Studies on the growth responses of virulent *Treponema hyodysenteriae* in liquid medium

by

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INTRODUCTION

The primary etiologic agent of swine dysentery has been recently cultivated in routine laboratory conditions. Treponema hyodysenteriae is an aerotolerant anaerobe and has earned recognition as being the only cultivatable pathogenic treponeme at this time. Propagation of the organism in vitro has opened many areas of research aimed towards the possible erradication of swine dysentery.

Because a practical method for the cultivation of T. hyodysenteriae was just recently described, little is known about the growth characteristics of this organism. The objectives of this project were to 1) evaluate and define practical methods for the quantitation of growth responses of T. hyodysenteriae, 2) define an optimal temperature and initial pH of the growth medium, and 3) initiate studies directed toward simplifying the complex growth medium of trypticase soy broth supplemented with fetal calf serum. It is hoped that this work will result in the improvement of growth in the complex growth medium with more predictable yields and stability of pathogenic laboratory isolates, and aid development of a defined growth medium that will support other pathogenic treponemes.

LITERATURE REVIEW

Swine Dysentery

Swine dysentery has been recognized and described as a specific disease since 1921 (Whiting et al., 1921). The course of this swine disease has been outlined (Harris and Glock, 1973) and well defined (Alexander and Taylor, 1969; Glock, 1971; Harris and Glock, 1973). The clinical signs of the disease include a rapidly debilitating condition characterized by diarrhea with loss of blood, and excess mucus in the feces. Pigs afflicted with dysentery often appear gaunt and depressed. The morbidity of weanling pigs (8-14 weeks old) is often more than 90% and mortality may be as great as 30% (Harris and Glock, 1973).

The gross appearance of the large intestine has been described (Glock and Harris, 1972; Harris and Glock, 1973). In acute cases of dysentery the intestinal wall and mesentery are edematous, the serosal surface is inflamed, and a muco-fibrinous exudate often adheres to the epithelial surface. In chronic cases of the disease, edema is less severe and a fibrinonecrotic pseudomembrane usually covers the mucosa (Harris and Glock, 1973). Microscopic examination of the lesions shows congested vessels near the lumen, edema of the lamina propria, and exhaustion and degeneration of both goblet and epithelial cells (Glock and Harris, 1972;

Harris and Glock, 1973).

The etiology of swine dysentery has been discussed (Akkermans and Pomper, 1973; Doyle, 1948; Harris, 1974). During the past quarter century, many workers considered V. coli to be the primary etiologic agent of swine dysentery (Sorensen, 1970). The actual role of V. coli was never defined because pure cultures did not reproduce a clinical disease consistent with that found in natural outbreaks (Andress and Barnum, 1968; Terpstra et al., 1968).

Spirochetes have been observed in the colonic contents of pigs with dysentery (Blakemore and Taylor, 1970; Carpenter and Larson, 1952; Glock, 1971; Harris et al., 1972a; Taylor, 1970; Tesouro, 1969). Electron micrographs have revealed bacteria which resemble Vibrio and large spirochetes within the colonic crypts of dysenteric pigs (Glock, 1971; Taylor and Blakemore, 1971). A large spirochete (Treponema hyodysenteriae) has been shown to invade both goblet and epithelial cells (Blakemore and Taylor, 1970; Glock, 1971; Glock and Harris, 1972; Glock et al., 1974; Taylor and Blakemore, 1971).

Although there is evidence which indicates that Vibrio and the large spirochetes play important roles in the etiology of swine dysentery (Hamdy and Glenn, 1974; Harris et al., 1972a; Sofrenovic and Olujic, 1974), it has been shown that pure cultures of a specific large spirochete

(T. hyodysenteriae) will produce the disease by oral inoculation of susceptible swine (Akkermans and Pomper, 1973; Harris et al., 1972a; Kinyon, 1974; Olujic et al., 1973; Taylor and Alexander, 1971; Taylor, 1972). The pathogenicity of various isolates of T. hyodysenteriae has been established (Kinyon, 1974) and one strain (B204) retains pathogenicity for swine after 23 in vitro passages in liquid medium (Joens, L. A., Iowa State University, personal communication, 1974).

Treponema hyodysenteriae is accepted as the primary etiologic agent of swine dysentery by many workers, but the possible synergistic roles of other organisms, such as Vibrio and normal intestinal flora in swine, remain to be explained. Taylor (1972) has reproduced swine dysentery in vibrio-free swine with the Type-1 spirochete. However, cultures of T. hyodysenteriae have not produced dysentery in pigs which have no intestinal flora. Brandenburg (1974) and Harris et al. (1972b) attempted to induce the disease in germ-free animals with T. hyodysenteriae and with combinations of the spirochete and Vibrio or Peptostreptococcus, but were not successful. The characteristic clinical signs and lesions of dysentery were not observed in the germ-free swine; however, it has been reported that T. hyodysenteriae and Vibrio coli establish in the colons of the animals (Harris et al., 1972b; Meyer et al., 1974a). Dysentery also

did not occur in gnotobiotic swine when infected with Escherichia coli, Lactobacillus, Vibrio coli, and Clostridium in combination with T. hyodysenteriae isolate B78 (Meyer et al., 1974b). In 1975, Meyer et al. reported that clinical dysentery in gnotobiotic piglets was produced by several swine anaerobes which included two Bacterioides and two fusiforms plus T. hyodysenteriae isolate B78. Disease did not occur with anaerobes alone or with the spirochetes alone, which indicates that a) a mixed infection is required for disease production, b) T. hyodysenteriae is dependent upon the anaerobes for environmental and/or nutritional requirements necessary for pathogenicity.

Differentiation of Dysentery Spirochetes

The primary etiologic agent of swine dysentery has been referred to as Type a spirochete (Taylor, 1970), Type-1 spirochete (Taylor, 1972), Borrelia-like organism (Harris and Glock, 1971), and large spirochete (Harris et al., 1972c). A smaller type of spirochete has been often observed in the colonic contents of dysenteric swine. The organisms have been referred to as Type b (Taylor, 1970) and Type-2 spirochetes (Taylor, 1972), PF (pig feces) strains (Smibert, 1971), PF-7 strain (Smibert and Claterbaugh, 1972), PN-5 strain (Saheb and Berthiaume, 1973), and small spirochetes (Harris et al., 1972c).

The two types of spirochetes are easily differentiated by morphology. The smaller spirochetes are tightly coiled and 4-7 μm in length and 0.24-0.30 μm in diameter. One or two axial fibrils originate at each end of the protoplasmic cylinder (Harris, et al., 1972c; Saheb and Berthiaume, 1973). The large, loosely coiled organisms have been described as 6-8.5 μm in length and 0.32-0.38 μm in diameter with 7-9 axial fibrils inserted at each end (Harris et al., 1972c). Kinyon (1974) determined the average dimensions of 21 isolates of T. hyodysenteriae-like organisms to be 8.5 μm in length and 0.317 μm in diameter.

The small spirochetes have varied metabolic characteristics. Several produce short-chained fatty acids from carbohydrates (Smibert, 1973); some require rumen fluid for growth (Smibert, 1971) while others require serum (Harris and Kinyon, 1974).

The large spirochete associated with swine dysentery was initially characterized by Harris et al. (1972c). The organism was observed to be motile, β-hemolytic, and negative for cytochrome oxidase and catalase. It stained weakly gram-negative and produced a small amount of acetic acid from glucose. The organism was maintained but would not grow well in peptone-yeast broth (PY) containing 0.15% agar, glucose, diacetyl tartaric acid ester of tallow monoglycerides (TEM-4T), and rabbit serum (Harris et al., 1972c).

The spirochete was classified in the genus Treponema on the basis of morphology and its location in the host. Because the large dysentery isolate differed from other species of the Treponema, these workers named the organism Treponema hyodysenteriae.

Organisms with morphologies similar to that of T. hyodysenteriae have been observed in normal pigs, but these isolates were not pathogenic for swine in transmission studies (Taylor, 1972; Kinyon, 1974). Large spirochetes have not been observed in swine enteric diseases other than swine dysentery (Akkermans and Pomper, 1973). Small spirochetes have been observed in the feces of diseased swine, but were not shown to be associated with the lesions of swine dysentery (Glock, 1971).

Classification of Spirochetes

The currently accepted classification for the order, Spirochaetales, has been summarized by Smibert (1973, 1974). These slender, flexuous microbes are helically coiled and are 5-500 μm long and 0.1-3 μm wide. They stain gram-negative, but are best observed by darkfield or phase-contrast microscopy. Spirochetes are motile, with a rapid whirling along the long axis of the cell, flexion, and movement in a corkscrew or serpentine manner. Spirochetes are saprophytic or free-living in nature or are parasitic. Some

spirochetes are pathogenic for human beings and animals.

The five genera are briefly described as follows (Smibert, 1973, 1974):

Leptospira are thin and tightly coiled with one or both ends hooked. Some are pathogenic to humans and animals, others are saprophytes; all are aerobes.

Spirochaeta are free-living in nature. They are facultative anaerobes, some are anaerobes, but are not strict anaerobes.

Borrelia cause relapsing fever in human beings and are transmitted by lice and ticks. These blood-borne organisms are not very strict anaerobes.

Cristispira are found in the intestinal tracts of molluscs but have not been cultured. As the largest of the spirochetes, they have 50 to several hundred axial fibrils which form a ridge or "crista".

Treponema are found in the intestines, oral cavity, and genital regions of humans and animals; some are pathogenic. These organisms range from strict anaerobes to aerotolerant anaerobes.

Classification of the Treponemes

For decades, host-associated spirochetes were classified on the basis of source, staining characteristics, and morphology as viewed by a light microscope (Breed et al., 1957;

Prevot, 1966). Listgarten and Socransky (1965) used the electron microscope as an aid in differentiation of treponemes based upon the size of the protoplasmic cylinder, the number of axial fibrils, and the structure of the outer envelope. Socransky et al. (1964) also noted differences in the biochemical and nutritional characteristics of two human oral (HO) treponemes. In 1969, Socransky et al. compared the morphology and biochemical characteristics of 30 oral isolates and classified them as strains of T. denticola, T. macrodentium, or T. oralis, a proposed new species.

Although Smibert (1973, 1974) has retained the generic separation of the Spirochaetales based upon 1) location in nature or in the host; 2) morphology; and 3) relationship to oxygen, the classification of spirochetes has focused more on biochemical characteristics. A number of the Treponema have been characterized (Smibert, 1971). Smibert (1973) has divided the genus into two groups. The species in Group One include noncultivatable pathogens; in Group Two are all species which have been cultured in vitro.

Group One

Treponema pallidum is the cause of venereal and congenital syphilis in monkeys and human beings. Treponema pertenue commonly causes yaws, which is a human skin disease

peculiar to subtropical countries; Treponema carateum is the cause of pinta or carate in humans. Treponema paraluis-cuniculi (synonym: T. cuniculi) causes a benign, syphilis-like disease in rabbits and produces cutaneous lesions in the genital-perineal area and around the eyes, ears, and nose. Two unnamed pathogens of Group One may be variants or subspecies of T. pallidum (the agent associated with endemic syphilis) and of T. pertenue (the agent associated with a disease in primates) (Smibert, 1973).

The species of Group One have been distinguished on the basis of lesions produced in laboratory animals. Pathogenic T. pallidum strains are propagated by intra-testicular inoculation of rabbits (Small and Newman, 1972; Weber, 1960). Under anaerobic conditions T. pallidum may remain motile for a week in a medium composed of phosphate buffer, saline, pyruvate, and serum constituents (Clark, 1962); however, no virulent strains of this organism have been cultured in vitro. Virulent strains of T. pertenue can be propagated in puncture wounds of the skin of hamsters, rabbits, or chimpanzees; T. carateum can be propagated by intradermal inoculation of chimpanzees, but is not virulent in rabbits, hamsters, or guinea pigs (Smibert, 1973).

Group Two

As early as 1948, it was proposed that five cultivated strains of T. pallidum be reclassified. Eagle and Germuth (1948) considered that the Nichols, Noguchi, Reiter, Kazaan, and Kroo strains should not be classified as T. pallidum because they were not pathogenic in rabbits and were morphologically different from the virulent strains. However, the initial classification of these strains and other treponemes persisted until more convincing evidence was accumulated.

Smibert (1973, 1974) has summarized the currently accepted changes in the classification of the Treponema. Since they have been cultivated in vitro, the formerly recognized avirulent strains of T. pallidum have been reclassified as either T. phagedenis or as T. refringens and have been assigned to Group Two. Because of metabolic similarities, the Reiter treponeme, English Reiter, and the Kazaan strains are classified as biotypes of T. phagedenis. The Noguchi strain and avirulent Nichols strains of T. pallidum, as well as T. calligyrum and T. minutum are included in T. refringens. The strains formerly designated as T. microdentium, T. commondii, and T. ambiguum have been reclassified as biotypes of T. denticola.

In addition to the avirulent strains formerly classified as T. pallidum, Smibert's Group Two includes the cultivatable

oral treponemes and some fecal isolates. Some of the oral species are: T. macrodentium, T. orale, T. denticola, T. scoliodontum, T. buccale, T. trimerodontum, T. mucosum (associated with alveolar pyorrhea), and T. vincentii (associated with Vincent's angina). Also in Group Two is the pathogen T. hyodysenteriae, the primary etiologic agent of swine dysentery (Glock, 1971; Harris et al., 1972c; Kinyon, 1974; Taylor and Alexander, 1971). Other species which have not been well documented in the literature are listed by Smibert (1974).

Morphology and Ultrastructure of Treponemes

By definition all the spirochetes possess:

1) a helically coiled protoplasmic cylinder bounded by a plasma membrane and cell wall complex;

2) one or more helically coiled axial filaments inserted at each terminus of the cylinder;

3) an outer envelope (sheath) that surrounds both the axial filaments and the protoplasmic cylinder (Pillot and Ryter, 1965). Noteworthy exceptions to these basic criteria are T. pallidum, T. pertenue, and T. denticola, which have been observed to be flat waves rather than helical coils (Cox, 1972).

Treponemes are 5-20 μm long and 0.09-0.5 μm in diameter (Smibert, 1973). The avirulent nichols biotype of T.

refringens has an irregular coiling pattern, with cytoplasmic evaginations or "blebs", and a granular layer of material around the cytoplasmic membrane and the sharply tapered ends. With three axial filaments originating at each terminus and overlapping in the middle of the protoplasmic cylinder, the arrangement of the fibrils may be designated as "3-6-3" (Jepsen et al., 1968). Nonpathogenic strains of T. pallidum have been shown to possess a trilaminar cell wall (Ryter and Pillot, 1963; Ovcinnikov and Delektorskij, 1967). Virulent T. pallidum may have two three-layered membranes around the protoplasmic cylinder (Jackson and Black, 1971a; Wiegand et al., 1972); however, the semirigid membrane has been considered analogous to the bacterial cell wall (Jepsen et al., 1968; Kawata and Inoue, 1964; Pillot and Ryter, 1965).

Structures similar to ribosomes have been observed within the protoplasmic cylinder (Jepsen et al., 1968; Ovcinnikov and Delektorskij, 1969b). So-called nuclei (Kawata and Inoue, 1964; Ovcinnikov and Delektorskij, 1969b), vacuoles (Jepsen et al., 1968; Ovcinnikov and Delektorskij, 1969b), and mesosomes (Kawata and Inoue, 1964; Jepsen et al., 1968; Wiegand et al., 1972) have been reported as treponemal organelles. The mesosomes, which may protrude through openings in the outer envelope (Wiegand et al., 1972) may serve as sites for antinuclear

antibodies and result in the beading phenomenon observed with some anti-gamma-globulins (Krause et al., 1971).

Virulent T. pallidum have dense tapered structures at both ends, designated as "nose pieces" (Wiegand et al., 1972), which may aid treponemes to enter host cells (Jepsen et al., 1968; Ovcinnikov and Delektorskij, 1969a). Intracellular organisms have been reported to be short and thick with few spirals and frequently folded upon themselves (Lauderdale and Goldman, 1972). Azar et al. (1970) have stated that T. pallidum may be "stored" within certain cells with the retention of antigenicity, viability, and pathogenicity.

The substructural pattern of the axial filaments has been compared to that of bacterial flagella (Jackson and Black, 1971b; Jepsen et al., 1968; Listgarten and Socransky, 1964; Pillot and Ryter, 1965). Deep filaments are attached to the inside of the cell membrane and may contribute to motility as well as to the structure and elasticity of the protoplasmic cylinder (Wiegand et al., 1972).

Nauman et al. (1969) hypothesized that electrical impulses travel along axial filaments to activate the helical portion of the striated tubules, which causes the cell to rotate upon its axis and results in movement. A theoretical explanation of normal and abnormal motility observed in spirochetes has been presented (Jahn and Landman, 1965). In

1972, Wang and Jahn explained their hydrodynamic theory which mathematically allows spirochetes to swim the way they do. The torque created by movement of an organism without an inert head is balanced by self-rotation of the body.

The treponemal outer envelope (OE) is a layered structure which completely encases the organism (Jepsen et al., 1968; Ryter and Pillot, 1963). The OE is probably more flexible and more easily ruptured than the cell wall (Kawata and Inoue, 1964). The envelopes of intact T. refringens biotype nichols resembles the OE of the oral treponemes (Jackson and Black, 1971a; Listgarten and Socransky, 1964). The polygonal arrangement of subunits is similar to the hexagonal lipoprotein and lipopolysaccharide layer in the cell wall of the Micrococcus (Work and Griffiths, 1968). Adverse growing conditions such as the lack of nutrients or the addition of small amounts of penicillin or immune sera may cause organisms to coil tightly, become encased in the OE and appear to be cysts (Lauderdale and Goldman, 1972; Ovcinnikov and Delektorskij, 1969b). The amorphous mucoid layer which surrounds virulent T. pallidum (Jepsen et al., 1968; Wiegand et al., 1972) may protect the antigenic sites on the envelope and may account for the poor antibody response against T. pallidum (Wiegand et al., 1972).

The OE of treponemes has been compared to the bacterial capsule, cell membrane (Bladen and Hampp, 1964), and cell wall (Jepsen et al., 1968; Listgarten and Socransky, 1964; Ryter and Pillot, 1963). Unlike a capsule, the envelope is flexible, vital to the cell, and chemically complex. The location, flexibility, and carbohydrate content of the envelope are also in contrast to a typical cell membrane. The OE differs from a cell wall in that it lacks mucopeptide, is not rigid, and does not give the cell its shape. The helical shape of spirochetes is due to the peptidoglycan layer in the cell wall, which surrounds the protoplasmic cylinder (Jackson and Black, 1971a; Johnson et al., 1973); the protoplasmic cylinder remains helical after the OE has been removed.

Composition of Spirochetes

The composition and metabolism of spirochetes have not been thoroughly studied because many of these organisms have not been cultivated *in vitro*. The most information concerning the *Treponema* has been obtained from oral treponemes and the biotypes of *T. phagedenis* (Smibert, 1973). Nothing about the composition of *T. hyodysenteriae* is known.

The *Treponema* are rich in lipids, with 14-20% of the dry weight being lipid material (Johnson et al., 1970a; Pillot and Faure, 1969). This is approximately equal to the

amount of lipid in the Leptospira (Johnson et al., 1970b; Kondo and Ueta, 1972) and about 10% less than the amount found in the Spirochaeta (Joseph, 1972). Allen et al. (1971) have reported that amino acids contribute 41-54% of the cellular material in the Reiter treponeme. Livermore and Johnson (1974) found that treponemes have glucose or galactose as major carbohydrate components. The major phospholipids are phosphatidyl choline and phosphatidyl ethanolamine. None of the treponemes had a detectable amount of phosphatidic acid (Livermore and Johnson, 1974). The polar lipids of Treponema and of Spirochaeta consist of 51.3% phospholipids and 48.7% glycolipids in mean mole percents (Livermore and Johnson, 1974). The major phospholipid found in Leptospira is phosphatidyl ethanolamine (60-70%), however, no glycolipid or phosphatidyl choline is present; the Spirochaeta contain glycolipid, but no phosphatidyl ethanolamine or phosphatidyl choline (Livermore and Johnson, 1974).

Smibert (1973) has summarized information concerning the carbohydrate and deoxyribonucleic acid (DNA) compositions of spirochetes. The guanine plus cytosine content of some of the species studied are as follows:

Spriochaeta 56-67% (Canale-Parola et al., 1968);

Leptospira 34-19% (Haapala et al., 1969);

Treponema 36-46% (Rathlev and Pfau, 1964; Smibert, 1971; Socransky et al., 1969).

The DNA homology between species has been proposed as a possible aid to distinguish species of the Treponema (Livermore, 1974).

Metabolism and Fatty Acid Composition of Spirochetes

The Borrelia (Smibert, 1973) and the Spirochaeta (Hespell and Canale-Parola, 1970) ferment glucose via the Embden-Meyerhoff pathway. Carbohydrates are metabolized to acetate, carbon dioxide (CO_2), and hydrogen (H_2) by a phosphoroclastic mechanism with acetyl-CoA and acetyl phosphate as intermediates in Spirochaeta (Hespell and Canale-Parola, 1970). The free-living Spirochaeta do not require long-chained fatty acids for growth because they can synthesize these compounds from acetate (Joseph, 1972; Meyer and Meyer, 1969; Meyer and Meyer, 1971). The major portion of fatty acids in Spirochaeta are normal and branched acids with 14-17 carbon atoms (Joseph, 1972; Meyer and Meyer, 1971).

The Leptospira cannot utilize carbohydrates or amino acids for energy and probably do not possess kinases for activation of intermediates (eg. pyruvate) or glucose (Baseman and Cox, 1969a). These aerobic spirochetes have a

tricarboxylic acid cycle and an electron transport system (Baseman and Cox, 1969b) and are capable of reverse glycolysis (Baseman and Cox, 1969a). Most pathogenic Leptospires require long-chained fatty acids or alcohols for growth (Johnson and Walby, 1972; Smibert, 1973). These organisms are characterized by the ability to β-oxidize long-chained fatty acids as sole sources of carbon and energy (Henneberry and Cox, 1970; Smibert, 1973). Most pathogenic Leptospira require a chain length of at least 15 carbon atoms (Johnson and Walby, 1972; Staneck et al., 1973) because they cannot elongate fatty acids (Johnson et al., 1970b). Unsaturated fatty acids are not required because the Leptospira can desaturate palmitic and stearic acids to the monounsaturates by a mechanism involving molecular oxygen (Johnson et al., 1970b); a number of pathogenic serotypes will grow well in a simple medium of salts, Tween 80, and bovine serum albumin as the detoxifying lipid-carrier (Ellinghausen and McCullough, 1965). Carbon dioxide is produced from the oxidation of saturated and unsaturated fatty acids (Henneberry and Cox, 1970). The lipid composition of the Leptospira reflects the fatty acids available in the growth medium (Kondo and Ueta, 1972; Johnson et al., 1970b).

The Treponema have been considered as three metabolic groups:

1) carbohydrate fermenters,

2) amino acid fermenters, and

3) those which utilize carbohydrates and amino acids for energy (Livermore, 1974).

The carbohydrate fermenters include T. macrodentium, HO, PF, and bovine rumen (BR) strains, which produce acetate and other products from glucose (Smibert, 1971; Smibert, 1973; Smibert and Claterbaugh, 1972; Socransky et al., 1969). Treponema refringens, T. vincentii, T. oralis, and T. scoliodontum produce acetate and other acids from amino acid fermentation (Smibert, 1971; Smibert, 1973). Although T. phagedenis primarily utilizes glucose (Allen et al., 1971) and T. denticola primarily amino acids (Hespell and Canale-Parola, 1971), both of these organisms can ferment carbohydrates and amino acids (Smibert, 1973). In T. denticola amino acid fermentation is probably by an initial conversion to pyruvate with subsequent metabolism to acetate, CO_2, and H_2 by a phosphoroclastic mechanism (Hespell and Canale-Parola, 1971) similar to that found in Spirochaeta (Hespell and Canale-Parola, 1970); these workers also demonstrated glycolysis in T. denticola.

Like the Leptospira, treponemes are capable of utilizing long-chained fatty acids to synthesize all of their complex polar lipids (Johnson et al., 1970b). The Treponema require long-chained fatty acids or short-chained volatile

acids for growth (Johnson and Eggebraten, 1971; Meyer and Meyer, 1971; Oyama et al., 1953; Smibert and Claterbaugh, 1972) because they cannot alter chain length or desaturate fatty acids (Johnson et al., 1970a). The reiterii and kazan biotypes of T. phagedenis both require a saturate of at least 14 carbon atoms and a mono-, di-, or trienoic unsaturate of at least 15 carbon atoms (Johnson and Eggebraten, 1971). The requirement for the pair of fatty acids by kazan-5 could be substituted for by trans-9-octadecenoic acid (Johnson et al., 1970a).

Kinyon (1974) observed that T. hyodysenteriae ferments carbohydrates with the production of H_2, CO_2, and small amounts of acetic and butyric acids from glucose. The organism requires serum for good growth and is stimulated by hydrogen (Kinyon, 1974), though propagation of T. hyodysenteriae in a broth medium with deoxygenated CO_2 as the anaerobic atmosphere has been reported (Kinyon and Harris, 1974). Although T. hyodysenteriae is metabolically similar to T. refringens biotype refringens (Holdeman and Moore, 1973), there is sufficient information to characterize this swine pathogen as a separate species (Kinyon, 1974). Isolates pathogenic for swine were generally found to be indole positive; nonpathogenic isolates were indole negative; type of hemolysis and fermentation of carbohydrates may also be useful criteria for distinguishing nonpathogenic from

pathogenic isolates.

Knowledge about the anaerobic respiration of treponemes is very scant. Kawata (1967) reported the presence of cytochromes a_2 and b in T. phagedenis biotype reiterii. Virulent T. pallidum may possess an electron transport system as evidenced by the observed uptake of oxygen (Cox and Barber, 1974).

Treponema hyodysenteriae is an oxygen tolerant anaerobe. This swine pathogen, which is negative for catalase, lipase, urease, and cytochrome oxidase tests (Kinyon, 1974), has been observed to take up oxygen in the growth medium (Harris et al., 1975). Because the Type-1 spirochete could not be isolated in prereduced media (Taylor, 1972), it is probable that strict anaerobic conditions are not optimal for growth of this treponeme even though it has been cultivated in prereduced tripticase soy broth supplemented with serum (Kinyon and Harris, 1974). It has been noted that isolation procedures for T. hyodysenteriae need not be anaerobic if blood agar medium is freshly prepared and immediately incubated in an atmosphere of hydrogen and carbon dioxide (Harris et al., 1972c; Kinyon, 1974).

Substrate Specificity Among the Treponemes

In addition to certain lipids, other substrates which either stimulate or are specifically required for growth have been reported. Isobutyric acid is required by T. denticola (Hardy and Munro, 1966; Socransky et al., 1964) for which DL-2-methyl butyric acid will substitute, but not n-butyric, iso-valeric, or 2-methyl valeric acids (Hardy and Munro, 1966). Socransky et al. (1964) found that putrescine and spermine would replace isobutyric acid; their conclusion was that the polyamines functioned in the maintenance of the cellular integrity of T. denticola, but were not incorporated into the cell wall. A treponeme isolated from rumen fluid has a requirement for iso-valeric acid (Wegner and Foster, 1960). A nonpathogenic pig feces isolate (PR-7) requires iso-butyric and n-valeric acids and growth is stimulated by caproic acids (Smibert and Claterbaugh, 1972). It is probable that organisms which require only short-chained fatty acids can synthesize long-chained acids from them (Smibert, 1973).

Many of the cultivatable treponemes either require cocarboxylase for growth or exhibit stimulation of growth (Smibert, 1971, 1973). Power and Pelczar (1959) noted growth stimulation of the Reiter treponeme by TEM-4T, which essentially contains palmitic, stearic, oleic, and linoleic acids. They also observed increases of the cell crop with

the individual fatty acids as supplements, with the exception of linoleic acid. Growth of the Reiter treponeme is accelerated by cytosine, uracil, and adenine, but thymine is not utilized (Steinman et al., 1952; Allen et al., 1971).

Hardy and Munro (1966) found that bicarbonate stimulated the growth of T. microdentium and that long-chained fatty acids in serum were inhibitory. However, 0.1% bicarbonate sufficiently neutralizes the toxic effects of 10% serum to allow good growth of the organism in a serum supplemented medium. Iso-valerate or n-butyrate could not replace the iso-butyrate requirement for either of these oral strains (Hardy and Munro, 1966; Socransky et al., 1964). The bicarbonate is probably required for the synthesis of long-chained fatty acids (Hardy and Munro, 1966).

Lipid Composition of Treponemes

It has been shown that T. phagedenis biotypes reiterii (Meyer and Meyer, 1971) and kazan (Johnson et al., 1970a) do not elongate, retroconvert, or desaturate fatty acids provided in growth media. These organisms incorporate fatty acids directly from the medium (Meyer and Meyer, 1971; Johnson et al., 1970a). The fatty acids of many treponemes have been found to reflect the fatty acids provided in the growth medium by serum (Cohen et al., 1970; Vaczi et al., 1966). The oral spirochetes, T. vincentii and T. denticola,

contain branched fatty acids in significant amounts (Cohen et al., 1970).

Of the total amount of lipids in the Treponema, 55-65% is phosphatidyl choline and 5% is cardiolipin (Faure and Pillot, 1960). The lipid of the reiterii biotype of T. phagedenis is approximately 25% monogalactyl diglyceride, 33% phosphatidyl choline, 8% choline plasmalogen, 7% phosphatidyl glycerol, and 6% cardiolipin (Meyer and Meyer, 1971). The glycolipid has been identified as 1-(O-beta-D-galactopyranosyl)-2,3-diglyceride (Livermore, 1974). The lipid of T. phagedenis biotype kazan-5 is reported as 45-55% galactopyranosyl diglyceride, 30-40% phosphatidyl choline, 5-10% phosphatidyl ethanolamine, and traces of cardiolipin (Johnson et al., 1970a). Livermore and Johnson (1970), identified the glycolipid as 1-(O-α-D glactopyranosyl)-2,3-diglyceride.

Anaerobic Methodology and Anaerobiosis

Because many spirochetes are anaerobic organisms, researchers have been concerned with the development of methods for reducing the oxygen tension of culture media and for maintaining an anaerobic environment. Physical methods to decrease oxygen tension have included boiling the medium, addition of reducing substances, and evacuation of containers and flushing them with inert gases (Hall, 1929).

Unfortunately the most elaborate combinations of these pioneer methods did not allow for the use of solid media.

With the advent of the anaerobic combustion jar designed by McIntosh and Fildes (1916), use of solid media and improved isolation techniques were possible. The history of the anaerobic jar has been reviewed (Sonnenwirth, 1972). A noteworthy safety modification by Stokes (1958) employed a palladium catalyst which did not require heating for activity. Brewer and Allgier (1966) introduced a gas-generating packet as a convenient and practical means for producing a "semianaerobic" environment so that solid media could be used in small laboratories.

Glove boxes have been designed with air locks to provide working space suitable for isolation and transfer of organisms in an anaerobic environment (Aranki et al., 1969; Leach et al., 1971; Rosebury and Reynolds, 1964). Variations of the first anaerobic chamber have been reviewed and the commonly used methods for isolating anaerobes have been compared (Dowell, 1972; Killgore et al., 1973).

The roll-tube technique for cultivation of rumen bacteria (Hungate, 1950) has proved to be a significant contribution to anaerobic methodology. This technique provides a low oxidation-reduction potential (Eh) and an oxygen-free atmosphere. It also prevents the reoxidation of organic substances in the medium, which minimizes the production of

peroxides. In 1966, the Hungate method was modified and described by Moore as the Virginia Polytechnic Institute (VPI) method. The VPI method employs prereduced, anaerobically sterilized (PRAS) media. Oxygen is excluded from culture medium and the specimen by a controlled stream of gases, which circulates within the culture tube during all manipulations. Both the Hungate and VPI methods allow for the cultivation of strict anaerobes.

The importance of proper collection, transport, and handling of specimens has been emphasized (Ellner et al., 1973; Holdeman and Moore, 1973). Since Loesche (1969) noted the differences in oxygen sensitivities among oral treponemes, more attention has been focused on the procedures used with treponemes. Sensitivity to aerobic environments has been observed for various treponemes (Bryant, 1952; Hanson and Cannefax, 1965; Harris et al., 1972c; Socransky et al., 1969; Smibert, 1971, 1973).

The Eh of a medium limits anaerobic growth and is related to the pH. When bacteria multiply in a static broth culture, the culture becomes reducing due to the accumulation of metabolites. Oxidizing agents are electrophilic, whereas reducing agents tend to give up electrons. If the electron potential is measured with a platinum electrode and a hydrogen reference electrode, electrons will be given up to the platinum electrode and the flow of electrons will

be toward the hydrogen electrode. Because the platinum electrode has a negative potential in relation to the hydrogen electrode, the Eh of the culture will be measured as negative millivolts (-mv). The measurement is an indication of the proportion of oxidized to reduced forms present (Hentges and Maier, 1972). A decrease of one pH unit causes the Eh to become more positive by 57.7 mv (Clark and Cohen, 1923).

As the re-dox potential was increased, Hanke and Bailey (1945) observed that the lag phase for Clostridium welchii also increased. The limiting potential may be influenced by the concentration of NaCl (Mead, 1969); Cl. welchii would grow at Eh values of +194 to +238 at 0.5%, but not 5% NaCl unless the Eh was decreased. O'Brien and Morris (1971) observed the uninhibited growth of Cl. acetobutylicum at an Eh of +340 mv if oxygen was excluded from the medium. Strains of Bacterioides are similarly unaffected by high oxidation-reduction potentials if oxygen is excluded from the growth medium (Hentges and Maier, 1972). The requirement for a low and controlled Eh for the growth of some treponemes has been reported (Socransky et al., 1964).

The reasons why oxygen prevents obligate anaerobes from growing are not known, but there are several theories about anaerobiosis. The mechanism of growth inhibition probably

involves the maintenance of certain enzymes in an oxidized state so that they cannot function in some essential reductive reactions (Davis, 1973). The poisoning activity of oxygen has been attributed to the accumulation of hydrogen peroxide (H_2O_2) because catalase, which destroys H_2O_2, is present only in aerobes; however, some aerotolerant anaerobes also lack catalase and are not poisoned (McCord et al., 1971).

The mechanisms for aerotolerance by some anaerobes are not well established. O'Brien and Morris (1971) have speculated that Clostridium acetobutylicum may tolerate a small amount of oxygen by sacrificing NADH to form water and NAD. If the supply of NADH becomes exhausted, as might occur with a prolonged exposure to oxygen, other metabolic functions which require NADH will stop and growth will cease.

Other findings indicate that, rather than H_2O_2, the metabolic poison is a very reactive free radical of molecular oxygen called superoxide (O_2^-) which results from the univalent reduction of oxygen by flavoproteins. Workers have concluded that superoxide dismutase, which destroys the toxic product by the reaction

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

(McCord and Fridovich, 1968), is present in aerobes and

aerotolerant anaerobes, but not in obligate anaerobes (McCord et al., 1971). Contrary to the conclusions of McCord et al. (1971) and Gregory and Fridovich (1974), superoxide dismutase has been detected in some obligate anaerobes (Yousten et al., 1975). Work with superoxide dismutases has been reviewed (Fridovich, 1974). The enzyme may function similarly in eucaryotes and procaryotes, but the actual role of superoxide dismutase in relation to anaerobiosis remains to be clarified.

Isolation and Propagation of Treponemes

Isolation methods for treponemes have relied upon the small diameters and motility of these organisms (Hardy et al., 1963). Some of the more popular methods used to isolate spirochetes from other bacteria include dilutions (Kast and Kalmer, 1940; Kinyon, 1974) and filtration Chandler and Clark, 1970; Hardy et al., 1964; Harris et al., 1972c; Taylor, 1972; Wichelhausen and Wichelhausen, 1942). Solid isolation media containing polymixin B, nalidixic acid, cycloserine, furacine, or sulfonamides have been used (Smibert, 1973). Bryant (1952) used "spinner tubes" to isolate colonies of a rumen organism. Various plated media have been used to aid separation of treponemes from other anaerobic species (Socransky et al., 1959; Hardy et al., 1963; Harris et al., 1972c).

Kinyon (1974) reported more rapid growth of T. hyodysenteriae on TSA with 5% bovine blood when incubated at 42C than when incubated at 37C. Blood agar with 400 µg/ml spectinomycin incubated at 42C has proved instrumental in the isolation of small numbers of T. hyodysenteriae from contaminating swine flora (Songer et al., 1975) and has greatly simplified the method of isolation previously used (Kinyon, 1974).

Some of the cultivatable treponemes, such as T. denticola (Socransky et al., 1969), do not grow well on solid media; however, colonial growth of other treponemes has been described (Hanson and Cannefax, 1965; Hardy et al., 1963; Harris et al., 1972c). Hanson and Cannefax (1965) have described five types of colonial morphology in four cultures of Treponema. The appearance of growth and hemolysis by T. hyodysenteriae grown on solid medium has been described (Harris et al., 1972c). This organism has been isolated on agar medium supplemented with blood from bovine, horse, sheep (Taylor and Alexander, 1971); porcine, rabbit, and human (Kinyon, 1974) sources. The hemolysis of sheep blood by a dysentery isolate has been described as a double zone, with more complete hemolysis in the inner zone. It has also been observed that swine dysentery isolates exhibit a more complete hemolysis of blood than isolates from nondysentery sources (Kinyon, 1974; Taylor, 1972).

Media used for the cultivation of avirulent treponemes have been complex in composition. The media generally consisted of peptones, a reducing agent such as thioglycollate, and tissue extracts or serum (Little and Subbarow, 1945; Wilcox and Guthe, 1966). Other animal fluids such as plasma, ascitic, and hydrocoelic fluids were also used by early workers in unsuccessful attempts to cultivate virulent T. pallidum (Kast and Kolmer, 1940). Little and Subbarow (1945) cultivated the avirulent Reiter treponeme with the albumin fraction of serum as a substitute for whole serum in a liver extract medium.

Steinman and others (1952, 1953, 1954) have grown the Reiter treponeme in a medium of 13 amino acids, three vitamines, a pyrimidine, glucose, inorganic salts, and serum albumin. Allen et al. (1971) have cultured the avirulent organism in thioglycollate medium supplemented with 10% rabbit serum.

Various researchers have attempted to use serum substitutes as growth factors. Davis and Dubos (1947) and Oyama et al. (1953) showed that albumin is a detoxifying lipid carrier. The essential growth factor for the Reiter strain was replaced with oleic acid or Tween 80 (Oyama et al., 1953). At pH 7.4 a lipid:protein ratio of 4:1 gave optimal growth. Ratios greater than 4:1 resulted in lipid toxicity. Crystalline β-lactalbumin was found to have

the same qualitative ability as serum albumin in the promotion of growth; neither protein nor the fatty acid supplement alone could replace the serum requirement. Socransky and Hubersak (1967) found that the α-2 globulin fraction would replace the serum requirement of T. macrodentium, whereas the albumin fraction was a poor substitute. In 1967, Rajkovic reported the cultivation of T. refringens biotype noguchii in a serum and albumin-free medium. Apparently this treponeme could tolerate the low concentration of Tween 80 used in the medium without the benefit of a detoxifying protein.

In addition to preference for animal sera or preparations of serum albumin as media supplements, yeast extract has been often included as a source of required vitamins and cofactors (Steinman et al., 1954; Smibert and Claterbaugh, 1972). Smibert and Claterbaugh (1972) reported a semidefined medium for a small spirochete isolated from a case of swine dysentery. The organism (PR-7) required nine vitamins and cofactors, a fermentable carbohydrate, heme, ammonium sulfate, and carbon dioxide. Isobutyrate, n-valerate, acetate, and pyruvate were also required.

It was not possible to cultivate isolates of organisms similar to T. hyodysenteriae in anaerobic media such as PY, PY+glucose, E, and thioglycollate; supplemented with bovine blood or plasma, rabbit serum, TEM-4T, or preparations of

filtered colonic mucosa (Kinyon, 1974). Harris et al. (1972c) noted that the spirochetes would not grow in tryptose broth. However, hydrogen stimulation of growth was observed and the organisms were propagated in unbuffered medium supplemented with fetal calf serum in an anaerobic atmosphere of hydrogen and carbon dioxide (Kinyon and Harris, 1974). Because T. hyodysenteriae would not grow in medium supplemented with rumen fluid (Kinyon, 1974), the probable nutritional value of serum is presumed to be long-chained fatty acids.

MATERIALS AND METHODS

General Culture Media

Solid

Trypticase soy agar[1] prepared as 2% and 4% (W/V) solutions was sterilized in an autoclave (15 minutes at 121C) and cooled in a 50C water bath. The medium was supplemented with 5% (V/V) citrated bovine blood (1 g citrate/100 ml blood) just before plates were poured. Bovine blood obtained from the same animal[2] was stored at 4C and used within one month after collection. Fresh blood agar plates (4% W/V) were used within 12 hours post preparation for viability determinations in quantitative growth studies. For routine determinations, any plates used 12 hours post preparation were held reduced in GasPak jars[1] with H_2:CO_2 (Brewer and Allgier, 1966) generator envelopes[1] and used within 24 hours.

Liquid

Thioglycollate[3] broth prepared according to the manufacturer's directions was dispensed in 7 ml amounts in screw capped tubes and autoclaved.

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[1]BBL, Division of Bioquest, Cockeysville, Maryland.

[2]Iowa State University, Veterinary Clinic Hospital.

[3]Difco Laboratories, Detroit, Michigan.

Trypticase soy broth without dextrose (TSB)[1] was prepared aerobically (Kinyon and Harris, 1974) and by the prereduced anaerobically sterilized method (Holdeman and Moore, 1973) without cysteine or resazurin (PRAS-CF-TSB). All media prepared by these methods were autoclaved in presses[2] for 15 minutes at 121C. The final pH was determined by immersion of a single pH probe[3] into randomly selected tubes; media ± 0.05 of the desired pH were used.

Unbuffered aerobic TSB was the general basal medium used to propagate and study growth responses of T. hyodysenteriae. Powdered medium was rehydrated according to the manufacturer's directions in 300 ml glass distilled H_2O[4] in a 1000 ml Erlenmeyer flask. The desired pH was adjusted with 8M NaOH and 5M HCl. The medium was heated with stirring and boiled for 10 minutes; it was cooled at room temperature for 15 minutes and then pipetted in 5, 8, or 10 ml amounts to stoppered anaerobe tubes[2] (18 x 142 mm).

The PRAS-CF-TSB was similarly prepared with glass distilled H_2O, in 300 ml amounts in a 500 ml round-bottom

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[1]Lot No. 11774, BBL, Division of Bioquest, Cockeysville, Maryland.

[2]Bellco Glass Inc., Vineland, New Jersey.

[3]Coleman Instruments, Maywood, Illinois.

[4]Bellco Glass Inc., Vineland, New Jersey; kindly supplied by Dr. H. C. Ellinghausen, NADC, Ames, Iowa.

flask, mixed, and boiled. The medium was cooled in an ice bath and bubbled with a stream of deoxygenated CO_2. The pH was adjusted to approximately 0.5 unit less than the pH desired; gas flow was changed to deoxygenated N_2 and the medium was automatically dispensed[1] in the desired amounts to stoppered anaerobe tubes.

Deoxygenation of gas was accomplished by passing it through a glass column filled with copper turnings heated to 350C. Addition of supplements and inoculation of sterile medium was done anaerobically under a flow of deoxygenated gas[2] using an anaerobic inoculator and the techniques previously described by Holdeman and Moore (1973).

Modified basal liquid media

Growth of T. hyodysenteriae in modifications of trypticase soy basal medium was studied. Modifications of the basal medium included a) a decreased concentration of TSB, b) two alternate methods of preparation, c) the two major components of TSB as basal medium, d) medium to which various salts solutions and buffer solutions were added, and e) various serum supplements.

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[1]Bellco Glass Inc., Vineland, New Jersey.

[2]Matheson Gas Products, Division of Will Ross, Inc., Joliet, Illinois.

Decreased concentration of TSB

Aerobic trypticase soy medium was prepared as one-half the concentration (½X TSB) recommended by the manufacturer. Sterile Na_2CO_3 equilibrated with deoxygenated CO_2 was aseptically added before inoculation.

Alternate methods of preparation of TSB

Aerobic medium (TSB-M) was prepared by combining the major components in commercial TSB as follows: 5.1 g trypticase, 0.9 g phytone, 0.75 g KH_2PO_4, 1.5 g NaCl in 300 ml glass distilled H_2O.

Filtered and gassed trypticase soy broth (FG-TSB) was prepared by dissolving 8.25 g powder in 300 ml warm glass distilled H_2O that had been boiled for 10 minutes and cooled for 15 minutes; the pH was adjusted to 7.5. The solution was passed through a 0.45 μm filter[1] flushed with deoxygenated N_2, via application of 3 lbs vacuum and 2 lbs pressure N_2. The sterile medium was aseptically collected in 50 ml syringes and transferred under a flow of a deoxygenated mixture of 50% H_2 and 50% CO_2 (H_2:CO_2) to a sterile 500 ml Erlenmeyer flask which was closed with a vented rubber stopper. The medium was bubbled for 20 minutes with deoxygenated H_2:CO_2 that was filtered through

[1]Falcon Plastics, Division of Becton, Dickinson and Co., Los Angeles, California.

a 0.45 μm cellulose acetate filter[1]. The flask was supported in a slanted position in an open-front hood that had a positive flow of filtered air. Medium was aseptically pipetted in 8 ml amounts to sterile anaerobe tubes which were held and stoppered under a flow of deoxygenated H_2:CO_2.

Trypticase and phytone media

Aerobic trypticase[2] and PRAS-CF trypticase were prepared with 5.1 g and 10.2 g (2X) trypticase in 300 ml glass distilled H_2O. These preparations were made with and without 0.75 g KH_2PO_4 and 1.5 g NaCl.

Aerobic phytone[2] and PRAS-CF phytone were prepared with 0.9 g and 1.8 g (2X) phytone in 300 ml glass distilled H_2O. These preparations were made with and without 0.75 g KH_2PO_4 and 1.5 g NaCl.

Buffered TSB

Aerobic TSB with 4 ml VPI salts (Holdeman and Moore, 1973) per 100 ml medium was prepared with 1.7 g trypticase and 0.3 g phytone. The salts solution consisted of the

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[1]Millipore Corporation, Bedford, Massachusetts.

[2]BBL, Division of Bioquest, Cockeysville, Maryland.

following components in g/100 ml:

$CaCl_2$	0.02
$MgSO_4 \cdot 7H_2O$	0.02
K_2HPO_4	0.10
KH_2PO_4	0.10
NaCl	0.20

Sterile sodium carbonate was added prior to inoculation.

Aerobic TSB with Medium-10 salts (Caldwell and Bryant, 1966) was prepared with 1.7 g trypticase, 0.3 g phytone, 4 ml solution A, and 4 ml solution B per 100 ml glass distilled H_2O.

Solution A: 0.6 g K_2HPO_4 in 100 ml

Solution B: in g/100 ml

KH_2PO_4	0.6
$(NH_4)_2SO_4$	0.6
NaCl	1.2
$MgSO_4 \cdot 7H_2O$	0.25

Aerobic TSB with Bryant's salts (Bryant, 1974) was prepared with 1.7 g trypticase, 0.3 g phytone, 0.04 g $(NH_4)_2SO_4$, 0.0004 g $FeSO_4 \cdot 7H_2O$, and 0.5 ml mineral solution per 100 ml glass distilled H_2O.

Mineral solution: in g/100 ml

KH_2PO_4	1.8
NaCl	1.8

$CaCl_2 \cdot 6H_2O$	0.053
$MnCl_2 \cdot 4H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	0.002

Salts and buffer solutions

Several different salt solutions and buffers were prepared and used as follows (Livermore, 1974):

S-1 solution: in g/100 ml glass distilled H_2O

tetrasodiumethylene - diaminetetracetate (EDTA)[1]	0.2
$CaCl_2$	0.75
$MgCl_2$	2.5
$FeSO_4 \cdot 7H_2O$	0.1

S-z solution: in g/100 ml glass distilled H_2O

NH_4Cl	0.1
KH_2PO_4	0.1
$MgSO_4$	0.05
$CaCl_2$	0.05

The S-1 solution was used as 0.1% and 0.2% final concentrations with sterile 1M phosphate buffer (pH 7.4) which was added prior to inoculation to final concentrations of 2×10^{-2}M or 6×10^{-2}M. The S-1 solution was also used with

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

sterile 0.01M tris(hydroxymethyl) aminomethane[1] buffer (pH 7.4). The S-z solution was used as 0.1% and 0.2% final concentrations with filtered sterilized $NaHCO_3$ and CO_2-equilibrated Na_2CO_3 in final concentrations of 0.05% and 0.2%, respectively.

T-sp solution: in g/100 ml of glass distilled H_2O

$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.13
NaCl	1.0
$(NH_4)_2SO_4$	5.0

The T-sp solution was used as 0.1% and 0.5% final concentrations with 0.1 ml/10 ml medium of 1M phosphate-5% $NaHCO_3$ buffer (pH 7.4). The buffer was added prior to inoculation and included the following components in g/100 ml glass distilled H_2O:

KH_2PO_4	13.93
K_2HPO_4	2.73
$NaHCO_3$	5.0

Sodium carbonate[2] was anaerobically prepared as a 10% solution by a method similar to that described by

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[1]Eastman Kodak Co., Rochester, New York.

[2]Fisher Scientific Co., Fair Lawn, New Jersey.

Bryant and Burkey (1953). Approximately 30 g Na_2CO_3 per 300 ml glass distilled H_2O were heated in a 500 ml round-bottom flask. Deoxygenated N_2 was circulated above the solution while it was boiled for 10 minutes. The solution was allowed to cool three minutes with circulated N_2, rubber-stoppered and autoclaved. After autoclaving, the buffer was cooled to room temperature, bubbled for 15 minutes with deoxygenated CO_2, and pipetted in 1 ml amounts to sterile rubber-stoppered tubes under a flow of deoxygenated CO_2.

The diluent used for viability determinations and direct total counts was 0.01M phosphate buffered saline (PBS), pH 7.2-7.4 (Kinyon, 1974). The PBS was prepared by combining 85.7 ml 0.1M Na_2HPO_4 with 14.3 ml 0.1M KH_2PO_4 and 900 ml 0.85% NaCl and then dispensed in 9 ml amounts in screw-capped tubes and autoclaved.

Serum and medium supplements

Sterile fetal calf serum (FCS)[1] was obtained in 500 ml amounts; dialyzed fetal calf serum (D-FCS)[1] and immunoprecipitin tested fetal calf serum (GG-free FCS)[1] were obtained in 100 ml amounts. Sera were dispensed into sterile screw-capped tubes by means of a sterile Cornwall syringe.

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[1]Grand Island Biological Co., Grand Island, New York.

The tubes of sera were incubated 12 hours at 37C to check for contamination; sterile sera were stored at -20C until used and added to sterile medium at the time of inoculation.

Waymouth medium with 5 μg per ml of Tween 80 (Morrison and Jenkin, 1972) (Wo_5),[1] Wo_5 with 2000 μg bovine serum albumin per ml ($Wo_5 + BSA_{2000}$),[1] lactalbumin hydrolysate with Hank's salt solution (LHL),[2] and NCTC-135 Medium[2] were prepared by pregassing 100 ml amounts in 100 ml sterile bottles as described for FG-TSB. Wo_5 medium was prepared with and without 50 mg cysteine hydrochloride[3] per 100 ml medium.

Minimal Eagle's medium F-15 (MEM)[2] was prepared in 100 ml amounts according to the manufacturer's directions as single strength (1X) and 10X with 1X buffer. The medium was filter sterilized and gassed as described for FG-TSB.

The protocol used for testing combinations of FCS and semidefined substitutes with 10X MEM was as follows:

ml 10X MEM base	5	4	3	2	1
FCS	0.5	1.0	2.0	3.0	4.0
Wo_5	0.5	1.0	2.0	3.0	4.0
$Wo_5 + BSA_{2000}$	0.5	1.0	2.0	3.0	4.0
TSB pH 8.1	0.5	1.0	2.0	3.0	4.0

[1]Kindly supplied by Dr. H. M. Jenkin, The Hormel Institute, Austin, Minneosta.

[2]Grand Island Biological Co., Grand Island, New York.

[3]Sigma Chemical Co., St. Louis, Missouri.

Wo_5 + BSA_{2000} was similarly used as the base in combinations with FSC, Wo_5 and TSB pH 8.1.

Fatty acid poor bovine albumin (FP-BSA) and Fraction V Pentex (BSA) powders[1] were prepared as 20% solutions in glass distilled H_2O by gentle mixing at 16C until powders were completely dissolved; the pH was adjusted to 7.4. Albumin solutions were sterilized by filteration through 0.45 μm cellulose acetate filters. Albumins were used as 1% final concentrations or mixed with 10% sterile stock solutions of Tweens[2] 80, 60, and 40 to give final concentrations of 1% albumin, and 0.05%, 0.1%, or 0.15% of a Tween per 100 ml medium.

Short-chained fatty acids were mixed as follows (Livermore, 1974): 1 ml each of isobutryic,[3] n-valeric,[2] and n-caproic[3] acids dissolved in 100 ml glass distilled H_2O. The autoclaved mixture was used at final concentrations of 0.05%, 0.1%, or 0.15% with and without 1% BSA per 10 ml medium.

Filter sterilized 10% solutions of glucose, lactose, fructose, and maltose were used as supplements at 0.1% and 0.2% final concentrations per 10 ml medium.

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[1]Miles Laboratories, Inc., Elkhart, Indiana.

[2]Sigma Chemical Co., St. Louis, Missouri.

[3]J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Growth Studies

Bacteria

The two virulent isolates of T. hyodysenteriae used in these studies were kindly supplied by J. M. Kinyon (Iowa State University). Isolate B140 was recovered from a dysentery pig raised in Minnesota and isolate B204 was recovered from a dysentery pig raised in Iowa; both have been shown to be pathogenic for specific pathogen-free (SPF) swine (Kinyon, 1974). Frozen cultures were rechecked for purity on blood agar and in thioglycollate broth. Zones of growth on blood agar were transferred as agar plugs to aerobic TSB supplemented with 10% (V/V) FCS. Gas-producing cultures 24-36 hours old were frozen at -80C under deoxygenated H_2:CO_2 in 1 ml amounts in sterile rubber-stoppered tubes. These frozen stock cultures were thawed at 37C-39C and transferred directly to aerobic TSB supplemented with 10% FCS (TSB-FCS) under deoxygenated H_2:CO_2. Cultures were incubated in slanted racks at 37C-39C in circulated air incubators (28"x18"x18").

Inoculum

Studies were performed with cultures between 10-20 in vitro passages. Isolate B204 was used for the majority of the growth studies. For general growth studies, the inoculum was serial passaged 3-4 times in TSB-FCS under

the conditions to be studied before data were collected. For studies on growth conditions, inoculum was passaged once in TSB pH 7.0 or 7.5 with 8% FCS at 37C-39C and then directly to the experimental conditions. Inoculum used for substitution studies was from a culture of isolate B204 transferred to aerobic medium without FCS to minimize carry-over of serum. Tubes of control medium with and without FCS were also inoculated. Inoculum was routinely checked for contamination by inoculating thioglycollate broth after transfers were made. Wet mounts of inoculum and cultures were observed for the presence of T. hyodysenteriae and for contaminants by use of a phase microscope[1] (Leitz, with Heine condenser) at a magnification of 630X.

Viability determinations

Estimates of viable cell numbers in inoculum were routinely made at the time of transfer. One ml of culture was serially diluted ten-fold in PBS to 10^{-6}. Samples were thoroughly streaked onto one quarter of a blood agar plate as 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} dilutions with a 0.01 ml calibrated platinum loop (Kinyon, 1974). Each dilution series was plated in duplicate. Plates were immediately incubated in 80% H_2 and 20% CO_2 with 5 lbs vacuum in vented

[1]Ernst Leitz, Wetzlar, West Germany.

GasPak jars with cold palladium catalyst at 37C or 42C. The atmosphere was obtained by evacuating the jars three times to 25 lbs vacuum and filling with H_2. The jars were evacuated to 25 lbs vacuum once more, filled with H_2 to 10 lbs vacuum, then filled to 5 lbs vacuum with CO_2 (Kinyon, 1974). Methylene blue indicators were observed to be colorless. GasPak jars were opened every two days and plates were examined for growth evidenced by areas or zones of hemolysis. Plates were placed in the 80% H_2 and 20% CO_2 atmosphere and incubated within 1-2 hours after opening.

Total cell counts

Estimates of total cell numbers were done by direct counts under dark-field microscopy[1] at a magnification of 450X in Petroff-Hausser counting chambers[2] using standard techniques. Generally counts were made from the 1:10 dilution of a PBS dilution series which was also used for a viability determination. Counts were done in duplicate or triplicate and the average number of cells in the sample was determined.

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[1]Ernst Leitz, Wetzlar, West Germany.

[2]Hausser Scientific, Blue Bell, Pennsylvania.

Nephelometer readings

The nephelometer readings were performed by means of a Coleman 7 nephelometer.[1] The instrument was adjusted to zero with uninoculated TSB-FCS or appropriate medium and calibrated to 84 with an Ellinghausen 28 turbidity standard[2] (Roessler and Brewer, 1967). For experiments employing standardized inoculum, the culture was adjusted to the desired nephelometer readings and the total cell number of the 1:10 dilution in PBS was determined; with a Petroff-Hausser chamber; an estimate of the viable cell numbers was also made. The adjusted inoculum was then diluted ten-fold in series of medium to be tested and incubated at the appropriate temperature. Thus, four methods of quantitation were used to verify the quality of inoculum within the limits of accuracy imposed by ten-fold dilutions and initial sampling of the culture.

Standardization of inoculum

Cultures initiated with identical inoculum were incubated at 37C; nephelometer readings were observed periodically. Five of the cultures were selected at nephelometer readings of 30, 40, 50, 60 and 70 units; these were

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[1]Coleman Instruments, Maywood, Illinois.

[2]Kindly supplied by Dr. H. C. Ellinghausen, NADC, Ames, Iowa.

standardized to readings of 30 nephelometer units and total cell counts were done immediately. Standardization of 10 ml inoculum was accomplished by diluting the cultures with aerobic TSB in the proportions indicated by a simple formula:

$$\frac{\text{culture reading}}{20} = X; \quad \frac{10}{X} = \text{ml culture};$$

$$10 - \frac{10}{X} = \text{ml diluent.}$$

Standardized inocula (10%, V/V) were transferred to three culture tubes of TSB supplemented with 10% (V/V) FCS, and then were incubated at 37C. Nephelometer readings of each group were made approximately every five hours for three days.

The average readings for each group of three cultures were plotted versus time of incubation. The data were subjected by computer to a regression analysis (Steele and Torrie, 1960) and the average slopes representing the cultures initiated with five different sources of standardized inocula were compared to determine if differences in growth rates had resulted due to the sources of inoculum.

Development of a method for optimization of growth

Treponema hyodysenteriae isolates B140 and B204 were transferred to TSB-FCS at initial pH values of 5.4, 6.0, 6.5, 7.0, 7.5 and 8.0. Replicate sets of cultures were

incubated at 34C, 37C, 39C, and 42C. Nephelometer readings of maximum growth that occurred in medium at different pH values were determined; maxima for cultures incubated at 37C and 39C were graphed for each pH. Viability determinations of cultures used for inoculum were performed; plates for corresponding sets of cultures were incubated at 37C, 39C, or 42C.

A 10% (V/V) pooled inoculum of isolate B140 was transferred to TSB-FCS at initial pH values of 6.5, 6.8, 7.0, 7.25, 7.5, 7.8, and 8.0. Duplicate sets of cultures were incubated at 37C and at 39C. Maximum growth of cultures was determined by nephelometry and the decreases of the pH of medium were determined. A 10% (V/V) pooled inoculum of isolate B204 was passaged to TSB-FCS at initial pH values 6.75, 7.0, 7.25, 7.5, 7.75, 8.1, 8.25, and 8.6 for four serial passages. Duplicate sets of cultures were incubated at 37C and at 39C. Decreases in the pH values of the medium were determined during incubation. Nephelometer readings and viable determinations of the cultures were performed. Culture plates were incubated at 37C or at 39C. Nephelometer readings of several cultures were plotted versus time to determine the effect of pH the medium on growth.

Isolate B204 was passaged in TSB with FCS at initial pH 7.5 and incubated at 42C, 38C, and 34C. Total cell

counts or viability determinations were performed on standardized inocula. Inoculum derived from a 10^{-2} dilution was serially diluted 1:10 in TSB supplemented with 8% FCS. Each series was incubated for seven days and data from four passages of duplicate series incubated at the three different temperatures were summarized for comparison.

An experiment to compare growth in TSB with 8% FCS at initial pH values 6.5, 7.0, 7.5, 8.0 and 8.5 incubated at 38C was similarly conducted. Standardized inocula derived from a 10^{-2} dilution were employed and each dilution series was done in duplicate. Data from four passages were pooled and the effects on the initiation of growth in TSB with 8% FCS at the different pH values were compared.

Growth responses

The growth of isolate B204 in aerobic TSB and in PRAS-TSB supplemented with 10% FCS under CO_2 and under H_2:CO_2 were compared for six passages with 10% (V/V) inoculum. The average nephelometer readings for two passages of duplicate cultures were plotted for comparison of the growth responses related to the anaerobic atmospheres. Subsequent serial passages under these cultural conditions were made.

The total protein of fetal calf serum routinely used to supplement TSB was determined by the method of Lowry et

al. (1951). The effects upon growth of cultures supplemented with 10% fetal calf serum heated at 80C and at 56C for 15, 30, 45, 60, and 90 minutes were determined. Serum heated to approximately 100C for 5, 10, 15, 20, and 30 minutes was centrifuged at 10,000 xG at 4C for 60 minutes. Cultures in TSB supplemented with 1% FCS were transferred to TSB supplemented with 1% of filter sterilized supernatant.

Isolate B204 inoculum was serially diluted in TSB supplemented with 6%, 5%, 4%, 3%, 2%, 1% FCS and in unsupplemented TSB. Cultures were observed for growth for a period of five days.

Growth at 38C of serially diluted inoculum in medium supplemented with 10% GG-free and 10% D-FCS sera was compared to growth in medium supplemented with untreated fetal calf serum. Cultures were observed for growth for a period of five days.

Transmission

Four six-week-old SPF Duroc swine were obtained from Sam Kennedy, Clear Lake, Iowa. The herd has been free of swine dysentery for seven years and uses drug-free feed. The pigs were paired and housed in two prefumigated concrete isolation pens at a temperature of 25-30C and fed a ration of pig grower (16%) protein daily for six days. Water was

available ad libitum throughout the experiment.

Feed was withheld for 24-36 hours prior to inoculation. Two pigs were inoculated via stomach tube once a day for two days with 50 ml of a 24-36 hour culture of isolate B204 at 20 in vitro passages. The other two pigs were held as uninoculated control animals. The inoculum was grown in TSB at initial pH 7.5, supplemented with 8% FCS and incubated at 38C. After the second inoculation the pigs were again fed a daily ration of feed for the remainder of the study.

The pigs were observed daily for fecal consistency (normal, soft, loose, or watery); fecal composition (normal, mucus, blood, or mucus and blood); general condition (normal, depressed, gaunt, or moribund). Wet mounts in PBS of rectal swab specimens were observed by phase microscopy for the presence of T. hyodysenteriae, Vibrio, or small spirochetes in the feces before inoculation and periodically after inoculation. Rectal swab specimens were collected on sterile, dry, cotton, tipped applicators which were immersed in 0.5 ml PBS and held at 4C until observations were made.

After 40 days the experiment was terminated. The pigs were electrocuted, then exsanguinated. Postmortem observations of stomach, small intestine, colon, cecum, rectum, and other organs were recorded.

Scrapings of the colonic mucosa were observed by phase microscopy and streaked on blood agar containing 400 μg per ml spectinomycin (Songer et al., 1975) and on tergitol-7[1] medium. Samples of colonic tissue were added to 20 ml tetrathionate[1] broth in screw-capped tubes and subsequently streaked on brilliant green agar.

The blood agar plates were incubated in 80% H_2:20% CO_2 atmosphere at 42C; the tergitol-7, tetrathionate broth, and brilliant green cultures were incubated aerobically at 37C for detection of Salmonella spp.

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[1]Difco Laboratories, Detroit, Michigan.

RESULTS

Quantitative Methods

Correlation of nephelometry, direct total cell counts, and viability determinations

The nephelometer readings for growth responses of three cultures were determined at three-hour intervals during 70 hours incubation at 37C. Direct total cell counts and viability determinations were done at 6-8 hour intervals during 96 hours incubation. Total numbers were similar to viable numbers until approximately 60 hours incubation after which the viable numbers decreased (Table 1).

Data to determine the relationships among the three methods used to quantitate the growth of T. hyodysenteriae in three replicate cultures are presented in two conventional forms. Figure 1 shows that viable cell determinations are parallel to corresponding total cell counts and that these values do not have a linear relationship to an arithmetic plot of nephelometer readings.

A linear relationship of the $\log_{10}$ of total cell numbers or $\log_{10}$ of viability determinations is observed when cell mass measured in nephelometric units is plotted as a logarithmic function (Figure 2).

Figure 3 shows the parallel relationship of nephelometer readings to total cell numbers when data are plotted

Table 1. The increase of total cell numbers per ml and viable cell numbers per ml determined for 3 replicate cultures of *T. hyodysenteriae* isolate B204

Incubation Time in Hours	Average Total Cell Numbers	Average Viable Cell Numbers	Range of Total Cell Numbers[a]		Range of Viable Cell Numbers[b]	
			Minimum	Maximum	Minimum	Maximum
0	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
6	1.6×10^7	1.3×10^7	1.5×10^7	1.8×10^7	8×10^6	2×10^7
12	1.7×10^7	1.8×10^7	2×10^7	2.1×10^7	1×10^7	3×10^7
18	3.1×10^7	1.9×10^7	2.7×10^7	3.7×10^7	1.5×10^7	3×10^7
24	3.8×10^7	4.4×10^7 (2)[c]	3.3×10^7	4×10^7	1.5×10^7	5×10^7
30	5.3×10^7	5×10^7	5.1×10^7	5.8×10^7	4×10^7	6×10^7
36	7.6×10^7	5×10^7	6.9×10^7	7.5×10^7	4×10^7	6×10^7
48	8.5×10^7	3.5×10^7 (2)	8×10^7	9.3×10^7	1×10^7	6×10^7
54	1.1×10^8 (1)[c]	8.3×10^7	1.1×10^8	1.1×10^8	4×10^7	2×10^8
60	1.2×10^8	1.4×10^8	1.1×10^8	1.2×10^8	1×10^8	2×10^8
72	1.4×10^8 (1)	7×10^7	1.4×10^8	1.4×10^8	4×10^7	9×10^7
78	1.5×10^8 (2)	8.5×10^7	1.4×10^8	1.6×10^8	7×10^7	1×10^8
90	1.4×10^8 (2)	8×10^7	1.4×10^8	1.4×10^8	7×10^7	9×10^7
96	1.4×10^8 (2)	1.5×10^7	1.4×10^8	1.4×10^8	4×10^6	3×10^7

[a]Ranges are ± 25% of the average.

[b]Ranges are ± 50% of the average.

[c]Parentheses designate number of samples other than 3.

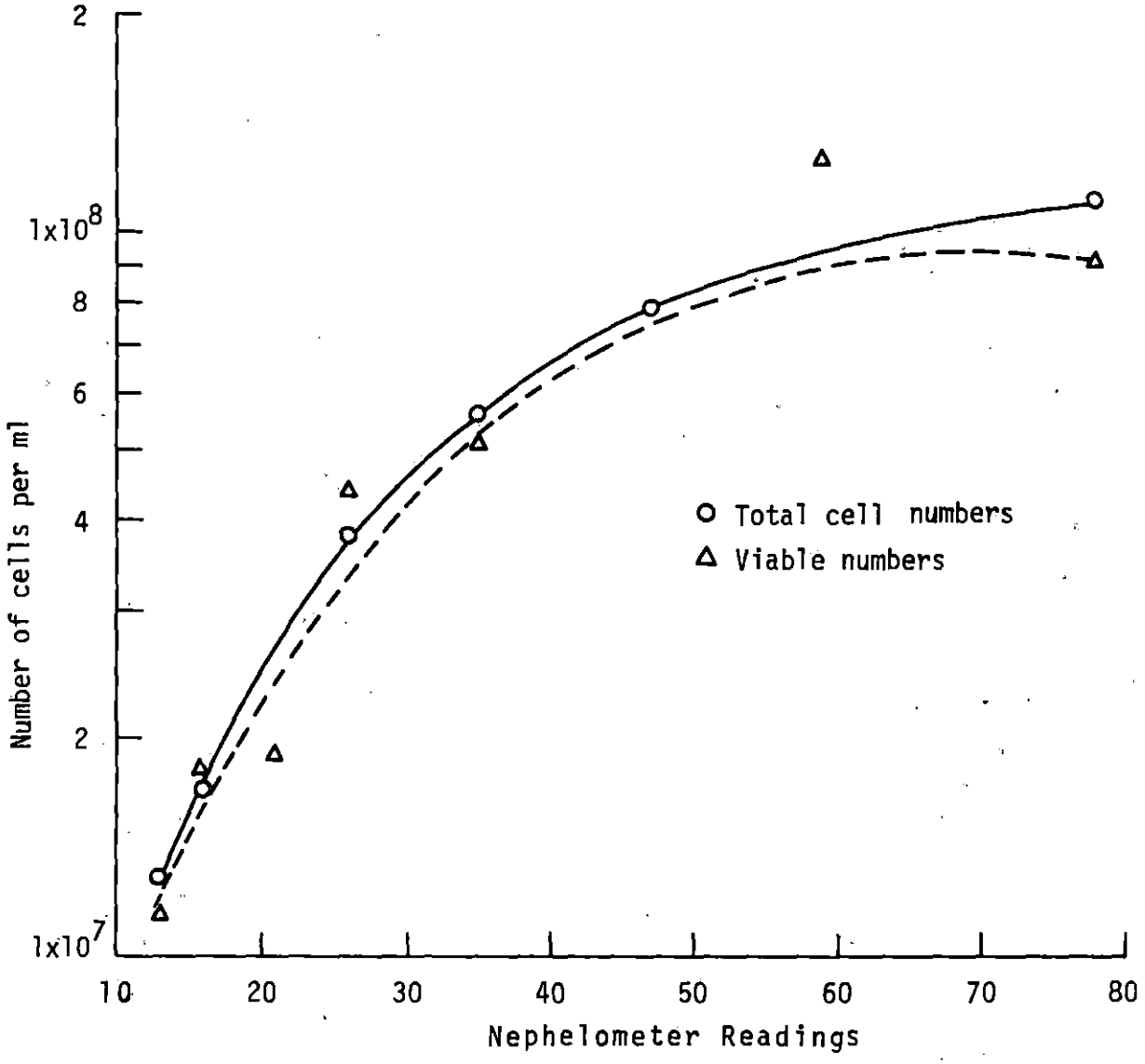


Figure 1. Relationship between log numbers of cells and an arithmetic scale of corresponding nephelometer readings during growth of T. hyodysenteriae. Data indicate the mean values

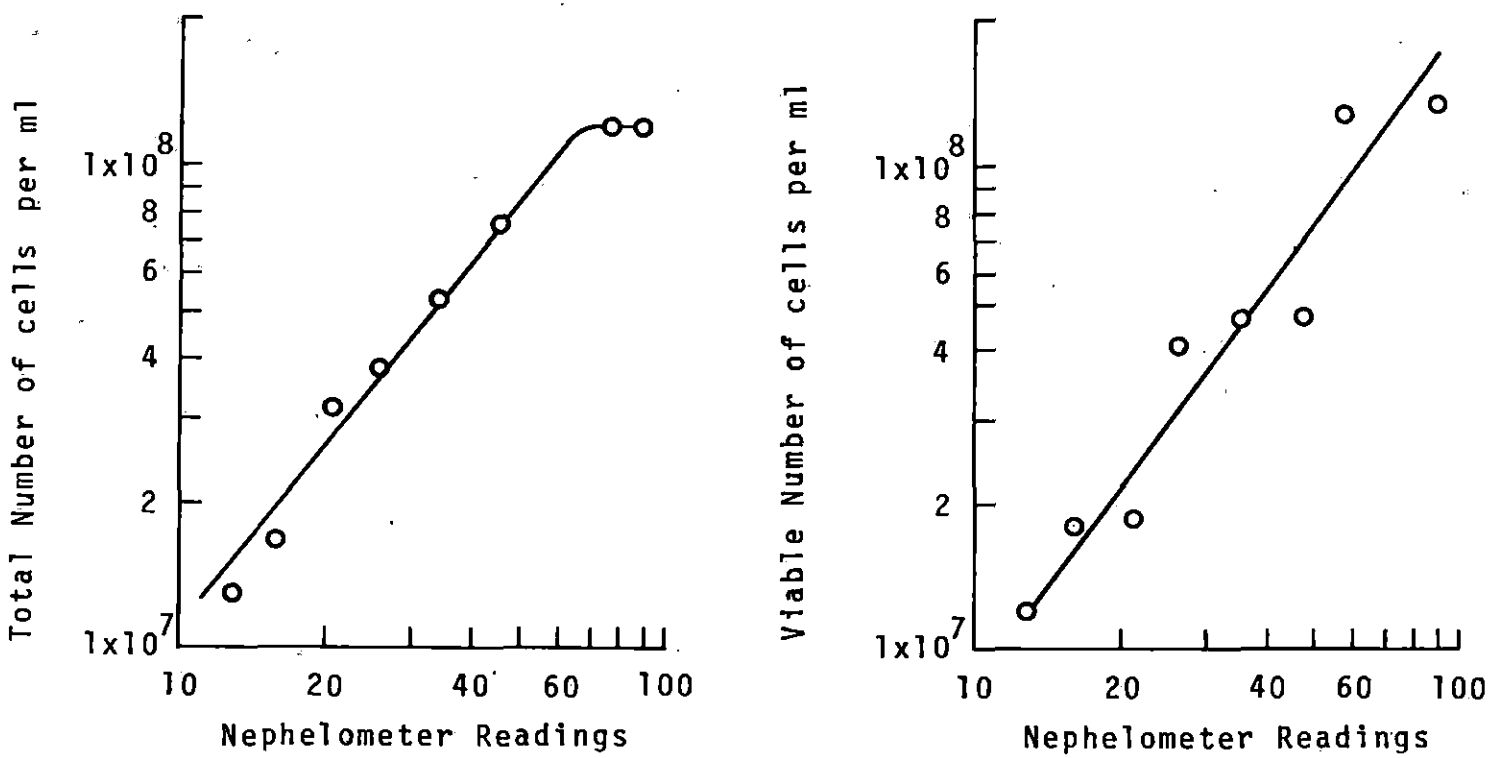


Figure 2. Relationships of total numbers and viable numbers of cells to a logarithmic scale of corresponding nephelometer readings. Data indicate the mean values

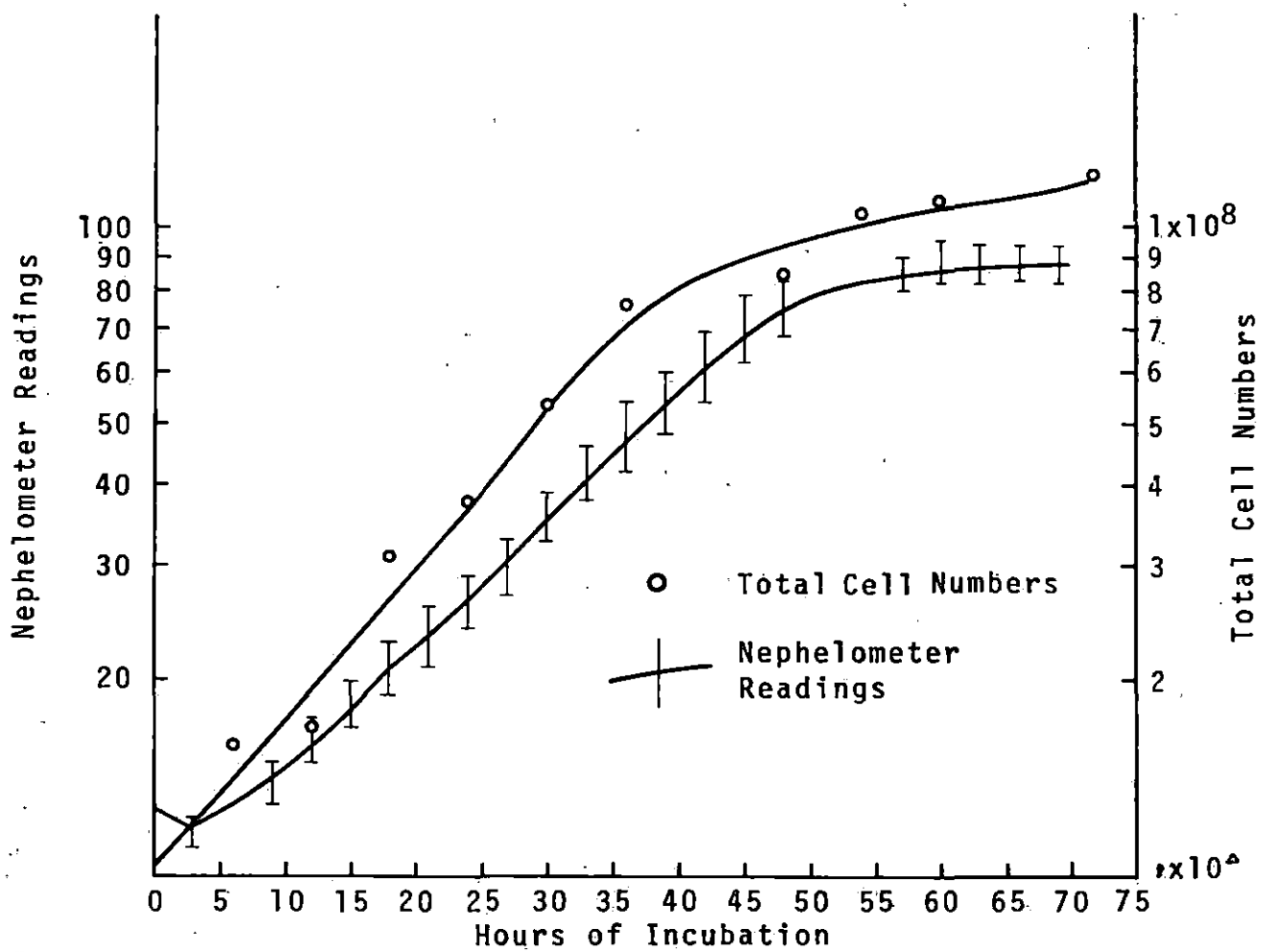


Figure 3. Relationship of total cell numbers to corresponding nephelometer readings versus hours of incubation. Bars indicate ranges and the solid line represents mean values of nephelometer readings. Data indicate mean values of total cells per ml with the best fit line

on logarithmic scales versus time. Doubling time as indicated by either method is approximately 12 hours.

A growth curve for T. hyodysenteriae measured by nephelometry is given in Figure 4.

These data clearly indicate a direct and linear relationship between total cell numbers determined by direct cell counts and the total cell mass determined by nephelometer readings. In this manuscript, trends of growth will be shown by arithmetic plots of nephelometer readings versus time, thus showing real differences in measurements of cell mass.

Standardization of inoculum from different culture densities measured in nephelometer units

Cultures used for inoculum were transferred after 28-40 hours incubation at 37C at nephelometer readings of 30, 40, 50, 60, or 70. The quality of the standardized inocula is presented in Table 2. Growth patterns of three replicate tubes initiated from the five different inoculum sources are shown in Figure 5. The graphs indicate an average maximum nephelometer range of 63-68. All five groups exhibited a lag within 0-15 hours and doubling of cell mass occurred every 10 hours (Figure 6). Increases in the cell mass measured nephelometrically terminated between 45-52 hours incubation for four of the five groups. The set of cultures

Figure 4. Growth curve for three cultures of T. hyodysenteriae. Bars indicate the ranges; the solid line represents mean values of nephelometer readings for three cultures

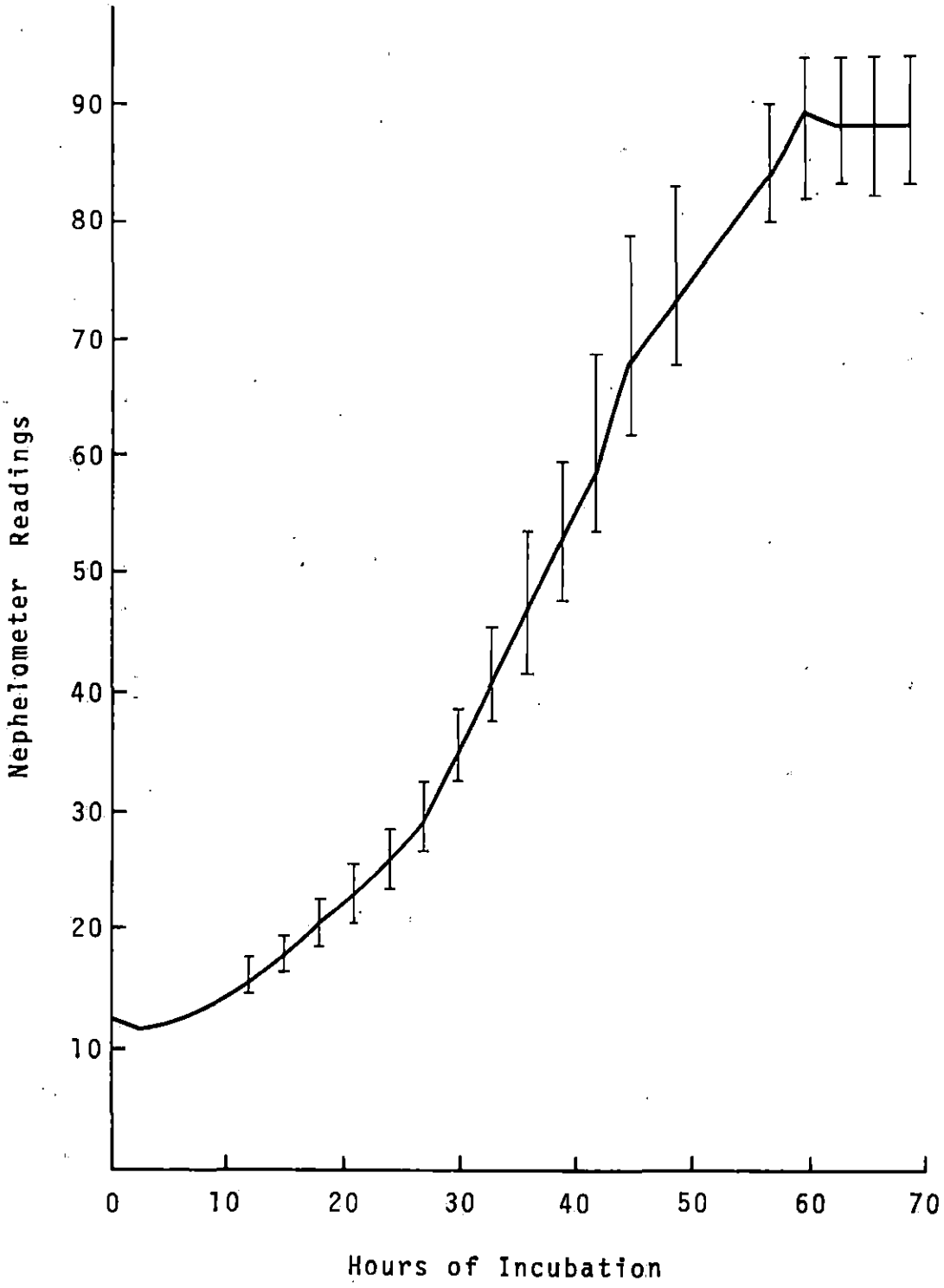

Nephelometer Readings
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
Hours of Incubation

Table 2. Quality of standardized inoculum of T. hyodysenteriae isolate B204 from cultures with different nephelometer readings

Culture	Age in Hours	Nephelometer Readings	Dilution Ratio	Total cell Numbers
1	28	30/30[a]	10:0[b]	5×10^{7}[c]
2	33	40/30	5:1.5	7.4×10^{7}
3	36.5	50/30	4:3	8.6×10^{7}
4	38	60/30	3.5:3.5	7.5×10^{7}
5	40	70/30	3:4	1×10^{8}

[a]Numerator designates nephelometer reading of the culture; denominator designates nephelometer reading of the inoculum.

[b]ml culture:ml TSB diluent.

[c]Numbers are averages from 3 counts of 1:10 dilutions of the standardized inoculum.

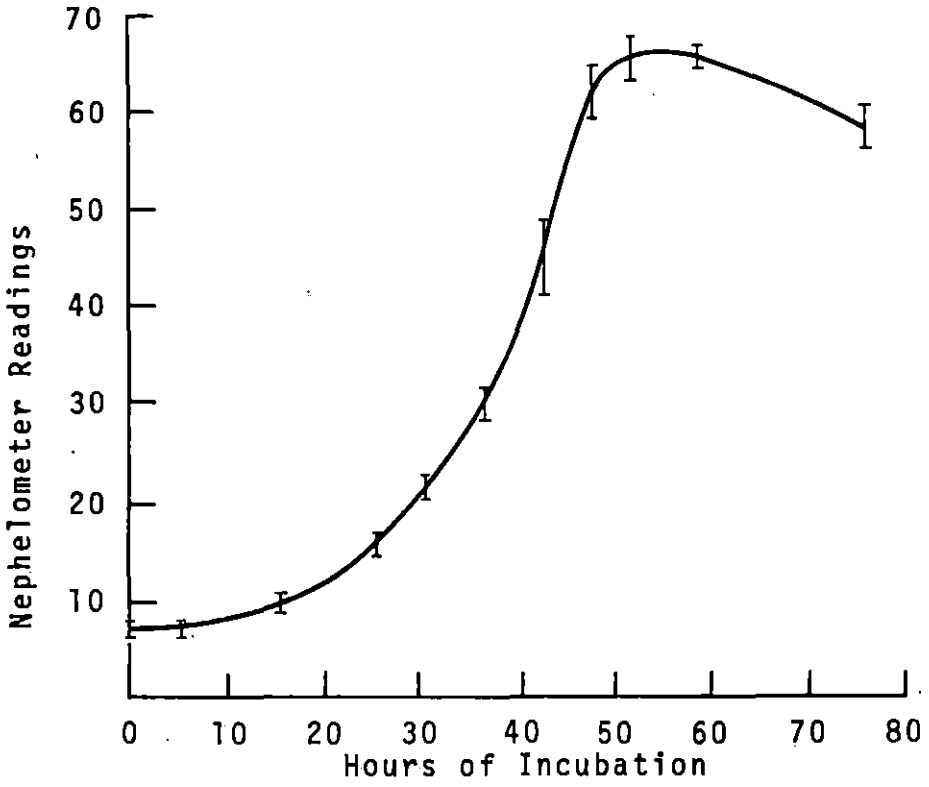
initiated with inoculum standardized from a culture with a nephelometer reading of 70 terminated after approximately 40 hours incubation.

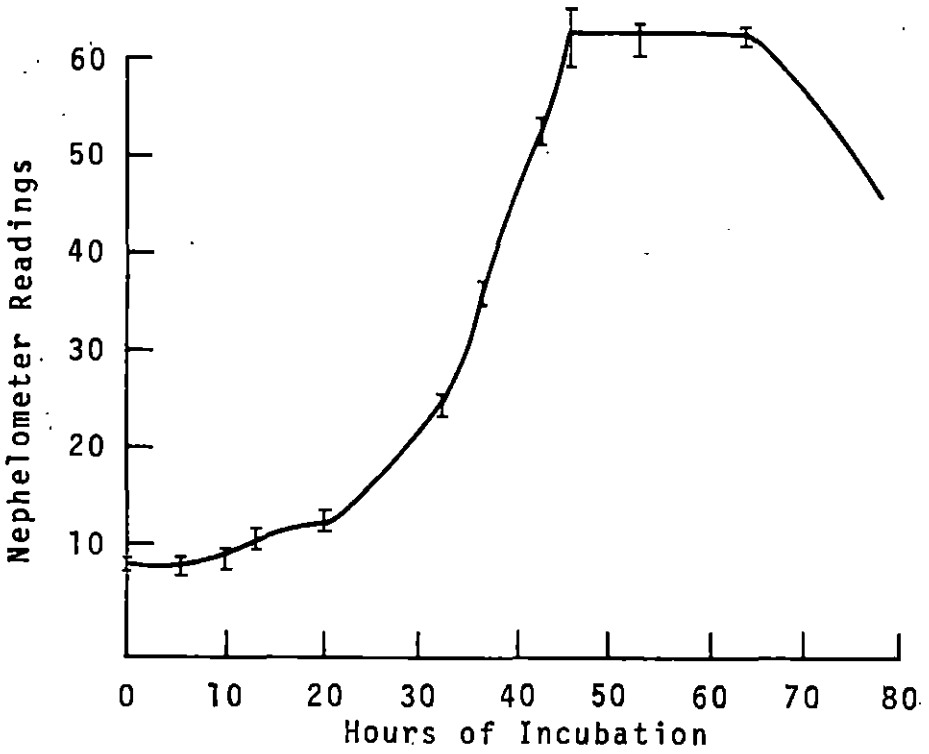
Regression analysis indicated that there were no significant differences among the average slopes obtained from the five groups initiated with standardized inocula. The group initiated with inoculum from the culture with a

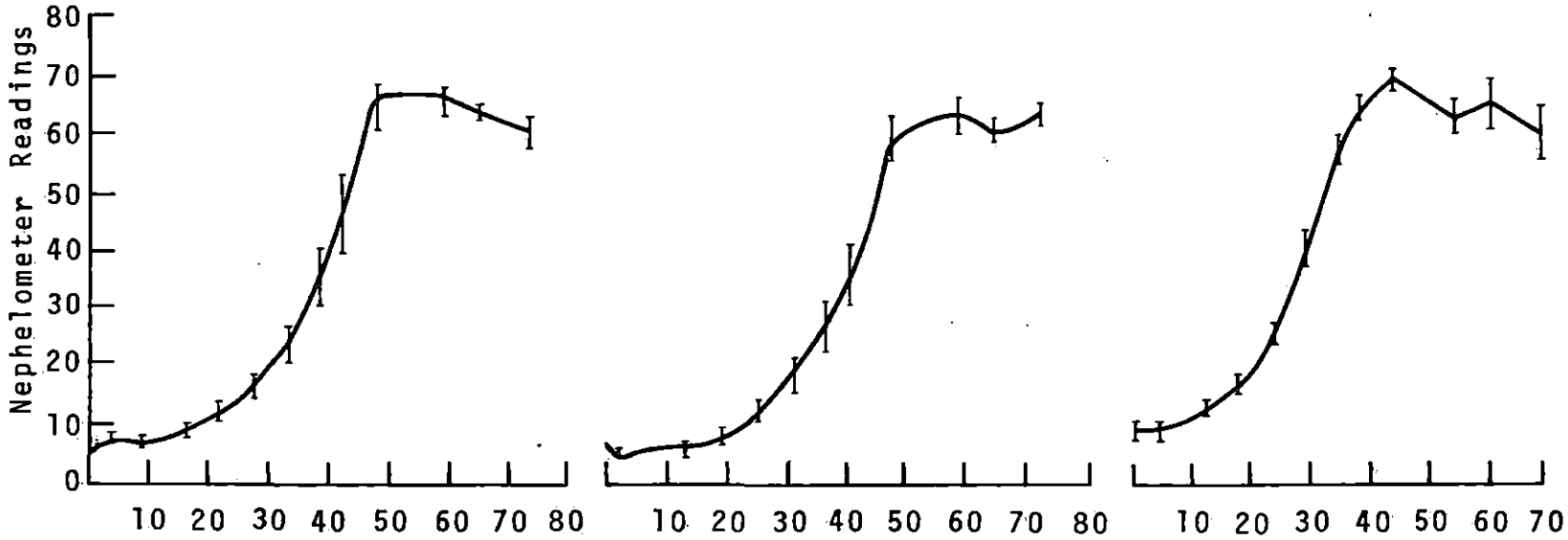
Figure 5. Growth responses of five groups of cultures initiated with standardized inoculum. Bars indicate ranges; solid lines represent mean values of nephelometer readings for each group

a) nephelometer reading of culture (NR) = 30, slope = 1.46

b) NR = 40, slope = 1.36


Nephelometer Readings
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Hours of Incubation


Nephelometer Readings
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Hours of Incubation



c) NR = 50, slope = 1.67 d) NR = 60, slope = 1.33 e) NR = 70, slope = 1.87

Figure 5 (continued)

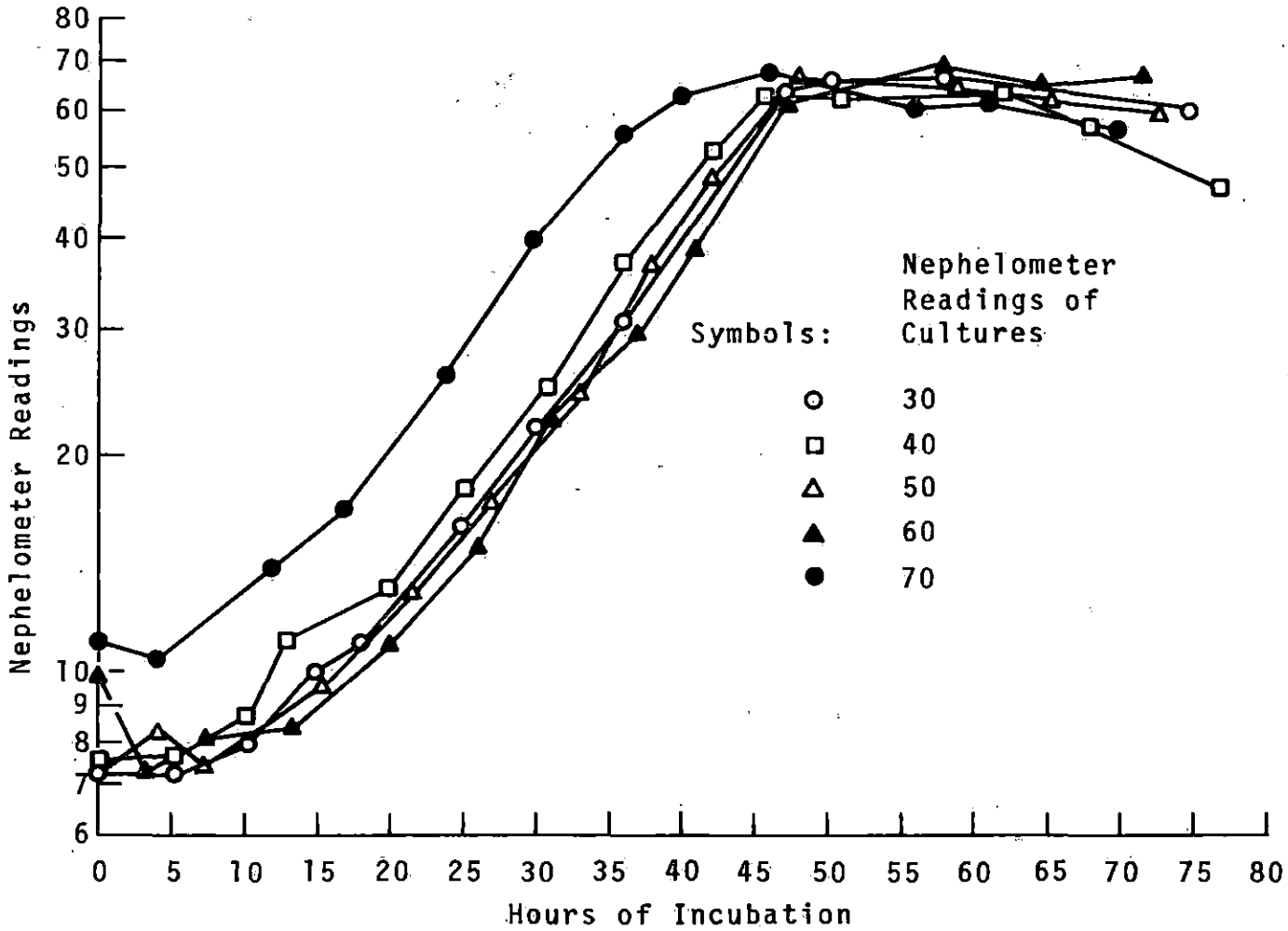


Figure 6. Relationship of growth responses of five groups of cultures initiated with standardized inoculum. Symbols represent means of nephelometer readings

nephelometer reading of 70 differed the most, but this difference was within the limits of tolerance ($P < 0.01$).

Definition of Optimal Temperature and pH

Growth at 34, 37, 39, and 42C in TSB-FCS at different initial pH values

Cultures of T. hyodysenteriae isolates B140 and B204 were initiated with approximately 1 x 10^7 viable cells per ml in TSB-FCS at initial pH values of 5.4-8.0 and grown at 37C. Subsequently they were inoculated to like medium of TSB-FCS at corresponding pH values and incubated at 34, 37, 39, and 42C.

Isolate B140 Growth of the first passage of T. hyodysenteriae isolate B140 did not occur in TSB-FCS at any pH after 80 hours incubation at 34C. Growth occurred in TSB-FCS at pH values 6.0-8.0 within 36 hours incubation at 37, 39, or 42C. Inoculum transferred to TSB-FCS at pH 5.4 did not grow at any of the temperatures. Subcultures incubated at 37C grew in TSB-FCS at pH 7.0 and 7.5 within 30 hours and those incubated at 39C grew in medium at pH 6.5-8.0 within 48 hours, but subcultures did not grow in TSB-FCS at any of the pH values incubated at 42C.

Isolate B204 The first subculture of T. hyodysenteriae isolate B204 grew in TSB-FCS at pH 7.5 after 80 hours incubation at 34C; the nephelometer reading was 16. Growth

of the first passages at 37, 39, and 42C occurred in TSB-FCS at pH 6.0-8.0 within 36 hours incubation; nephelometer readings ranged 40-60 after 48 hours. Inoculum transferred to TSB-FCS at initial pH 5.4 did not grow at any of the temperatures. Subcultures incubated at 37C reached maximum nephelometer readings after 36 hours; subcultures incubated at 39C grew in TSB at pH values 6.0, 7.0, 7.5, and 8.0 within 30 hours, and grew at pH 6.5 after 48 hours. Subcultures incubated at 42C grew in TSB-FCS at pH 6.5-8.0 within 36 hours, but subcultures in TSB-FCS at pH 6.0 did not grow at 37C or 42C.

The quality of unstandardized inocula used for the second passage of isolates B140 and B204 is presented in Table 3. Cultures incubated at 34C were not transferred, but the numbers of viable cells per ml were estimated. The presence of viable numbers in cultures with low nephelometer readings (less than 10) indicates maintenance rather than growth.

The nephelometer readings of the inocula (first passage) and maximum growth of the subcultures of isolates B140 and B204 incubated at 37C and 39C are presented in Figures 7 and 8. Nephelometer readings for inoculum of subcultures of B204 at 39C are not shown in Figure 8 because TSB-FCS at pH 6.5 and 7.0 was inadvertently missed during inoculation; therefore, the culture at pH 7.5 with

Table 3. Nephelometer readings and average numbers of viable cells per ml of first passage inoculum used for the second passage of T. hyodysenteriae, isolates B140 and B204, incubated at four temperatures

Isolate	Initial pH	Incubation Temperature and Ages of Cultures: 42C 48 Hours	
		Nephelometer Readings	Viable Cell Numbers
B140	8.0	10	2×10^4
	7.5	14	8×10^6
	7.0	19	3×10^6
	6.5	12	1×10^6
	6.0	10	1×10^5
	5.4	0[a]	$<10^2$
B204	8.0	47	8×10^5
	7.5	50	5×10^5
	7.0	17	1×10^7
	6.5	11	1×10^7
	6.0	21	1×10^4
	5.4	3[a]	2×10^3

[a]Not transferred.

[b]ND = not determined.

Incubation Temperature and Ages of Cultures					
39C 50 Hours		37C 55 Hours		34C 80 Hours	
Nephelometer Readings	Viable Cell Numbers	Nephelometer Readings	Viable Cell Numbers	Nephelometer Readings	Viable Cell Numbers
45	4×10^6	39	1×10^4	9[a]	1×10^7
43	4×10^6	46	1×10^5	5[a]	1×10^7
43	4×10^6	43	1×10^5	4[a]	1×10^5
29	5×10^5	39	1×10^5	5[a]	1×10^5
24	8×10^6	31	6×10^4	2[a]	1×10^5
0[a]	$<10^2$	1[a]	$<10^2$	0[a]	10^2
90	8×10^6	56	1×10^5	7[a]	2×10^6
100	4×10^6	65	ND[b]	16[a]	2×10^5
ND	1×10^5	21	2×10^6	2[a]	8×10^5
ND	2×10^6	28	2×10^5	1[a]	1×10^7
28	4×10^7	35	2×10^5	0[a]	5×10^5
2[a]	$<10^2$	2[a]	2×10^3	0[a]	2×10^3

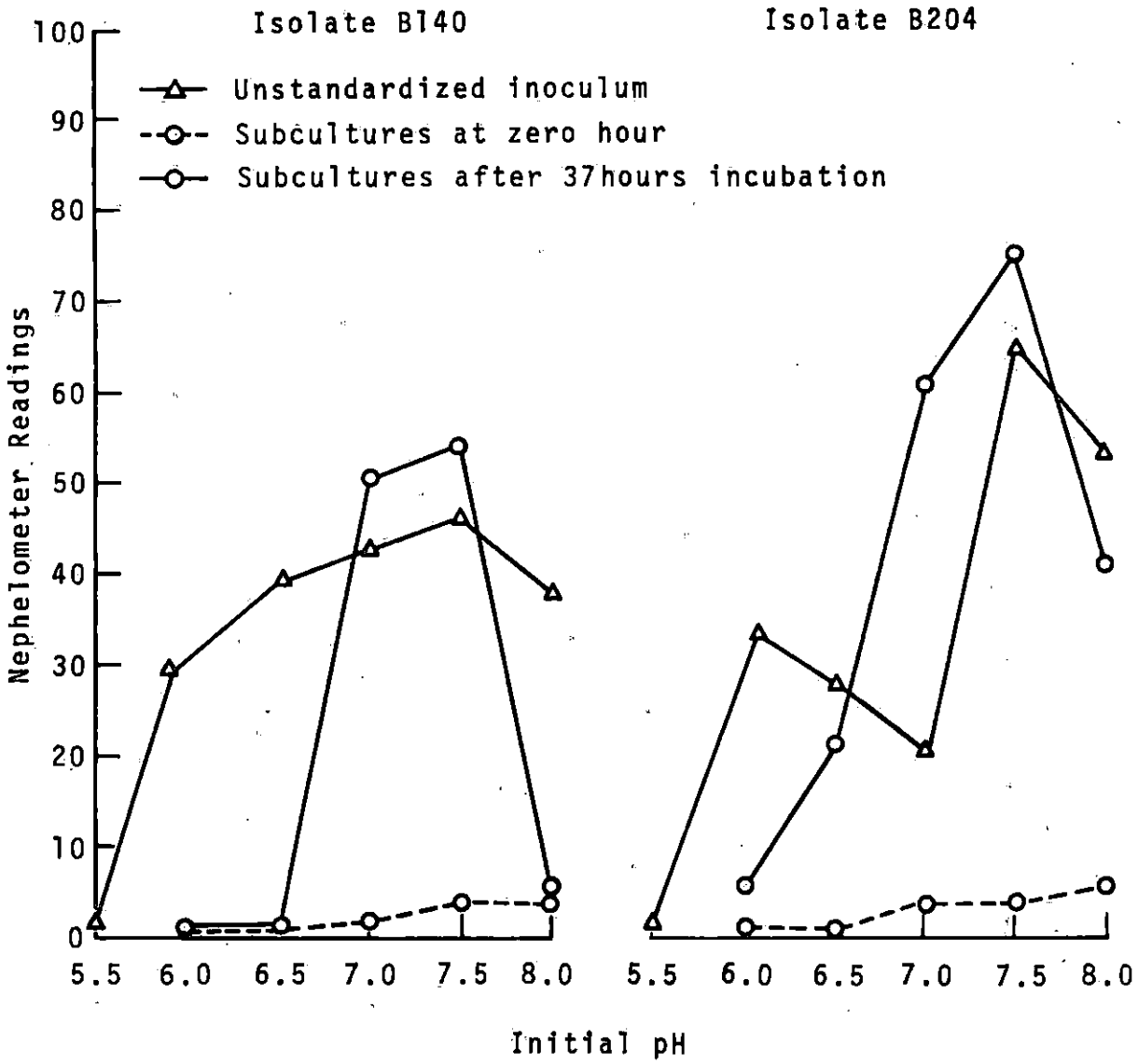


Figure 7. Nephelometer readings of the total growth of T. hyodysenteriae grown in TSB at different initial pH values supplemented with FCS and incubated at 37C

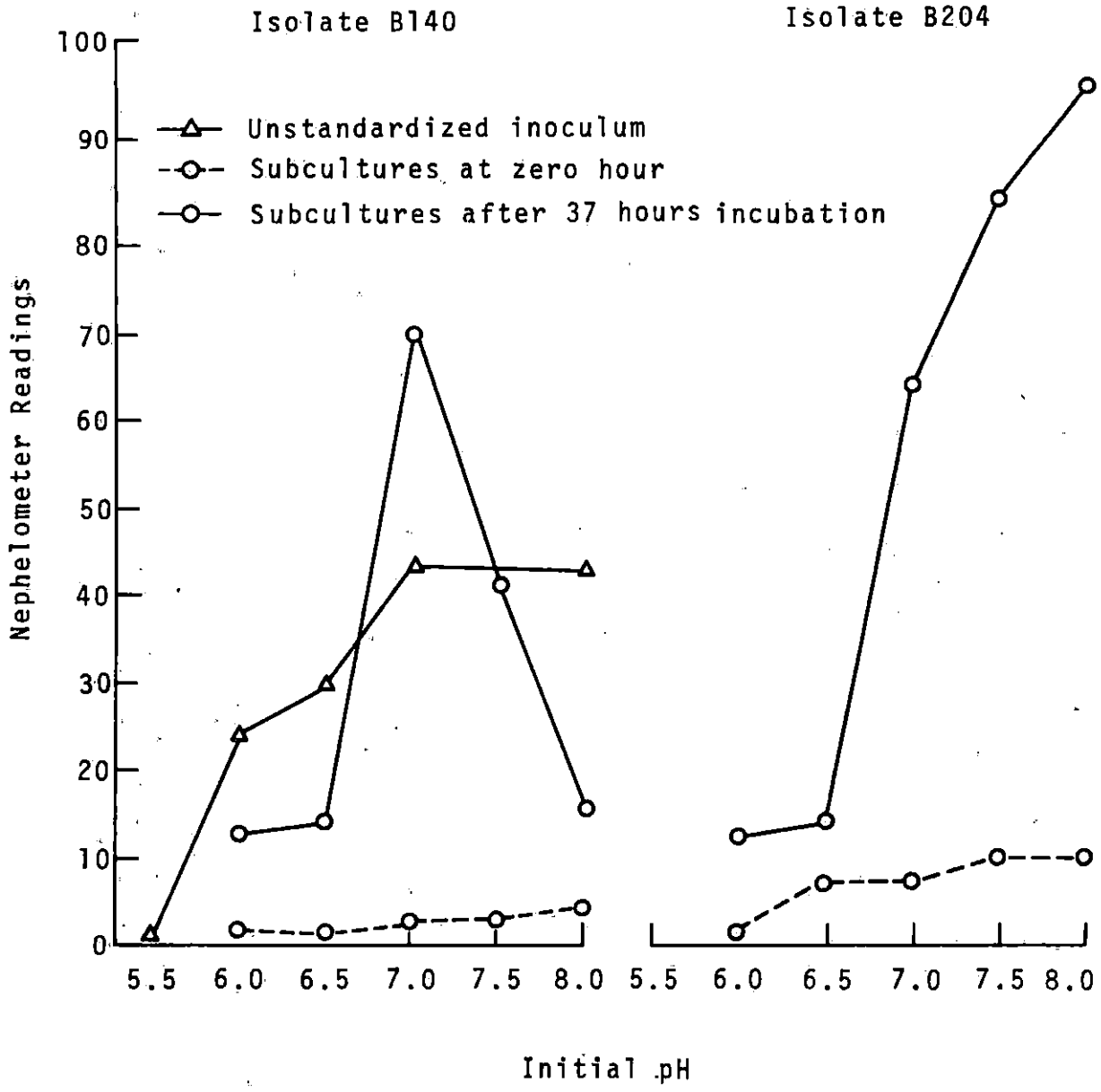


Figure 8. Nephelometer readings of the total growth of T. hyodysenteriae grown in TSB at different initial pH values supplemented with FCS and incubated at 39C

a nephelometer reading of 100 was used to inoculate TSB-FCS at pH 6.5 and 7.0. Inoculum for cultures at pH 8.0 and 6.0 had nephelometer readings of 93 and 28, respectively.

Nephelometer readings of the cultures of the second passage indicate that in this preliminary study, the optimal conditions for growth of isolate B140 are a pH of 7.0-7.5 and a temperature of 37C-39C. The optimal pH for growth of isolate B204 is a pH of 7.0-8.0 at 37C-39C and pH 7.5-8.0 at 42C. Comparison of the growth of isolates B140 and B204 on the basis of turbidity indicates that cultures grew best at 37C-39C in TSB-FCS at initial pH 7.0-8.0.

Growth of isolates B140 and B204 at 37C and 39C in medium with different initial pH values

Because growth of isolates B140 and B204 seemed to be best at 37C and 39C it was decided to repeat the study on growth in medium at initial pH values 6.5-8.6 incubated at 37 and 39C.

Isolate B140 The inocula used for subcultures incubated at 37C and 39C were grown in TSB-FCS at pH 7.0 and 7.5, respectively. Both cultures of inoculum were producing gas and were diluted to a nephelometer reading of 25; the 37C inoculum had approximately 5×10^5 viable cells per ml and the 39C inoculum had approximately 5×10^6 viable cells per ml. Cultures of the first passage

incubated at 37C grew in TSB-FCS at all the pH values tested. After 48 hours incubation cultures were standardized to nephelometer readings of 25-33 and transferred; the average number of viable cells of the inocula ranged 2 x 10^6 to 1 x 10^7 cells per ml. Subcultures incubated at 37C grew in TSB-FCS at pH 7.5 and 7.8; but did not grow in TSB-FCS at other pH values. After 56 hours incubation the pH values decreased from pH 7.8 to pH 6.75 and from pH 7.5 to pH 6.8. These cultures were also transferred; inoculum at pH 7.8 had a nephelometer reading of 28 and 5 x 10^6 viable cells per ml and inoculum at pH 7.5 had a nephelometer reading of 31 and 8 x 10^5 viable cells per ml. The subcultures grew within 30 hours to nephelometer readings of 60 and 30 in TSB-FCS at pH 7.8 and 7.5, respectively. Inoculum subcultured from pH 7.8 to 8.0 grew to a nephelometer reading of 42. Inoculum transferred to TSB-FCS at other initial pH values did not grow at 37C.

Cultures incubated at 39C grew in TSB-FCS at pH values of 6.5-7.8 within 40 hours; inoculum grown in TSB-FCS at pH 8.0 did not passage in TSB-FCS at pH 8.0. Subcultures grew in TSB-FCS at pH values of 6.5-7.8. Inoculum transferred from TSB-FCS at pH 7.8 to TSB-FCS at pH 8.0 also grew, but a second attempt to subculture in TSB-FCS at pH 8.0 failed. Nephelometer readings and viable numbers of cells of inocula used for subcultures in TSB-FCS at different

pH values are summarized in Table 4. Results of growth of the third subculture at 39C are given in Figure 9. Growth of B140 occurred in TSB-FCS at pH 6.8-7.8 at 39C. The effects of equilibration of TSB-FCS with H_2:CO_2 and growth of the organisms in medium which was incubated at 37C and 39C are presented in Table 5.

Isolate B204 The inocula for subcultures incubated at 37C and at 39C were grown in TSB-FCS at pH 7.0 and 7.5, respectively. Both cultures were producing gas and had nephelometer readings of 75. The 37C inoculum was diluted to a nephelometer reading of 26, and the 39C inoculum was diluted to a nephelometer reading of 38; both inocula had approximately 5×10^7 viable cells per ml. The quality of standardized inocula used for subsequent serial passages and the changes of the initial pH of TSB-FCS are presented in Tables 6 and 7, respectively.

Isolate B204 grew in TSB-FCS with initial pH values 6.75-8.6 for two subcultures at 37C. Cultures grown in TSB-FCS at 6.75 and at 7.0 were not transferred a third time because of slow growth. Cultures incubated at 39C grew in TSB-FCS at all the initial pH values tested and were transferred within 24 hours incubation.

Equilibration of uninoculated TSB-FCS with H_2:CO_2 minimized the effect of the initial pH values of the medium. The pH values of the different inocula were also significantly

Table 4. Quality of the inoculum used for the first and second serial passages of T. hyodysenteriae isolate B140 grown in TSB at different initial pH values and incubated at 39C

Initial pH	First Passage[a]		Second Passage[a]	
	Nephelometer Readings	Viable Cell Numbers	Nephelometer Readings	Viable Cell Numbers
8.0	ND[b]	ND	54/34[c]	5×10^6[d]
7.8	54/54	1×10^7	59/35	3×10^7
7.5	47/47	1×10^7	60/35	2×10^7
7.25	35/35	6×10^6	41/41	6×10^7
7.0	52/52	3×10^7	55/34	1×10^7
6.8	46/46	1×10^6	47/32	5×10^6
6.5	45/45	5×10^7	32/32	2×10^6

[a]Cultures were 48 hours old at time of transfer.

[b]ND = not determined.

[c]Numerator designates nephelometer reading of the culture; denominator designates nephelometer reading of the inoculum.

[d]Numbers are the average approximations of viable cells per ml.

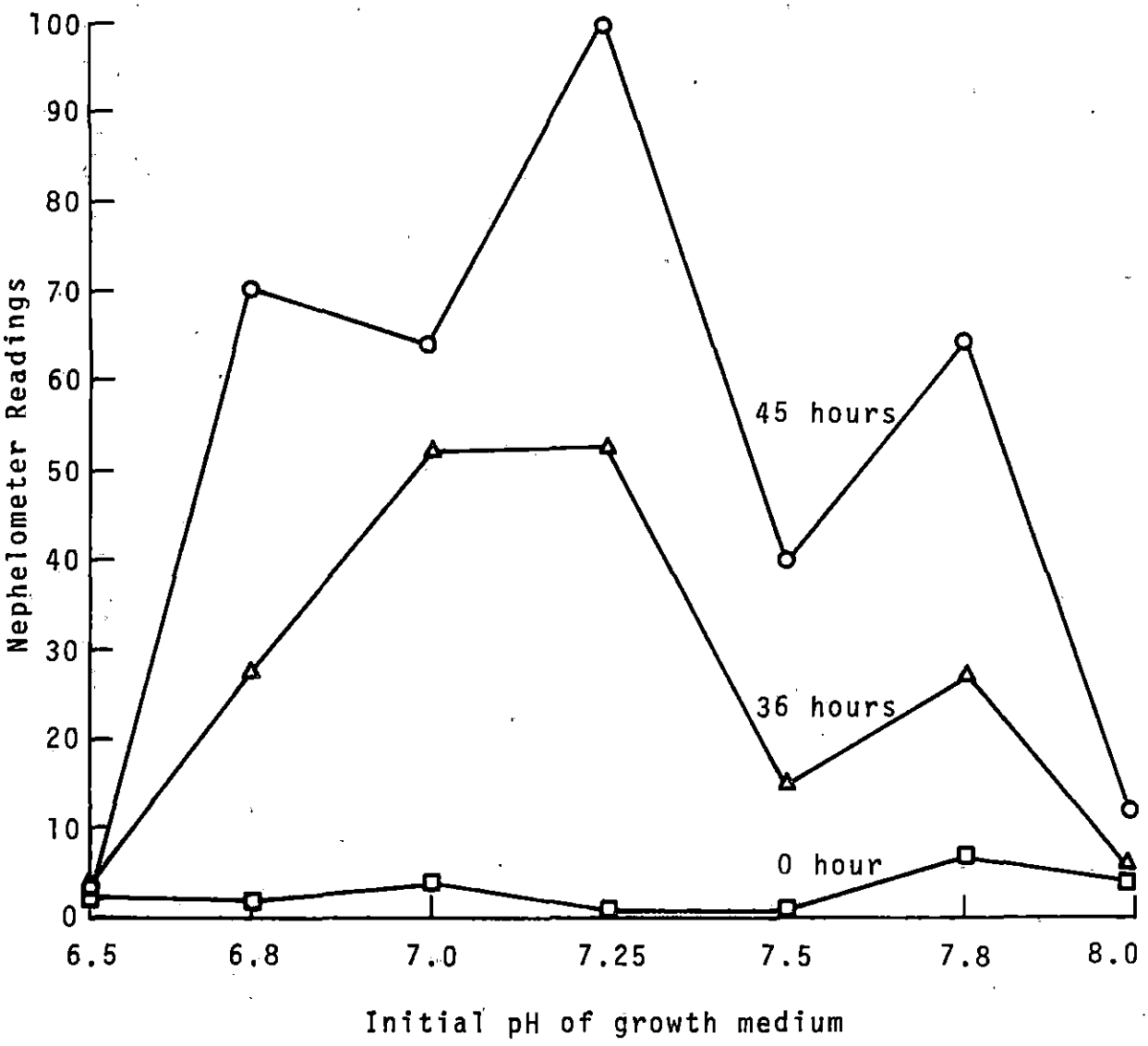


Figure 9. Average nephelometer readings of duplicate cultures of isolate B140 grown in TSB at different initial pH values supplemented with FCS and incubated at 39C

Table 5. Decreases in the pH of TSB equilibrated with H_2:CO_2 prior to inoculation and after 48 hours incubation at 37C and 39C of cultures of T. hyodysenteriae isolate B140

Initial pH	pH Before Inoculation		Culture pH After Inoculation	
	37C	39C	37C	39C
7.8	7.1[a]	7.15	6.75	6.6
7.5	7.0	7.0	6.8	6.3
7.25	6.8	6.9	6.9	6.75
7.0	6.7	6.8	6.45	6.6
6.8	6.55	6.7	ND[b]	6.4
6.5	6.3	6.5	6.2	6.4

[a]Average pH from two uninoculated tubes of TSB-FCS after 1-2 hours incubation at the appropriate temperature.

[b]ND = not determined.

decreased from the initial pH values. The inability of the growth medium to maintain the initial pH resulted in similar growth patterns for all the cultures so that an optimum pH was not defined (Figure 10). A pH of 8.6 incubated at 39C indicated a more rapid increase in mass for the fourth subculture of B204, but the inoculum had 10^8 cells per ml and was 24 hours old. Culture in medium at 7.25- were transferred after 16 hours incubation and had fewer

Table 6. Quality of T. hyodysenteriae isolate B204 inoculum used for 3 serial passages incubated at 37C and 39C

Passage Number	Initial pH	37C			39C		
		Age in Hours	Nephelometer Readings	Average Viable Number	Age in Hours	Nephelometer Readings	Average Viable Number
1	8.6	23	26/34[a]	ND[b]	23	48/42	4×10^{8}[c]
	8.25	23	29/37	ND	23	56/45	5×10^{7}
	8.1	23	35/36	ND	23	39/44	1×10^{8}
	7.75	23	36/36	ND	23	42/42	8×10^{7}
	7.5	23	42/36	ND	23	59/42	5×10^{7}
	7.25	23	41/35	ND	23	40/46	5×10^{7}
	7.0	30	35/35	ND	23	48/44	5×10^{7}
	6.75	50	29/29	ND	23	44/42	5×10^{7}

[a]Numerator designates nephelometer reading of the culture; denominator designates nephelometer reading of the inoculum.

[b]ND = not determined.

[c]Numbers are the average approximations of viable cells per ml.

Table 6 (Continued)

Passage Number	Initial pH	37C			39C		
		Age in Hours	Nephelometer Readings	Average Viable Number	Age in Hours	Nephelometer Readings	Average Viable Number
2	8.6	18	28/28	3×10^7	18	26/26	4×10^8
	8.25	18	33/33	5×10^7	18	26/26	4×10^7
	8.1	18	33/33	2×10^7	18	29/29	8×10^7
	7.75	18	34/34	3×10^7	18	29/29	3×10^7
	7.5	18	34/34	5×10^7	18	22/22	2×10^7
	7.25	18	33/33	2×10^7	18	26/26	2×10^7
	7.0	23	39/39	8×10^6	18	25/25	1×10^8
	6.75	23	24/24	2×10^7	22	20/20	5×10^7
3	8.6	19	41/41	2×10^7	24	20/20	5×10^7
	8.25	19	45/45	1×10^8	18	27/27	8×10^7
	8.0	19	38/38	7×10^7	16	27/27	2×10^7
	7.75	19	32/32	5×10^7	16	29/29	6×10^7
	7.5	24	44/44	7×10^7	16	29/29	6×10^7
	7.25	24	35/35	5×10^7	16	28/28	1×10^8
	7.0	NA[d]	NA	NA	24	27/27	8×10^7
	6.25	NA	NA	NA	24	15/15	2×10^7

[d]NA = not applicable.

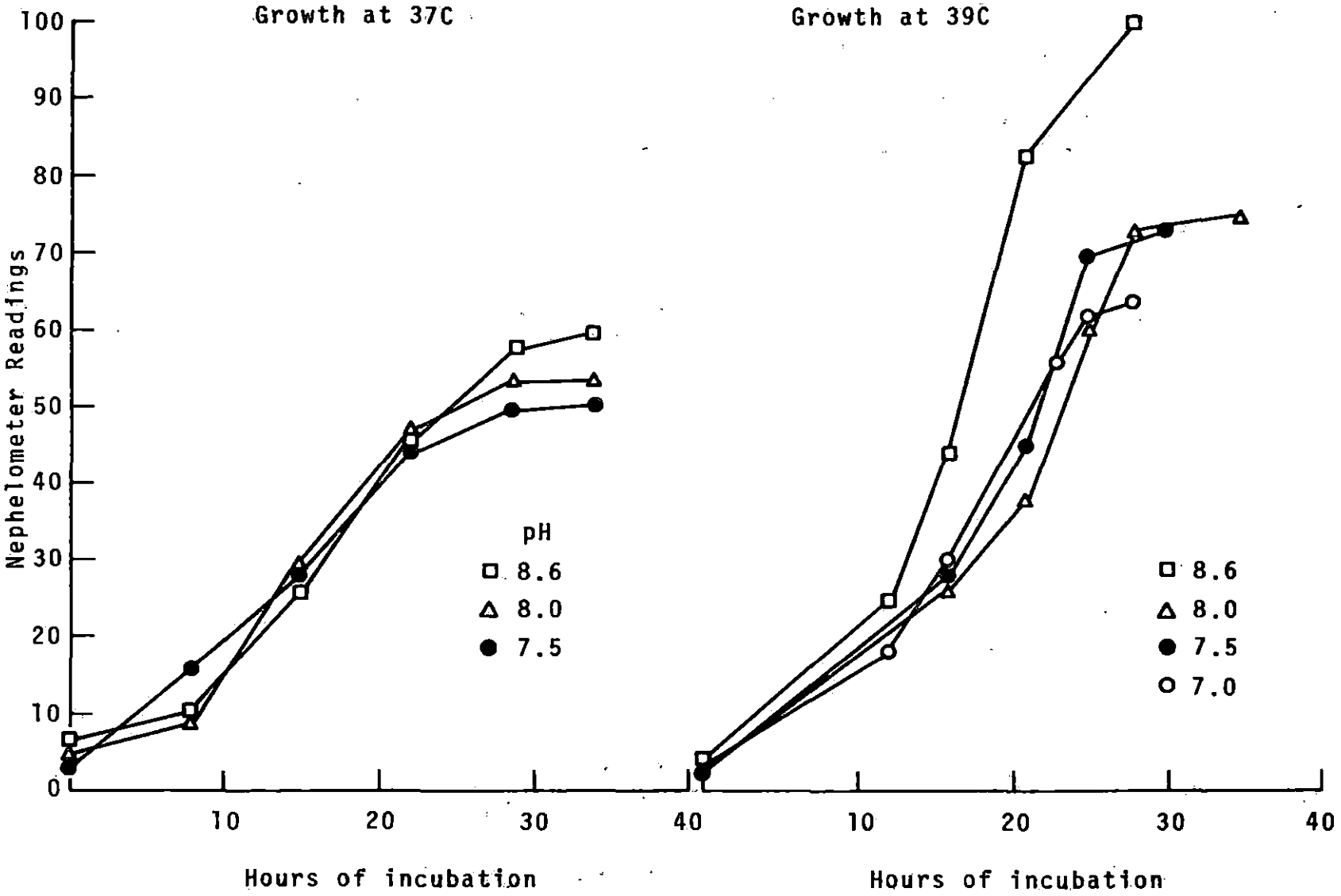
Table 7. Decreases in the pH of TSB equilibrated with H_2:CO_2 prior to inoculation and after 48 hours incubation at 37C and 39C of cultures of T. hyodysenteriae isolate B204

Initial pH	pH Before Inoculation		Culture pH After Incubation	
	37C	39C	37C	39C
8.6	7.2[a](2)[b]	7.4 (1)	6.9	7.15 (1)
8.25	7.2 (2)	7.2 (1)	6.8	6.95 (1)
8.1	7.1 (2)	7.0 (1)	6.8	6.85 (1)
7.75	7.0 (2)	6.95 (1)	6.75	6.85 (1)
7.5	6.9 (2)	6.8 (1)	6.64	6.8 (1)
7.25	6.8 (2)	6.82 (1)	6.6	6.7 (1)
7.0	6.9 (1)	6.65 (1)	6.5	6.6 (1)
6.75	6.6 (1)	6.6 (1)	6.4	6.6 (1)

[a]pH of uninoculated TSB-FCS after 1-2 hours incubation at the appropriate temperature.

[b]Parentheses indicate number of samples.

Figure 10. Growth of the third subculture of T. hyodysenteriae isolate B204 grown in TSB at different initial pH values supplemented with FCS. Cultures were initiated with standardized inoculum grown at 37C and at 39C


Growth at 37C
Growth at 39C
Nephelometer Readings
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
Hours of incubation
pH
8.6
8.0
7.5
7.0

viable cells per ml inoculum. Similar growth responses for isolate B140 in various pH values were also obtained and optimal conditions were not defined.

Serial dilution of inoculum as a method for optimization of incubation temperature and initial pH

Temperature Inoculum for the first subcultures in the first group was from an 18 hour culture with 5×10^8 cells per ml. Inoculum for first subcultures in the second group was from a 20 hour cultures with 1.4×10^8 cells per ml. A summary of the quality of standardized inocula grown in TSB with 8% FCS and used for the second passages in both groups is given in Table 8. Viable determinations ranged 5×10^7 to 3×10^8 cells per ml.

Cultures of the two groups were set up in duplicate series with standardized inocula and incubated at 42, 38, and 34C. The average number of days required for diluted inoculum to evidence growth when incubated at these three temperatures for four passages are presented in Table 9.

The culture series of the two subcultures in the first group incubated at 42C and at 34C were incubated at 38C after seven days incubation at the tested temperatures. There was no growth initiated in any of the cultures which had not evidenced growth after seven days incubation at 42C; however, within 2-3 days incubation at 38C, subcultures

Table 8. The quality of standardized inoculum of T. hyodysenteriae isolate B204 used for serial dilution in TSB incubated at different temperatures

Group	Incubation Temperature °C	Age in Hours	Nephelometer Readings	Total Cell Numbers
I	42	43	35/45[a]	2×10^8[b]
	38	44	40/42	1.3×10^8
	34	42	34/42	1×10^8
II	42	30	30/32	9.3×10^7
	38	42	32/32	1.5×10^8
	34	50	23/23	5.3×10^7

[a]Numerator designates the nephelometer readings of the culture; denominator designates the nephelometer reading of the inoculum used.

[b]Numbers represent averages of duplicate counts of 1:10 dilutions of the standardized inoculum.

previously incubated at 34C with an inoculum equal to or greater than 10^3 cells per ml showed visible growth.

Nephelometer readings of approximately 5-10 corresponded with first evidence of visible growth. The first cultures of all series in which growth was not observed after seven days incubation also had no evidence of spirochetes when observed by phase microscopy.

Table 9. Effect of incubation temperature on the initiation of growth and on the number of days required for growth of diluted inoculum of T. hyodysenteriae isolate B204

Inoculum Concentration	42C			38C			34C		
	Growth Ratio	Average Days	Range of Days	Growth Ratio	Average Days	Range of Days	Growth Ratio	Average Days	Range of Days
10^8	7/7[a]	1.6	0.5-3.5[b]	7/7	0.6	0.5-1.0	6/8	1.3	1.0-1.5
10^7	8/8	2.9	1.0-6.0	7/7	1.1	1.0-1.5	3/8	4.3	4.0-5.0
10^6	4/7	2.1	1.5-3.0	8/8	2.1	2.0-3.0	0/8	NA[c]	NA
10^5	2/6	3.0	(3.0)[d]	7/7	2.5	2.0-3.5	0/8	NA	NA
10^4	2/7	4.0	3.0-5.0	8/8	3.1	2.0-4.5	0/8	NA	NA
10^3	2/8	5.0	(5.0)	8/8	3.3	3.0-3.5	0/8	NA	NA
10^2	1/8	5.0	(5.0)	8/8	3.9	3.5-4.5	0/6	NA	NA
10^1	2/8	5.0	4.0-6.0	8/8	4.6	4.0-5.5	0/6	NA	NA
10^0	0.4	NA	NA	2/4	4.8	4.5-5.0	0/4	NA	NA
0	0.4	NA	NA	0/4	NA	NA	0/4	NA	NA

[a]Numerator designates number of cultures that evidence growth; denominator designates number of cultures inoculated.

[b]Minimum-maximum number of days

[c]NA = not applicable.

[d]Parentheses indicate that culture(s) equalled the average number of days.

Initial pH Inoculum for the first subculture in the first group was from a 20 hour culture with approximately 1.5×10^8 cells per ml. Inoculum for the first subculture in the second group was from a 20 hour culture with approximately 2×10^7 cells per ml. The quality of the standardized inocula used for the second passages in both groups is given in Table 10.

The duplicate series of each initial pH value tested for the ability of diluted inoculum to evidence growth were incubated at 38C. Results from the four passages of both groups are summarized in Table 11. Cultures in which growth was not observed after seven days incubation also had no evidence of spirochetes when observed by phase microscopy.

Growth Conditions and Responses to Variations of TSB-FCS and to Medium Substitutes

Growth of T. hyodysenteriae in atmospheres of H_2:CO_2 and CO_2

Preliminary observations indicated that an atmosphere of H_2:CO_2 allowed more rapid growth of the organisms than an atmosphere of CO_2 for three consecutive passages. Growth patterns from two serial passages of cultures under H_2:CO_2 and under CO_2 propagated in aerobically prepared TSB and in PRAS-CF-TSB are presented in Figures 11 and 12, respectively.

Table 10. The quality of standardized inoculum of T. hyodysenteriae isolate B204 used for serial dilutions in TSB at different initial pH values

Group	Initial pH	Age in Hours	Nephelometer Readings	Total Cell Numbers	Viable Cell Numbers
I	8.5	44	38/30[a]	1.3×10^{8}[b]	3×10^{7}[c]
	8.0	44	92/32	9×10^{7}	3×10^{7}
	7.5	44	63/30	1×10^{7}	5×10^{7}
	7.0	44	40/28	7.5×10^{7}	1×10^{6}
	6.5	44	45/32	7.5×10^{7}	ND[d]
II	8.5	31	30/30	8.8×10^{7}	1×10^{8}
	8.0	54[e]	29/29	1.3×10^{7}	1.5×10^{8}
	7.5	31	28/28	8.2×10^{7}	1×10^{8}
	7.0	31	43/33	1×10^{8}	1×10^{8}
	6.5	31	38/29	9×10^{7}	1×10^{8}

[a]Numerator designates the nephelometer readings of the culture; denominator designates the nephelometer reading of the inoculum used.

[b]Numbers represent averages of duplicate counts of 1:10 dilutions of the standardized inoculum.

[c]Numbers are the average approximations of viable cells per ml.

[d]ND = not determined.

[e]Culture was the 10^{-3} dilution of the inoculum.

Table 11. Effect of initial pH of TSB on the initiation of growth and on the number of days required for growth of diluted inoculum of T. hyodysenteriae isolate B204

Initial pH	8.5			8.0		
Inoculum Concentration	Growth Ratio	Average Days	Range of Days	Growth Ratio	Average Days	Range of Days
10^8	8/8[a]	0.5	(0.5)[b]	8/8	0.6	0.5-1.0[c]
10^7	8/8	1.2	1.0-1.5	7/7	1.4	1.0-2.0
10^6	8/8	1.7	1.5-2.5	6/6	1.8	1.5-2.0
10^5	6/6	2.3	2.0-3.0	7/7	2.5	2.0-3.0
10^4	8/8	3.0	2.5-3.5	7/7	3.0	2.5-3.5
10^3	7/8	3.6	3.0-4.0	6/6	3.7	3.0-4.0
10^2	7/7	4.1	3.5-5.0	7/7	4.0	(4.0)
10^1	3/7	4.7	4.0-5.0	5/7	4.9	4.5-5.0
10^0	2/7	5.0	4.0-5.5	2/7	5.5	5.0-6.0
0	0/8	NA	NA	0/7	NA	NA

[a]Numerator designates number of cultures that evidenced growth; denominator designates number of cultures inoculated.

[b]Parentheses indicate that the culture(s) equalled the average number of days.

[c]Minimum-maximum number of days.

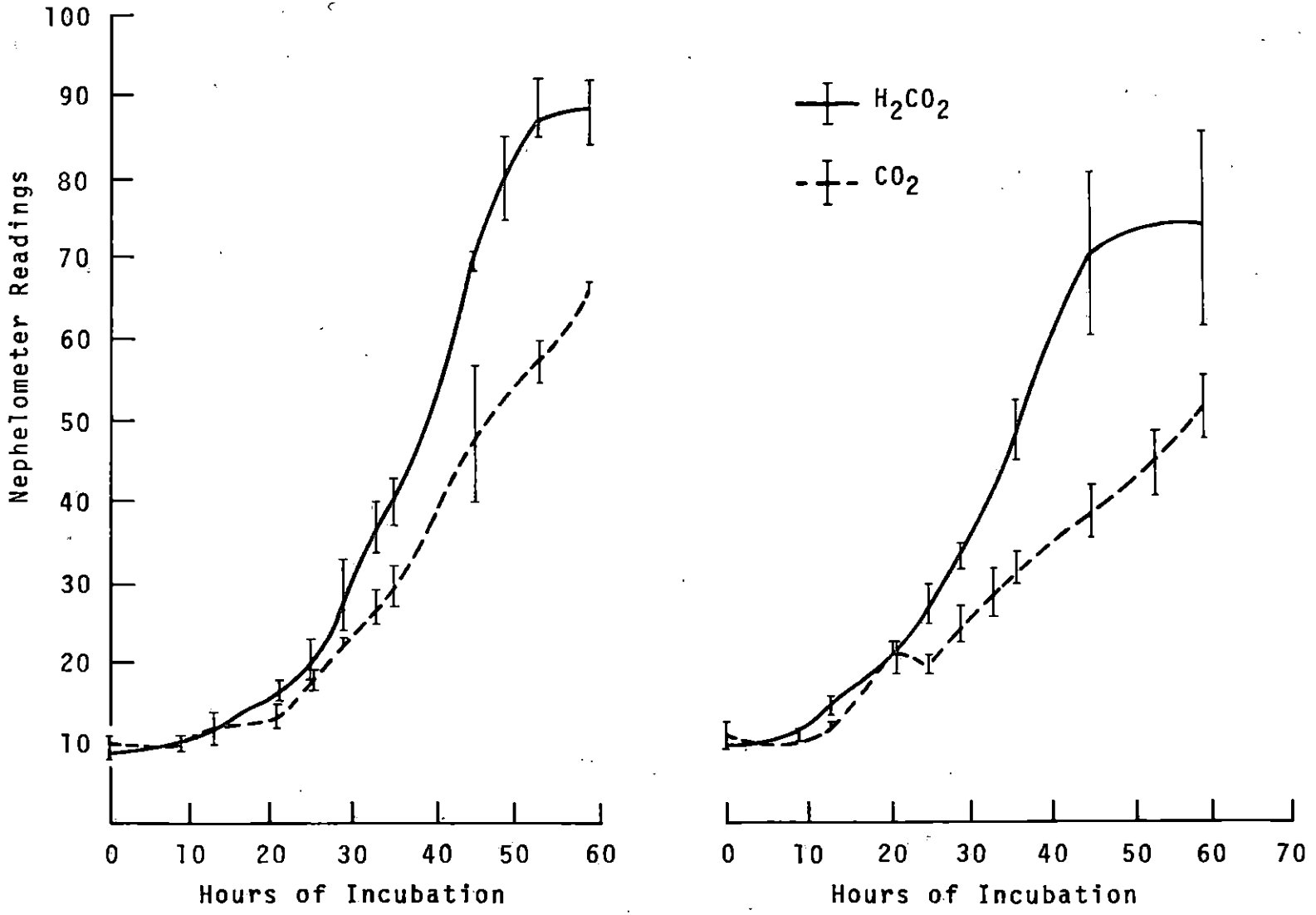
Table 11 (Continued)

7.5			7.0			6.5		
Growth Ratio	Average Days	Range of Days	Growth Ratio	Average Days	Range of Days	Growth Ratio	Average Days	Range of Days
8/8	0.5	(0.5)	7/7	0.7	0.5-1.0	6/6	0.6	0.5-1.0
8/8	0.8	0.5-1.0	6/6	1.1	0.1-1.5	8/8	0.9	0.5-1.0
8/8	1.6	1.5-2.0	6/6	1.5	(1.5)	6/6	1.7	1.5-2.0
8/8	2.5	2.0-3.0	6/6	2.0	(2.0)	7/7	2.2	2.0-2.5
8/8	2.9	2.5-3.5	6/6	2.5	2.0-3.0	6/7	3.1	2.5-3.5
7/7	3.7	3.5-4.0	6/6	3.3	3.0-4.0	6/8	4.1	3.0-3.5
7/7	3.8	3.5-4.0	6/6	3.7	3.0-4.0	5/8	4.3	3.5-5.0
7/8	4.2	3.5-5.0	4/6	4.3	4.0-5.0	2/6	4.3	4.0-4.5
4/8	4.8	4.0-6.0	0/6	NA[d]	NA	0/6	NA	NA
0/8	NA	NA	0/6	NA	NA	0/6	NA	NA

[d]NA = not applicable.

Figure 11. Growth of T. hyodysenteriae in aerobic TSB supplemented with FCS under H_2:CO_2 and under CO_2. The average slope for 14th and 17th passages under H_2:CO_2 is 2.17; the average slope for 17th passage cultures under CO_2 is 1.46

Figure 12. Growth of T. hyodysenteriae in PRAS-CF-TSB supplemented with FCS under H_2:CO_2 and CO_2. The average slope for 14th and 17th passages under H_2:CO_2 is 2.06; the average slopes for 17th passage cultures under CO_2 is 0.84


Nephelometer Readings
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
Hours of Incubation
H_2CO_2
CO_2
0
10
20
30
40
50
60
70
Hours of Incubation

Serial passage of the organisms in PRAS-CF-TSB became difficult after six transfers because the organisms sometimes failed to grow or exhibited lag periods of 36 hours or more. Cultures under an atmosphere of CO_2 grew for 4-5 serial passages. Cultures grew most consistently in TSB-FCS with H_2:CO_2 as the atmosphere.

Serum properties

The total protein in a representative sample of FCS was determined to be 37-41 mg/ml.

Growth in TSB supplemented with 10% (V/V) serum heated at 56C or at 80C was subcultured four times. Viability determinations for two of the serial passages are presented in Table 12. Subcultures often did not show evidence of gas production. Growth occurred for two passages in TSB supplemented with serum heated at 100C, but did not occur in TSB supplemented with the supernatants obtained by centrifugation.

Dilution of inoculum in TSB supplemented with different concentrations of FCS

Aerobic TSB supplemented with 6%, 5%, 4%, 3%, 2%, and 1% FCS was inoculated with 1 x 10^7 to 5 x 10^7 viable cells per ml which was then serially diluted in medium supplemented with the appropriate concentrations of serum. An unsupplemented series of aerobic TSB was similarly inoculated.

Table 12. Viable determinations for two passages of T. hyodysenteriae isolate B204 grown in TSB supplemented with FCS exposed to 80C and to 56C

Minutes Exposed	80C		56C	
	First Passage	Second Passage	First Passage	Second Passage
15	1×10^7[a]	1×10^8	1×10^6	1×10^7
30	7×10^7	2×10^8	1×10^7	1×10^7
45	7×10^7	5×10^7	1×10^6	ND[b]
60	5×10^7	7×10^7	7×10^7	2×10^7
90	6×10^7	1×10^7	1×10^7	3×10^7

[a]Numbers are the average approximations of viable cells per ml.

[b]ND = not determined.

Cultures were observed for visible evidence of growth every 12 hours. Results of the average number of days required for the diluted inoculum to evidence growth in medium supplemented with the various concentrations of FCS are summarized in Table 13.

Effects of D-FCS and GG-free serum

Treponema hyodysenteriae was passaged four times with 10% serum in aerobic TSB. Supplements used were D-FCS, GG-free FCS, and untreated FCS. The fifth and sixth

Table 13. Growth of inoculum of T. hyodysenteriae isolate B204 diluted in TSB supplemented with different concentrations of FCS

Inoculum Concentration	Percent fetal calf serum						
	6	5	4	3	2	1	0
10^7	1[a](3/3)[b]	1 (1/1)	ND[c]	1 (1/2)	1 (1/1)	1 (1/1)	1 (1/1)
10^6	2 (3/3)	2 (2/2)	2.5 (2/2)	1.5 (2/2)	1.5 (2/2)	3 (1/1)	NG[d](1/1)
10^5	3 (3/3)	3 (1/2)	3 (2/2)	3 (2/2)	4 (2/2)	NG (1/1)	NG (1/1)
10^4	3.3 (2/3)	NG (1/2)	3 (1/2)	NG (2/2)	NG (2/2)	NG (1/1)	NG (1/1)
10^3	7 (1/3)	NG (1/2)	4 (1/2)	NG (1/2)	NG (2/2)	NG (1/1)	NG (1/1)

[a]Numbers indicate the average number of days required for evidence of visible growth.

[b]Numerator indicates number of cultures that evidenced growth; denominator indicates number of cultures inoculated.

[c]ND = not determined.

[d]NG = no growth observed during 5 days incubation.

subcultures were inoculated as serial dilutions to compare the abilities of the sera to support growth (Table 14). Inoculum grown with dialyzed serum grew better when transferred to TSB with untreated serum as the supplement. Inoculum grown with GG-free serum grew as well when passaged to like whole medium or to TSB supplemented with untreated serum.

Responses to FCS and growth medium substitutes

None of the substrates attempted in these studies replaced the serum requirement of T. hyodysenteriae. Aerobic TSB with 1% BSA supplemented with 0.05%, 0.1%, or 0.2% of Tweens 80, 60, and 40 did not support growth of an inoculum of 1×10^6 to 1×10^7 viable cells per ml. Medium supplemented with 1% BSA or 1% BSA plus 0.05%, 0.1%, or 0.2% VFA mixture did not support growth of identical inoculum. Organisms maintained the best morphology in TSB with 1% BSA or FP-BSA. Cells incubated in TSB with other combinations appeared lysed or grossly distorted when viewed with phase microscopy.

Pregassed HLH supported growth of a 5% inoculum for three serial passages when 10% FCS was added. Combinations of HLH with 20%, 30%, or 50% serum allowed growth of only the first passage inoculum.

No growth occurred in 10X Wo_5 with or without 50 mg

Table 14. Comparison of D-FCS and GG-free FCS to untreated FCS (FCS-control) as serum supplements for diluted T. hyodysenteriae isolate B204

	Supplement	Inoculum Source	Log_{10} number of cells per ml in diluted inoculum				
			10^7	10^6	10^5	10^4	10^3
Trial 1	D-FCS	D-FCS	1[a]	3	3[b]	NG	NG
	FCS-Control	D-FCS	1	ND[c]	3	NG	NG
	GG-Free	GG-Free	1	3	3	3[b]	4
	FCS-Control	GG-Free	1	3	3	3	NG
	FCS-Control	FCS-Control	1	3	3	3[b]	ND
Trial 2	D-FCS	D-FCS	1	2	3	NG[d]	NG
	FCS-Control	D-FCS	1	2	3	3	NG
	GG-Free	GG-Free	1	3	3	3	4
	FCS-Control	GG-Free	1	3	3	3	4
	FCS-Control	FCS-Control	1	2	3	3	NG

[a]Numbers indicate the average number of days required for evidence of visible growth.

[b]Inoculum used for Trial 2.

[c]ND = not determined.

[d]NG = no growth observed during 5 days incubation.

cysteine/100 ml, 10X MEM, or Wo_5 + BSA_{2000} supplemented with FCS supported growth after 48-72 hours; gas bubbles were observed.

The best qualitative growth was supported by Wo_5 + BSA_{2000} supplemented with 60% or 40% FCS. Motile cells with good T. hyodysenteriae-like morphology were also observed in TSB pH 8.1 when supplemented with 20% Wo_5 + BSA_{2000}.

The NCTC-135 preparation allowed three serial passages when 10% (V/V) FCS and 0.01M tris were added; NCTC-135 did not support more than the first inoculum unless both FCS and buffer were added. The morphology in aerobic TSB plus 10% (V/V) NCTC-135 appeared better when 0.01M tris was also added. Gas bubbles were observed in TSB supplemented with 10% or 20% NCT-135 without tris.

Best results for the second and third passages in trypticase medium was observed with reduced trypticase plus 10% NCTC-135, 0.2% glucose, 0.02M tris, and 2% FCS. Aerobic trypticase with the same supplements did not support growth of the second passage inoculum; carbohydrates other than glucose did not enhance growth.

Growth in NCTC-135 supplemented with 2% (V/V) FCS, 0.02M tris, 0.1% Na_2CO_3, and 0.5% BSA was not enhanced by the addition of 0.2% glucose. Growth in NCTC-135 plus 2% FCS was not enhanced by the addition of 0.1% Na_2CO_3, 0.5%

BSA, or 0.2% glucose. Growth of the second passage in NCTC-135 plus 2% FCS occurred with 0.2% glucose or when 0.2% glucose plus 0.5% BSA were added; growth of the fourth passage did not occur.

Growth in modified basal medium

Growth of a 5% inoculum occurred for nine consecutive passages in FG-TSB supplemented with 8% FCS.

Medium prepared with ½X TSB and supplemented with 10% FCS supported growth after 48 hours for one passage when 0.2% CO_2-equilibrated Na_2CO_3 was added; no growth occurred without the buffer. In contrast, ½X TSA enriched with 5% citrated bovine blood allowed confluent growth with β-hemolysis after 24 hours incubation at 42C or at 37C.

The following media supplemented with FCS did not support growth of more than the 10% inoculum for the first passage: Aerobic phytone without KH_2PO_4, 2X aerobic phytone with or without KH_2PO_4, and 2X aerobic trypticase with or without KH_2PO_4. Motile cells with good morphology were subcultured twice in aerobic phytone plus KH_2PO_4 and four times in aerobic trypticase with or without KH_2PO_4 supplemented with 7% to 10% FCS.

A 10% inoculum did not passage more than once from aerobic TSB supplemented with 8% FCS to aerobic TSB-M supplemented with 8% FCS alone, or when either 0.15% or 0.2%

CO_2-equilibrated Na_2CO_3 was also added.

Of the more reduced modified basal medium, reduced phytone did not allow any evidence of growth of the inoculum for the first transfer, whereas reduced trypticase allowed two transfers of motile cells.

Reduced phytone with salts and reduced trypticase with or without salts supported growth for two serial passages; reduced phytone without salts, 2X reduced trypticase and 2X reduced phytone did not support growth of the first subculture.

Growth in aerobic TSB with 10% serum and a 5% inoculum (B204) did not occur after 84 hours incubation at 38C when salts and buffers were added as follows: S-1, S-z, T-sp, Bryant's salts (1974), tris-PO_4, or 0.05% $NaHCO_3$. The combinations which supported growth for three serial passages were: PO_4-CO_3 buffer with S-1, PO_4-CO_3 buffer with T-Sp, 0.05% $NaHCO_3$, and 0.01M tris. Tris-PO_4 buffer allowed five serial passages of a 10% inoculum.

Growth of a serially diluted inoculum of 1×10^7 to 1×10^8 viable cells per ml in trypticase and phytone medium supplemented with 6% serum occurred in only the first tube of the series after six days incubation when Caldwell-Bryant buffer was added. No growth occurred in any of the tubes when Bryant's salts (1974) with 0.2% CO_2-equilibrated Na_2CO_3, 0.05% $NaHCO_3$, 0.1M or 0.01M tris, 0.1% PO_4-CO_3, 0.05% $NaHCO_3$,

0.1% thioglycollate medium, or 0.1% tris-PO_4 buffer were added.

Growth of approximately 1 x 10^7 viable cells per ml occurred four times in TSB with VPI salts supplemented with 6% FCS and 0.2% CO_2-equilibrated Na_2CO_3; with 0.1% thioglycollate medium, 0.01M tris, or 0.001M tris growth occurred for three serial passages. Growth of identical inoculum occurred three times in TSB-VPI supplemented with 6% FCS and 0.22+ filtered $NaHCO_3$ alone or with 0.01M tris also added.

Growth in aerobic TSB-FCS buffered with CO_2-equilibrated Na_2CO_3

Growth of T. hyodysenteriae was successfully serial passaged eight times in aerobic TSB buffered with 0.2% CO_2-equilibrated Na_2CO_3 and supplemented with 10%, 8%, or 5% FCS. Inoculum, transferred after 24-36 hours incubation, ranged approximately 1 x 10^7 to 1 x 10^8 viable cells per ml. The cultures in buffered medium with initial pH 7.5 were pH 6.9 to 7.0 after growth; unbuffered cultures were pH 6.5 to 6.6 after growth. The uninoculated buffered medium maintained pH 7.15 to 7.25; unbuffered controls maintained pH 6.75. The cultures in buffered medium with initial pH 7.0 were pH 6.85 to 6.95 after growth; unbuffered cultures were pH 6.3 to 6.35 after growth.

Initiation of 5% inoculum in buffered medium at initial pH 7.5 or 7.0 was delayed compared to initiation and growth in unbuffered medium (Figures 13 and 14).

Repeated attempts to initiate growth in buffered or unbuffered medium with frozen buffered stock cultures were unsuccessful. Attempts to initiate growth in buffered medium with low passage unbuffered stock cultures were also unsuccessful.

Transmission

The pigs were held in the unit for six days and were observed to be normal before challenge with T. hyodysenteriae. Occasionally atypical large spirochetes and small spirochetes were observed in the feces. Approximately four days after the second challenge, both of the exposed pigs showed signs of diarrhea. One of the animals suffered from a watery diarrhea for two days; the other animal had a very loose diarrhea for two days. The abnormal feces consistency persisted for about four days then both pigs recovered; blood or mucus were not observed. No T. hyodysenteriae were observed from fecal samples by phase microscopy and none were isolated on the selective medium. Control animals remained normal throughout the experiment. The pigs were held in the pen for 40 days and then killed. The two control animals had normal digestive tracts; one animal had

a respiratory infection. The two exposed animals were completely normal and there were no gross lesions typical of swine dysentery. Selective media were negative for Salmonella.

Figure 13. Growth responses of cultures in unbuffered medium and in buffered medium at initial pH 7.5. Cultures were supplemented with 10%, 8%, or 5% FCS; 0.2% CO_2-equilibrated Na_2CO_3 was used as buffer

a) Responses with 10% FCS

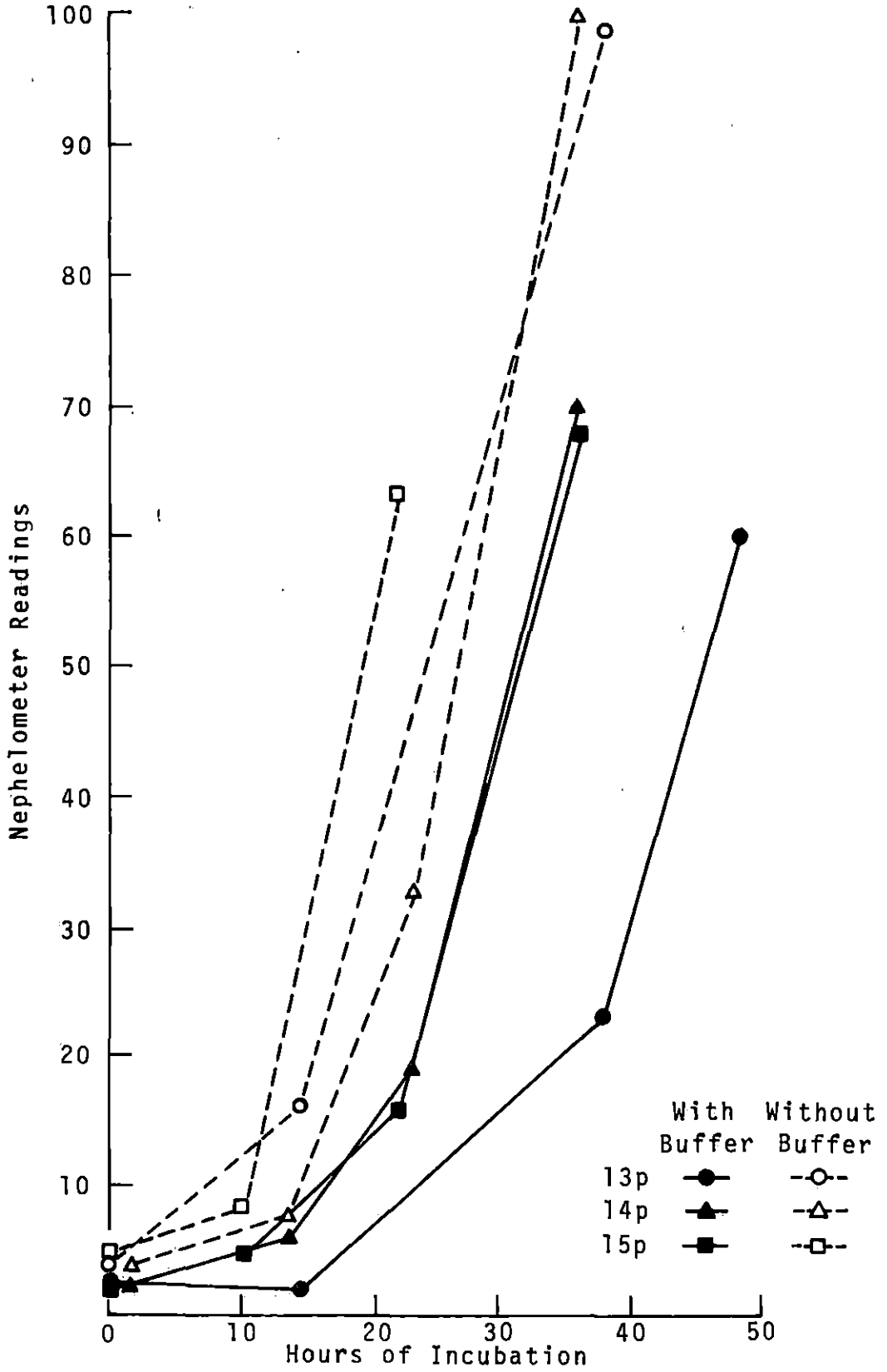

Nephelometer Readings
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
13p
14p
15p

Figure 13 (continued)

b) Responses with 8% FCS

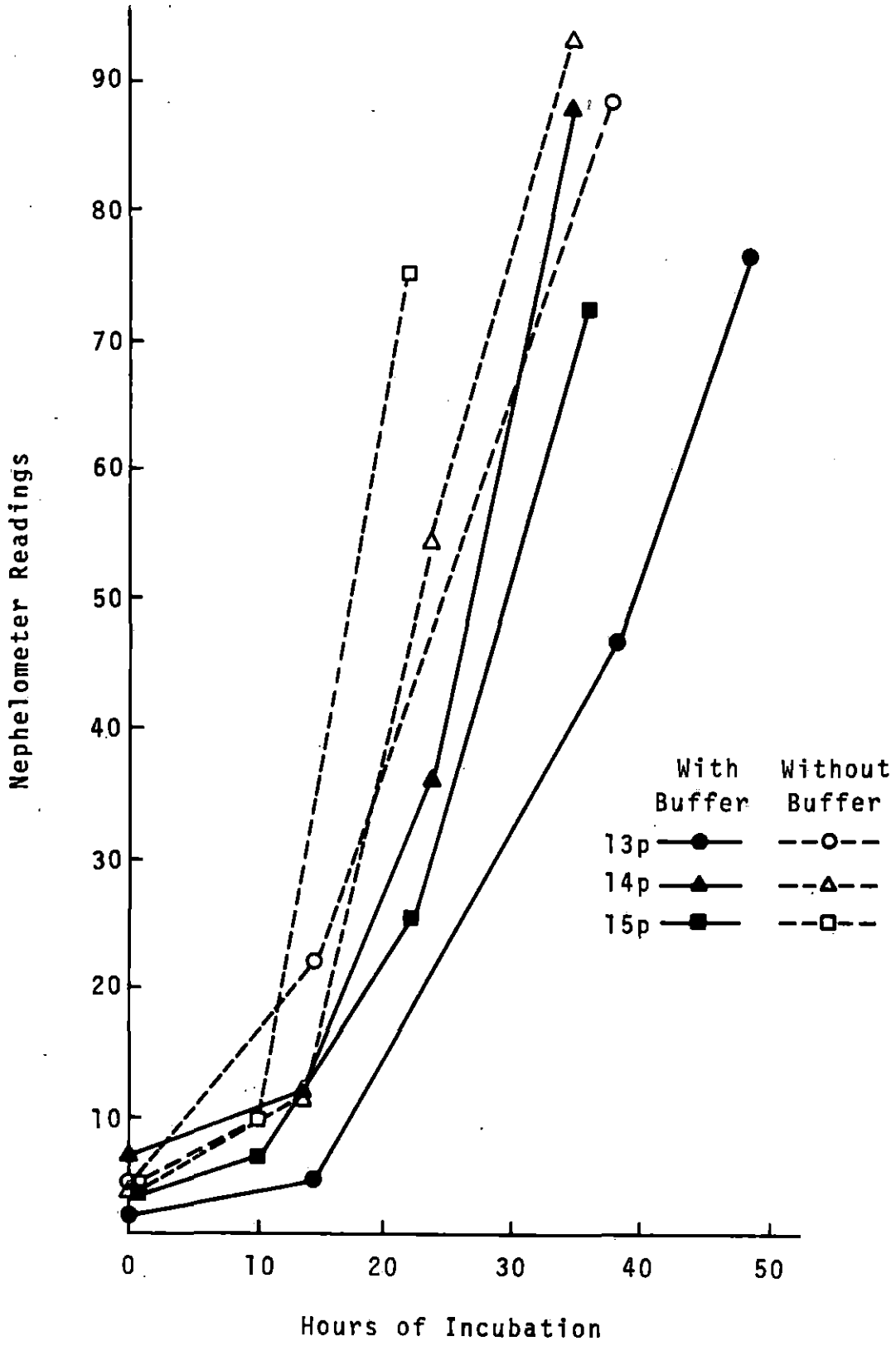

Nephelometer Readings
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
13p
14p
15p

Figure 13 (continued)

c) Responses with 5% FCS

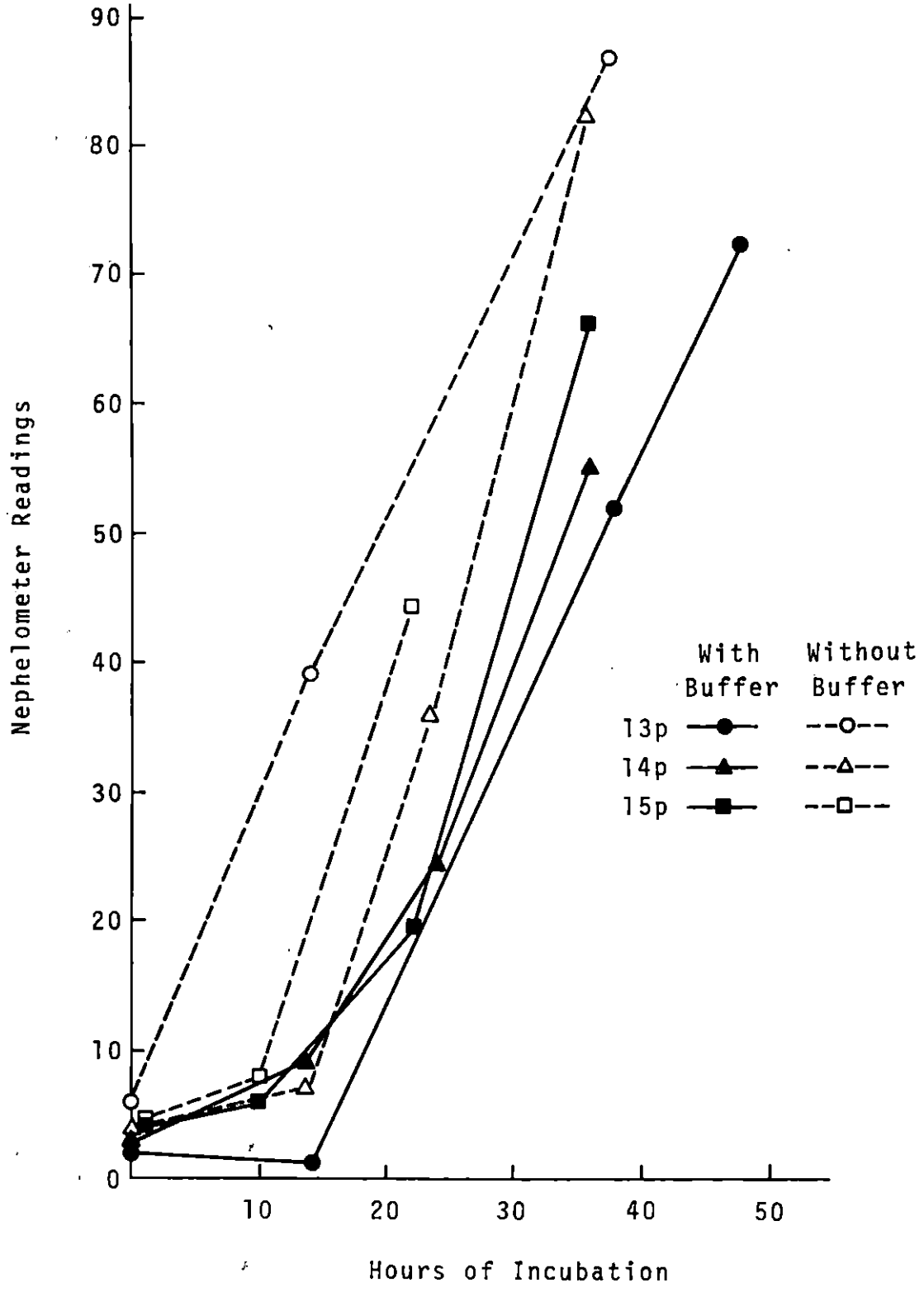

90
80
70
60
50
40
30
20
10
0
Nephelometer Readings
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
13p
14p
15p

Figure 14. Growth responses of cultures in unbuffered medium and in buffered medium at initial pH 7.0. Cultures were supplemented with 10%, 8%, or 5% FCS; 0.2% CO_2-equilibrated Na_2CO_3 was used as buffer

a) Responses with 10% FCS

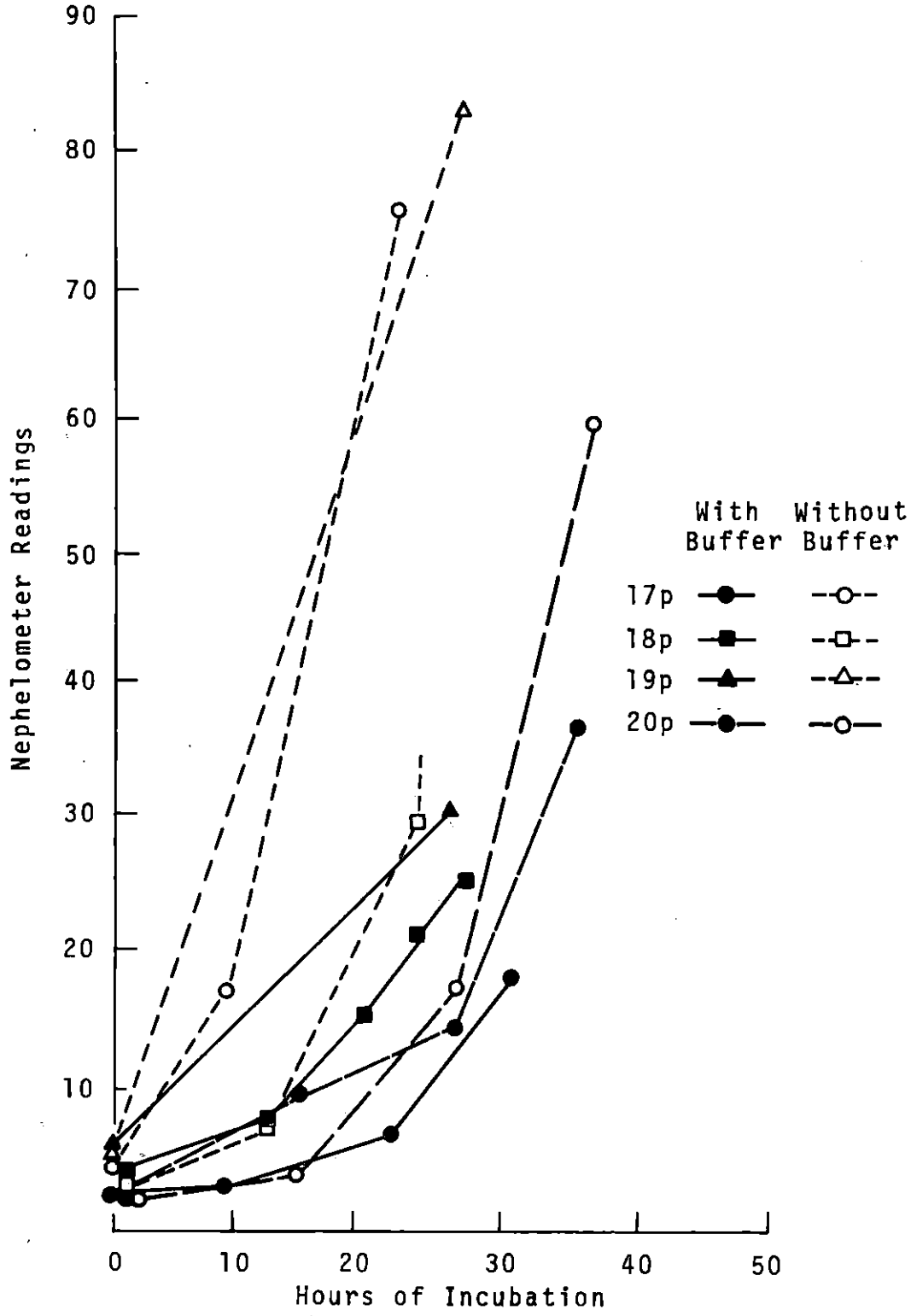

90
80
70
60
50
40
30
20
10
Nephelometer Readings
0
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
17p
18p
19p
20p

Figure 14 (continued)

b) Responses with 8% FCS

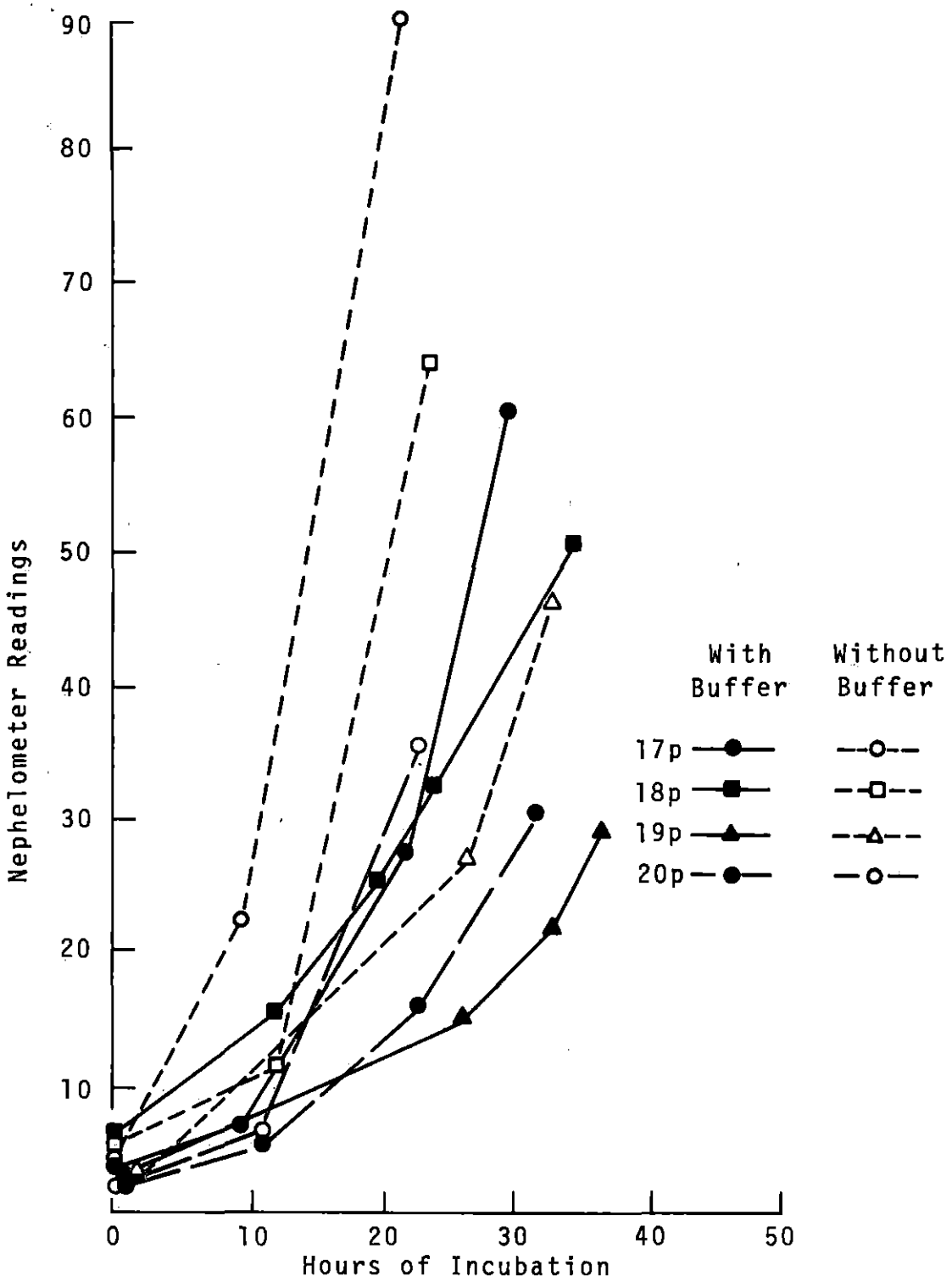
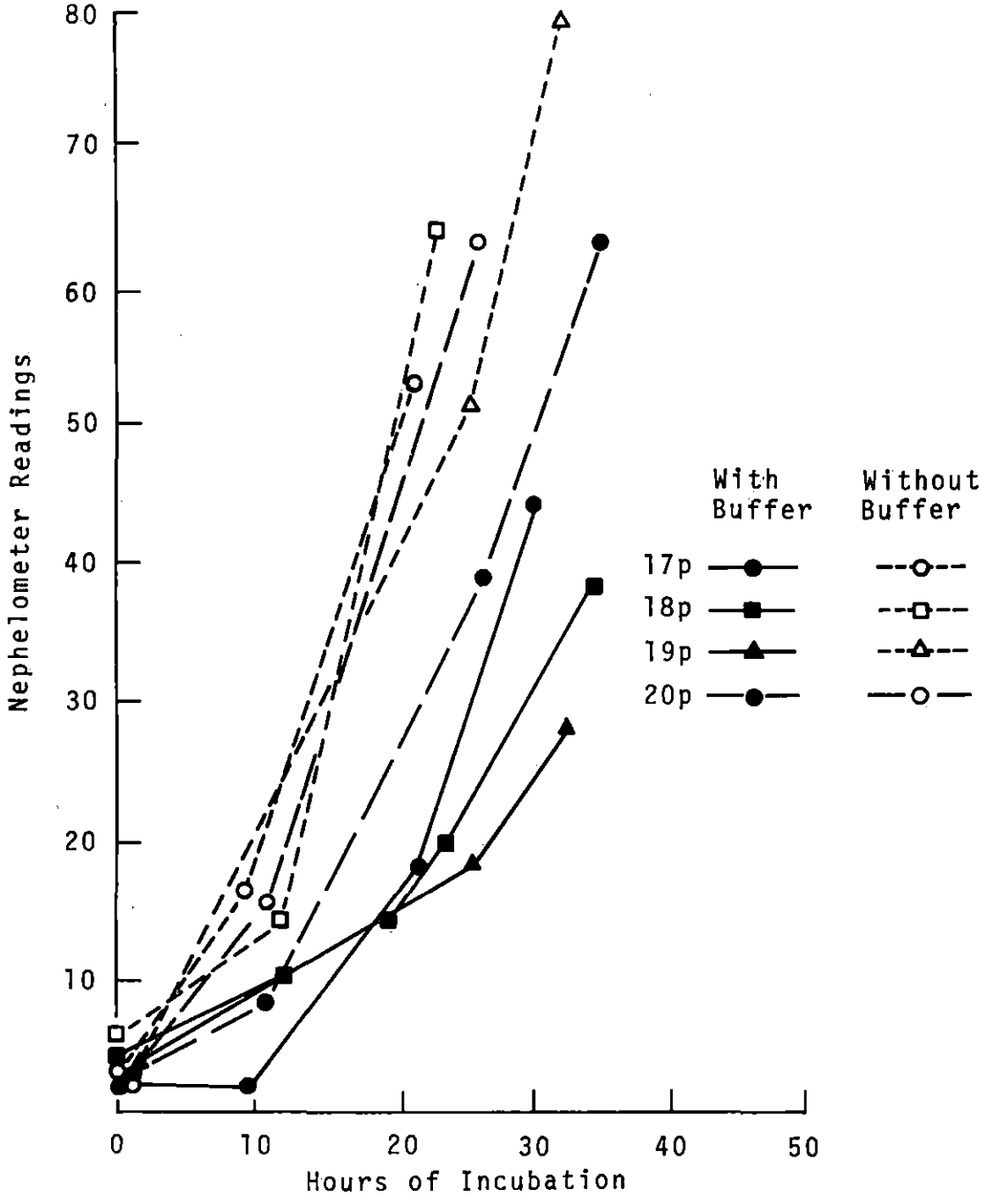

Nephelometer Readings
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
17p
18p
19p
20p

Figure 14 (continued)

c) Responses with 5% FCS


80
70
60
50
40
30
20
10
Nephelometer Readings
0
10
20
30
40
50
Hours of Incubation
With Buffer
Without Buffer
17p
18p
19p
20p

DISCUSSION

Studies on the quantitative methods for growth of T. hyodysenteriae have shown that nephelometry and direct cell counts are acceptable and simple methods for the measurement of growth. A comparison of total cell numbers and viable cell numbers per ml shows that the methods gave similar results (Table 1) and both methods showed similar trends throughout the study (Figures 1 and 2). One sample taken at 48 hours yielded a considerably lower average viable determination than the other sample, which resulted in an average viable number of less than half of the total number. This may have occurred because of a technical error in performing the dilutions or plating of the sample.

Doubling of cell mass occurred every 10-12 hours in these studies. This is considerably slower than the six hour rate determined for isolate B204 in PRAS-TSB (Kinyon, 1974). The differences in growth rates for the same organism may reflect the adaptability of the organism and are likely due to the differences in the reduced and aerobic TSB medium.

Because of a recent controversy concerning the correct method for plotting turbidimetric measurements of total growth (Costilow, 1975; Bryant, 1975; Dworkin, 1975), consideration of how to present data on growth of T. hyodysenteriae measured nephelometrically was in order. The

methods in question are logarithmic plots versus arithmetic plots of nephelometer readings to reflect increases in cell mass.

Logarithmic plots were used to determine growth rates and phases, as well as to determine if nephelometric measurements had a linear relationship to cell numbers. Once the relationship was established as being linear (Figure 2) and a direct parallel relationship between nephelometer readings and direct total cell counts was demonstrated (Figure 3) arithmetic plots were used to demonstrate real differences in cell mass. Tube cultures of T. hyodysenteriae consistently yielded maximum total cell numbers of less than 5×10^8 cells per ml. With the low yields of total mass obtained, real differences in measurements of mass were considered important for better visualization of the data. The arithmetic plots of turbidimetric measurements have been preferred and used for comparison of growth by other workers for similar reasons (Bryant, 1975; Ellinghausen, 1959, 1973a).

Standardization of inoculum further established nephelometry as a reliable tool for studying growth responses of T. hyodysenteriae. Inoculum standardized from a culture with a nephelometer reading of 70 grew more rapidly than cultures initiated with inoculum standardized from less dense cultures; however, the total cell number

per ml was also greater than the numbers in other standardized inoculum. Nephelometer readings of cultures had been done by first inverting cultures three times and then allowing the nephelometer hairline to come to rest before determining the reading of the culture; it is probable that the diluted inoculum was not equal to an exact reading of 30. Some variation in total cell counts of the other standardized inoculum was also observed, but initial readings of the transferred inoculum are approximately equal; initial readings of transferred cultures from inoculum with a reading of 70 are greater than those of the other groups (Figure 6). A comparison of the curves shows that cultures initiated with standardized inoculum from cultures with nephelometer readings 30-60 are almost identical (Figures 5 and 6). The most variation is observed in the lag and in the stationary phases.

It was hoped that cultures of nephelometer readings 30-50 could be accurately standardized to obtain repeatable growth responses for consecutive passages; however, as the organism was continually subcultured repeated curves were not always obtained. Very likely this indicates the need for some adaptation to the growth medium and conditions (Lamanna et al., 1973). Unfortunately this may be a difficult problem to solve because after 16-20 <u>in vitro</u> passages the organism was very predictable, but lost much of

its virulence. It is not advisable to sacrifice virulent cultures for predictability and convenience.

The unpredictability of the organism made determination of the optimal pH and optimal temperature difficult. The preliminary data obtained with B140 and B204 (Table 3) show that T. hyodysenteriae grows to a greater total cell mass at 37C-39C in TSB-FCS at near neutral or slightly alkaline pH (Figures 7 and 8). Very little increases in mass occurred at 34C, but viable cells were maintained for at least three days. Isolate B204 grew better than isolate B140 at 39C-42C. Viability determinations of the standardized B140 inoculum (Table 4) show an increase in cell numbers per ml as the medium pH increases from 6.5-7.25; a pH range 7.0-7.8 allowed best growth of B140. An increasing trend in viable cell number per ml of standardized B204 inoculum was not observed for increases of pH from 6.75-8.6. It is likely that equilibration of TSB-FCS with H_2:CO_2 minimized the effects of the medium pH (Tables 5 and 7) and large numbers of cells in the inoculum masked any differences in the ability of different pH values to support growth of T. hyodysenteriae. Similar growth curves for pH 7.5-8.6 at 37C and pH 7.0-8.0 at 39C were obtained for isolate B204 (Figure 10); pH 8.6 at 39C differs considerably and is not easily explained. The medium at pH 8.6, which decreased to pH 7.2-7.4 after equilibration,

consistently yielded greater viable cell numbers per ml than standardized medium at other pH values incubated at 39C, but this did not occur at 37C (Table 6) therefore, a pH of 8.6 incubated at 39C seemed to be optimal for B204. Medium at pH 8.6 formed a precipitate after autoclaving which made nephelometer readings difficult to make and probably inaccurate.

Inability to consistently obtain repeatable data for the optimal temperature and pH of the medium prompted the use of serial dilution of inoculum. Only three temperatures were tested with this method because there was a long-term shortage of incubator space at the time and preliminary trials indicated that growth was approximately equal at 37-39C. One incubator (42C) was in an unairconditioned room and the temperature fluctuated from 40-43C.

Only isolate B204 was used because of the technical difficulty encountered with using two different isolates. The method of serially diluting standardized inoculum in duplicate series clearly established 38C as an optimal temperature and indicated that pH 7.5 was an optimal initial pH, evidenced by the ability to consistently support growth initiated by small numbers of cells per ml. The method has been used to observe effects of different substrates on growth of the Leptospira, which also have low maximal yields (Ellinghausen, 1973b). It is not uncommon

for host-associated microbes to have a limited range of temperatures and of pH values at which growth will occur (Lamanna et al., 1973).

Growth of T. hyodysenteriae has not occurred at 30C (Kinyon, 1974) and was very limited at 34C and at 42C (Table 9). Of the temperatures tested, an optimal range of 37-39C could have eventually been determined by serial passage with large numbers of cells in the inoculum; however, data with low numbers of B204 in the inoculum readily and dramatically shows the sensitivity of the organism to incubation temperature (Table 9).

Evidence that cells remained viable at 34C even though growth did not occur (Table 3) was confirmed when diluted inoculum in cultures held at 34C for seven days with no evidence of growth, grew within two days incubation at 38C.

Incubation temperature affects primarily the growth rate and nutritional requirements of a bacterium. As with other mesophiles growth rate decreases rapidly about 42C. Because lipids are a major component in the treponemes the effect temperatures above 40C may have on lipids may influence growth of T. hyodysenteriae more than other mesophiles. As the temperature is increased the total lipid portion of the cell decreases and those present tend to be saturated fatty acids (Ingraham, 1962).

In general nutritional demands of bacteria increase

at greater than optimal temperatures. Growth in aerobic TSB-FCS is decreased at 42C, but is more rapid on blood agar at 42C than at 37C (Kinyon, 1974). The fetal calf serum used to supplement TSB contains minimal amounts of hemoglobin (18 mg%, determined by GIBCO[1]). Ability to grow on ½X TSA supplemented with 5% blood but not in ½X TSB-FCS seems to indicate factors present in blood that are required for growth of T. hyodysenteriae and that are compensated for by TSB in the liquid medium. It is also possible that the differences of solid medium versus liquid medium influences growth of this organism.

In contrast to the methods feasible for determining an optimal temperature, the optimal pH for B204 could not have been determined with large numbers of cells as inoculum. Because the medium was not buffered and responses to the initial pH were similar with large numbers of cells in the inoculum (Figure 10 and Table 11), definition of an optimal initial pH value was not possible. Growth curves with nephelometry have been used to define requirements of Leptospira (Ellinghausen, 1960). It seems that a large inoculum grows better at the more alkaline initial pH and may be due to neutralization of acidic

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[1]Quality control analysis by Grand Island Biological Co., Grand Island, New York.

metabolites produced by cultures of T. hyodysenteriae (Kinyon, 1974) because the pH of cultures decreased after growth (Table 7). Isolate B140 did not subculture well at 39C in pH greater than 7.8, which indicated that a practical and more definitive method was necessary to define optimal growth conditions.

The differences in growth observed for the two isolates under various conditions indicates the necessity of studying more than one strain or isolate of T. hyodysenteriae to define optimal growth conditions and substrate requirements. Biochemical differences also occur among isolates though major similarities exist among pathogenic isolates (Kinyon, 1974). A slightly alkaline optimal pH was expected because increases in the pH of the colonic contents of pigs with dysentery (Elazhary et al., 1973) and other enteric diseases (Kenworthy, 1973) have been reported.

As cultures approached the stationary phase of growth, round-bodies were qualitatively more numerous. These forms have been previously reported for T. hyodysenteriae (Kinyon, 1974) and for other spirochetes (Lauderdale and Goldman, 1972). They probably occur as growth limiting nutrients become depleted and metabolites accumulate.

Observation of better growth with deoxygenated H_2:CO_2 than with CO_2 in TSB or in PRAS-CF-TSB supplemented with FCS supports previous data on H_2 stimulation (Kinyon, 1974).

Growth under H_2:CO_2 was also better and more consistent in aerobic TSB than in PRAS-CF-TSB. This may be due to differences in Eh and dissolved gasses such as O_2 in the two preparations and definitely warrants thorough investigation. Oxygen up-take by T. hyodysenteriae has been observed (Harris et al., 1975). Paired combination of H_2, CO_2, and N_2 in different proportions may be helpful in defining requirements for specific atmospheres.

The determination of 37-41 mg/ml protein in FCS as a technical reference compares favorably with 34-35 mg/ml determined by GIBCO. A major growth supporting component in serum was found to be heat stable; it is probable that this major component is protein. Socransky and Hubersak (1967) found that heat stable α-globulin could replace the serum requirement for T. microdentium. Other physical properties of the growth factors in serum, such as extractability by organic solvents, need to be studied.

Dilution of inoculum in TSB supplemented with different serum concentrations emphasizes the FCS requirement of the organism, particularly when minimal inocula are employed. Inoculum of 10^7 cells per ml grew in medium with no serum supplement, but 10^6 cells per ml did not grow during five days incubation. Isolate B204 has been serial passaged six times in PRAS-TSB without FCS (Kinyon, 1974).

Excellent growth was observed with GG-free serum at 10%

concentration for eight serial passages. Although fetal calf serum is known to have low concentration of antibodies, perhaps globulins inhibit growth of T. hyodysenteriae. Comparison of growth with greater concentrations of sera and minimal inocula may indicate differences in tolerance of the organisms to these sera. Kinyon (1974) observed growth with 20% normal FCS, but growth yields in the presence of high concentrations of serum have not been quantitated nor have isolates been continually subcultured. On the other hand, bactericidal activity of fetal pig serum against some E. coli strains has been reported (Schwab and Reeves, 1966); Hardy and Munro (1966) have reported that 10% rabbit serum was toxic for T. microdentium.

Although serum requirements for other spirochetes have been replaced with albumin and fatty acids (Ellinghausen and McCullough, 1965; Johnson and Eggebraten, 1971; Oyama et al., 1953) BSA and Tweens or short-chained fatty acids did not replace the serum requirement for growth of T. hyodysenteriae. Perhaps concentrations less than 0.05% would not be toxic and should also be evaluated. Livermore (1974) observed that Tweens or fatty acid salts complexed with BSA were still toxic for T. refringens biotype refringens, to which T. hyodysenteriae is biochemically similar (Kinyon, 1974).

It was hoped that stimulation or enhancement of growth

might occur with tissue culture supplements because T. hyodysenteriae is closely associated with host cells (Glock et al., 1974). Spirochetes did not survive at all in these media unless FCS was added. Some stimulation was observed with Wo_5 + BSA_{2000} and NCTC-135 in TSB and as serum supplemented medium. Glucose may stimulate growth slightly in NCTC-135. Utilization of carbohydrate by T. hyodysenteriae has been reported (Kinyon, 1974) so that some stimulation in less than optimal medium was expected. Both NCTC-135 and Wo_5 + BSA_{2000} have low concentrations of Tween 80 which may be slightly stimulatory to growth.

The requirement for BSA by L. Pomona and L. canicola was eliminated by detoxified Tween 80 and acetate, pyruvate, and glycerol (Staneck et al., 1973). Davis and Dubos (1947) found that albumin would bind the fatty acids in Tween 80 and permit growth of Mycobacterium. Perhaps extraction of free fatty acids from Tween 80 with ether or purification by column chromatography would decrease the toxicity of the Tweens sufficiently for T. hyodysenteriae and should be evaluated. It is unlikely that the organisms would grow in the presence of fatty acids without the benefit of a protective binding protein such as BSA. The Tween concentrations employed may reduce the surface tension too much for T. hyodysenteriae; however, Stalheim (1966) observed that leptospiral growth increased as surface tension

decreased with increases of 0.005% to 0.1% Tween 80.

It was significant that growth occurred readily in serum supplemented FG-TSB because many of the supplements were not autoclavable and it was thought that filter sterilized supplements might introduce toxic levels of O_2 when added to test medium. Trypticase has more of the substrates required for growth than phytone and aerobic preparations supplemented with FCS supported better growth than the reduced preparations, but growth was not equivalent to that obtained in complete TSB-FCS.

Of the buffers that were tested in TSB-FCS, CO_2-equilibrated Na_2CO_3 allowed the best growth. Bryant (1952) observed erratic growth of a rumen spirochete with phosphate buffers and stable growth with CO_2-equilibrated bicarbonate buffer. This type of buffer has been used successfully and described by several investigators of anaerobic organisms (Hungate, 1966; Bryant and Burkey, 1953; Caldwell and Bryant, 1966).

Treponema hyodysenteriae passaged quite easily in buffered medium supplemented with 5% FCS. There were longer periods for the adjustment phase (lag phase) in buffered cultures (Figures 15 and 16), but the pH in buffered cultures was maintained better than in unbuffered cultures.

Hardy and Munro (1966) were able to replace a serum

requirement with bicarbonate. Bryant and Burkey (1953) showed a corresponding growth response by rumen spirochetes to increased concentrations of carbonate.

The concentration used was according to Hungate's recommendation (1966) for proportions of gases other than 100% CO_2. This is probably not the best concentration for T. hyodysenteriae because inoculum frozen at -80C in the presence of 0.2% buffer was unable to initiate growth in TSB-FCS. Low passage stock cultures were also very sensitive to this concentration of carbonate. Recovery of T. hyodysenteriae on blood agar has been possible after as long as two years at -80C (Kinyon, 1974).

It seems logical that buffered cultures should be stable to preservation by either freezing or lyophilization, otherwise the purpose of the buffer which is to stabilize culture growth and allow some predictability and manipulations without destroying viability or virulence is lost.

Inability of isolate B204 to induce dysentery in the baby pigs after 20 in vitro passages indicates that virulence of the organism can be easily lost. It is possible that very mild lesions occurred when the pigs had diarrhea; however, all of the typical signs of dysentery were not observed and any damage to tissue must have been very short-termed. It is possible that virulence is stressed more

when the organism is cultured in 10 ml volumes rather than 250 ml volumes from which isolate B204 was still virulent after 23 consecutive passages (Joens, L. A., Iowa State University, personal communication, 1974). It is therefore necessary that virulence of isolates and strains are periodically checked and maximum number of subcultures with retention of virulence in tubed medium be determined.

SUMMARY

Nephelometry and total cell counts have been shown to be accurate quantitative methods for the growth measurements of T. hyodysenteriae. Since the organism grows to low maximal yields in tube culture (less than 5×10^8 cells per ml), definition of optimal conditions was not directly possible with these quantitative methods. It was necessary to employ serially diluted inoculum to clearly define temperature and pH requirements. This technique may be helpful in evaluations of growth conditions and physical properties of the medium. It should also save time and aid in the screening of various substrates that might be required by T. hyodysenteriae for growth in minimal medium.

None of the substrates tested were shown to stimulate growth or be requirements for growth. Components in both trypticase and phytone are required and remain to be identified. The serum requirement was not replaced by any of the substitutes attempted. Of the buffer solutions tested, the best possible buffer was CO_2-equilibrated Na_2CO_3; all others did not allow consistent growth.

Inoculum of 1×10^8 viable cells per ml of 20th passage isolate B204 subcultured 12 times in TSB-FCS at initial pH 7.5 and grown at 38C induced diarrhea, but did not cause clinical swine dysentery in two young pigs.

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