehydrogenase activity was not found in this organism. Several possible sources of acetyl CoA are discussed as follows:

1. The formation of acetyl CoA by phosphotrans-acetylase reaction in this organism has been proven by previous experiment (Table XV). The substrate of the reaction is acetyl phosphate which may be derived from

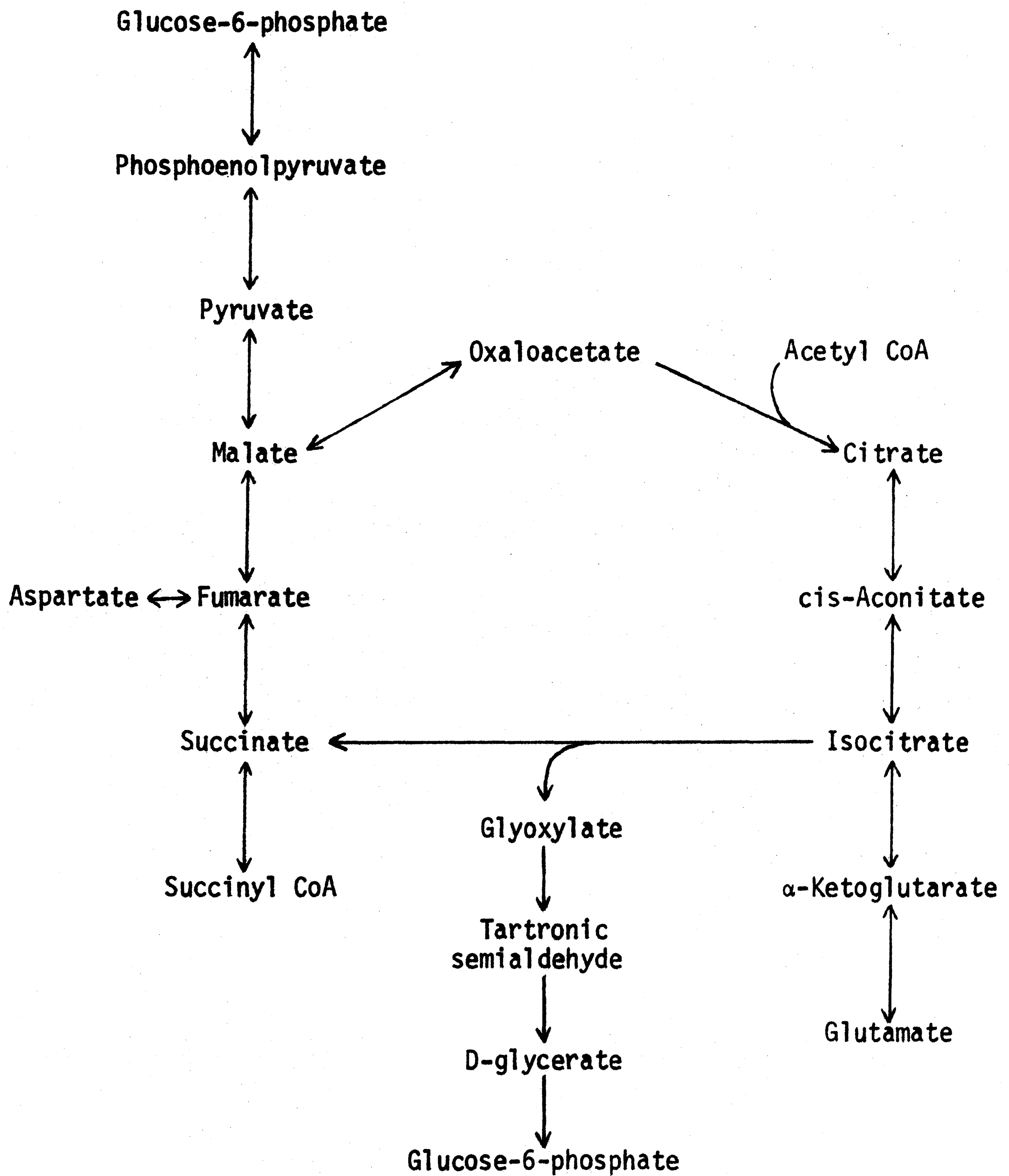
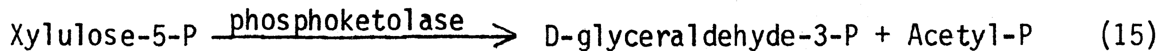


Figure 23. The proposed scheme for synthesis of glucose-6-phosphate in Campylobacter fetus.

xylulose-5-phosphate by phosphoketolase reaction (Table XIV), or may be obtained from phosphorylation of acetate by the acetokinase reaction. The reactions are presented as the following equations:

$$\text{Acetate} + \text{ATP} \xrightarrow{\text{Acetokinase}} \text{Acetyl-P} + \text{ADP} \qquad (14)$$

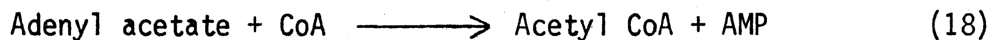


$$\text{Xylulose-5-P} \xrightarrow{\text{phosphoketolase}} \text{D-glyceraldehyde-3-P} + \text{Acetyl-P} \qquad (15)$$

$$\text{Acetyl-P} + \text{CoA} \xrightarrow{\text{phosphotransacetylase}} \text{Acetyl CoA} + \text{P} \qquad (16)$$

2. Acetyl CoA may be synthesized by acetyl CoA synthetase reaction. The two step reaction proposed by Berg (1956) is shown as follows:

$$\text{ATP} + \text{Acetate} \longrightarrow \text{Adenyl acetate} + \text{PP} \qquad (17)$$



$$\text{Adenyl acetate} + \text{CoA} \longrightarrow \text{Acetyl CoA} + \text{AMP} \qquad (18)$$

3. Acetyl CoA may be derived from acetoacetyl CoA or α-ketoacyl CoA, the degradation product of fatty acid by the reaction of acetoacetyl CoA thiolase (Goldman, 1954).

4. Acetyl CoA may be the product of the citrate cleavage enzyme (Plowman and Cleland, 1967) by the following reaction:

$$\text{Citrate} + \text{ATP} + \text{CoA} \longrightarrow \text{ADP} + \text{PP} + \text{Acetyl CoA} + \text{OAA} \qquad (19)$$

5. Several amino acids, namely, leucine, isoleucine phenylalanine and lysine may also be the source of acetyl CoA. When these amino acids degradate, one of the end products is either acetyl CoA, or the compounds acetaldehyde, or acetoacetyl CoA; and these may be converted into acetyl CoA.

SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the energy metabolism of 3 strains of *C. fetus*. A biphasic culture technique was employed to grow the culture and from which crude cellular extracts were prepared. Growth of the strains of *C. fetus* was extremely slow when basal medium was used. A slight improvement was obtained when the strains of *C. fetus* were grown in the basal medium with the addition of 0.5% lactate or acetate. 0.5% glucose in the basal medium stimulated the growth of *C. fetus* strains 482 and H840, but had no effect on that of strain PB1. 0.5% citrate in the basal medium slightly enhanced the growth of strain H840, had no effect on the growth of strain 482, and showed some inhibitory effect on that of strain PB1. The optimal pH for the biphasic growth of *C. fetus* ranged from pH 6.6 to 7.0.

Glucose was not utilized by *C. fetus*. However, growing *C. fetus* 482 in a basal medium + 0.5% glucose did produce a better growth response. This is probably due to some of the decomposed compounds of glucose which, having been subjected to Browning reaction in the medium during sterilization, effectively chelated the trace metal ions used as essential growth factors, and transported them into the cell. As a result, *C. fetus* grew better in the basal medium in which 0.5% glucose was added before sterilization.

Glucose uptake was not observed in this study; the organism probably does not have a glucose permease system. Furthermore, free glucose was not phosphorylated in the crude cellular extract by the reaction of glucokinase and the reaction of acetyl phosphate: hexose phosphotransferase. These

are probably the major reasons why C. fetus can not assimilate glucose.

The activities of certain key enzymes of the Embden-Meyerhof pathway were found in C. fetus; therefore, from pyruvate up to glucose-6-phosphate, the pathway is actively operating in the organism. Since C. fetus was known not to assimilate hexoses, several intermediates of citric acid cycle as well as compounds which very easily enter the cycle, were used as the carbon and energy sources for growth. As phosphorylated hexoses must be synthesized from these carbon sources, the Embden-Meyerhof scheme, consequently, served a synthetic function in the organism. This was also confirmed by the findings that a very active fructose diphosphatase and phosphoenolpyruvate synthetase reactions were found in the organism.

The oxidative portion of both the Hexose Monophosphate pathway and phosphoketolase pathway and the entire Entner Dudoroff pathway were not found in C. fetus; but the non-oxidative portions of both the Hexose Monophosphate and phosphoketolase pathways were found in the organism and are believed to compose the major pathway of pentose-5-phosphate metabolism in C. fetus.

Pyruvate is used as a carbon and energy source for the growth of C. fetus, but it was not oxidatively decarboxylated by pyruvate dehydrogenase in this organism. It was found that pyruvate was carboxylated by pyruvate carboxylase reaction in C. fetus H840 and by malic enzyme in C. fetus 482 and PB1. The carboxylated products of pyruvate were then oxidized by the citric acid cycle.

The citric acid cycle was actively operating except that α-ketoglutarate dehydrogenase was not detected in C. fetus 482 and PB1 and was very weak in H840. The gap in the cycle was supported by the reaction of isocitrate

lyase which cleaved isocitrate into glycoxylate and succinate from which the cycle was continued. Glyoxylate was unable to condense with acetyl CoA in _C. fetus_ since no malate synthase activity was found in this organism. Several possible metabolic roles of glyoxylate have been discussed.

Acetyl CoA, the essential compound for the initiation of citric acid cycle, was not derived from pyruvate in _C. fetus_. Most likely it comes from the degradation of amino acids and fatty acids or is directly obtained from acetate in the medium. The origin of acetyl CoA in _C. fetus_ remains an area for further study.

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METABOLIC PATHWAYS OF CAMPYLOBACTER FETUS

by

Ping Tu Wang

(ABSTRACT)

The energy metabolism of three strains of Camplyobacter fetus (i.e., C. fetus ss. fetus 482, C. fetus ss. intestinalis PB1, and C. fetus ss. jejuni H840) was investigated. A biphasic culture technique was employed to grow the culture, and from which crude cellular extracts were prepared. The growth of the organisms was extremely slow when a basal medium (BM) was used. At a level of 0.5% carbon source in BM, the growth of 482 was stimulated by glucose, lactate and acetate; citrate showed no effect. The growth of PB1 was stimulated by lactate and acetate; glucose had no effect and citrate inhibited the growth slightly. The growth of H840 was stimulated by glucose and citrate; acetate had no effect.

All enzyme assays were performed at 25 C in a Perkin-Elmer model 124 double beam spectrophotometer. The reaction mixture was 3 ml which contained the appropriate substrate, cofactors and properly diluted crude cellular extract. The specific activity of an enzyme was defined as μmoles of substrate transformed per minute per mg of protein.

When the strains of C. fetus were grown in an enriched medium, the citric acid cycle was actively operating except that α-ketoglutarate dehydrogenase was undetectable in 482 and PB1, and very weak in H840. The interruption in the cycle was supported by the reaction of isocitrate lyase which is a key enzyme of the glyoxylate bypass, and was found in the three

strains. Malate synthase was not detectable in either of them, but can be induced by growing the organisms in BM + 0.5% glucose or 0.5% acetate. No pyruvate dehydrogenase was found in either strain. Pyruvate was carboxylated by pyruvate carboxylase in H840 and by malic enzyme in 482 and PB1. All of them contained the activities of aspartase and glutamate dehydrogenase.

Glucose was not assimilated by C. fetus. α-methyl-D-glucoside was not absorbed by C. fetus 482 which was grown in the BM with or without glucose. It is believed that 482 does not possess a glucose permease system or that such a system can not be induced by growing the organism in a medium containing glucose. Furthermore, free glucose was not phosphorylated in 482 by glucokinase or acetylphosphate: hexose phosphotransferase.

The activities of 6 key enzymes of the Embden-Meyerhof pathway were all found in 482; therefore, the pathway is actively operating and presumably served as a synthetic scheme for the formation of hexose phosphate in the organism. The oxidative portion of both the hexose monophosphate pathway and phosphoketolase pathway, and the entire Entner-Dudoroff pathway were not found in 482. But the non-oxidative portions of the former pathways were present in 482 and is believed to compose the major pathway of pentose-5-phosphate metabolism in this organism.

There were activities of lactate dehydrogenase, reduced NAD and NADP dehydrogenases, and phosphotransacetylase found in 482. No ribokinase and gluconokinase were detectable in this organism.