THE EFFECT OF OXIDATION-REDUCTION POTENTIAL ON THE OUTGROWTH AND CHEMICAL INHIBITION OF *CLOSTRIDIUM BOTULINUM* TYPE E SPORES

by

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INTRODUCTION

Botulism has been recognized as a disease entity since 1793 (Geiger, 1941). Statistically it is not a significant public health problem. Only a score of such cases occur each year in the United States. On the other hand, the lethality rate of this disease (25% in 1974) is quite high compared to many other illnesses (USPHS, 1974a). In addition, as the commercial canning industry grows in volume and sophistication each year, the potential for the rapid and widespread distribution of a tainted product increases. Therefore, adequate processing and quality control measures must be continually emphasized and implemented. Food industry personnel and management must constantly be aware of the legal and moral consequences resulting from the distribution of adulterated or misbranded products. Also, of great economic importance to the food industry is the attendant adverse publicity which inevitably follows each outbreak of botulism.

The importance of the organism, Clostridium botulinum, and its hazard potential are reflected throughout the food industry. For example, all heat processes for low acid canned foods are based on a formula insuring the destruction of C. botulinum spores. The use of nitrite in the processed meats industry is based in part on its efficacy as an inhibitor of C. botulinum.

Other chemicals have been used to inhibit toxic and spoilage organisms in numerous types of foods. Such chemicals include sodium chloride, sucrose, glycerol, sodium benzoate, and substances used to

lower the pH of the product. As a result, many popular shelf stable products have been developed.

Extensive studies have been carried out to determine the levels of chemicals necessary to insure complete inhibition of C. botulinum. In such experiments variables other than the concentration of the specific chemical studied must be considered. These variables include: strain and serological type of the test organism, cell type (spore or vegetative), physiological condition of the inoculum (log phase or stationary, injured or uninjured), inoculum size, composition of the medium, incubation temperature, and oxidation-reduction potential (Eh) of the medium. Ideally these variables should be held at optimal growth conditions so that they will not constitute an additional inhibition. Several investigators have already shown synergistic inhibitive effects by pH and temperature, salt and temperature, pH and level of inoculum, pH and water activity, pH and Eh, salt and Eh, and others (Hanke and Bailey, 1945; Segner et al., 1966; Baird-Parker and Freame, 1967; Mead, 1969; Nassralla, 1971).

For more than a century anaerobic bacteria such as the clostridia have been known to require conditions of low oxygen concentration or low oxidation-reduction potential. Attempts to distinguish between a requirement for low Eh and a requirement for low oxygen concentration have met with difficulties since a change in dissolved oxygen generally results in a change in Eh. Also, because Eh per se is a reflection of the reducing and oxidizing systems present in the medium, a change in Eh develops from a concurrent change in chemical composition. If low

Eh is to be shown to be necessary or beneficial for the growth of anaerobes, it is obvious that several reducing systems must be studied to reveal a general tendency.

Food products are expected to exhibit a wide range of Eh values since each type varies in the amount and proportion of inherent reducing substances (i.e., reducing sugars, sulfhydryl containing amino acids and peptides, ascorbic acid). If the influence of oxidation-reduction potential determining substances on the growth of C. botulinum can be elucidated, the importance to the food industry is evident. The following study is an attempt to determine the effect (if any) of the oxidation-reduction potential and the oxygen concentration of the medium on the outgrowth and chemical inhibition of type E C. botulinum spores. In order to measure the Eh of the system and to prevent atmospheric oxygen from being an uncontrolled variable in this study, an airtight flask containing Eh and pH measuring electrodes was employed. In addition, strict anaerobic procedures and prereduced media were used.

REVIEW OF LITERATURE

A. *Clostridium botulinum* type E and Botulism

Clostridium botulinum types A, B, E, and F have been recognized as causing human cases of the foodborne intoxication called botulism. *C. botulinum* types C and D, while possibly involved in human cases, are of minor importance (Dolman, 1964). The organism is a gram positive, spore-forming, anaerobic bacillus. It is associated with a soil habitat and is found in all areas of the world. Type A is found more frequently in the soils of the western United States and New York, while type B occurs more frequently in the eastern states and Europe (Riemann, 1968). The distribution of type E spores seems to be confined more to estuarine waters (e.g. off the shores of northern Japan (Nakamura *et al.*, 1956), in Copenhagen Harbor (Johannsen, 1963), in the Great Lakes (Bott *et al.*, 1968), and on the Pacific (Eklund and Poysky, 1970), Eastern (Ward, 1970) and Gulf (Ward and Carroll, 1965) Coasts. It is not surprising that many aquatic and marine specimens contain type E spores. Bott *et al.* (1966) reported that 9% of fish from Lake Michigan and 57% from Green Bay contained viable type E spores. The ability of type E organisms to ferment sucrose and mannose aids in its differentiation from types A and B. Toxin neutralization tests are also utilized for identification. The vegetative organisms produce a type-specific, heat labile, protein exotoxin which is highly lethal to man by a neuro-paralytic mechanism (Riemann, 1968). The type E toxin has been shown to be activated by trypsin (Duff *et al.*, 1956). Outbreaks of botulism have occurred in a wide variety of foods; however, type E

has been primarily restricted to fish products— canned, dried or smoked (USPHS, 1974a). Toxin containing foods are generally devoid of large numbers of other bacteria. This may indicate that the organism cannot compete well with other microorganisms (Jay, 1970).

B. Growth and Chemical Inhibition of C. botulinum

The optimal growth temperature for types A and B is about 37 C (Ohye and Scott, 1953), whereas type E grows best at 30 C (Riemann, 1968). In addition, type E spores have been shown to be able to germinate, grow, and produce toxin at 3.3 C (Schmidt et al., 1961; Grailkoski and Kempe, 1963). Schmidt et al. (1961) obtained gas and toxin production at 3.3 C after 31 to 45 days incubation. Growth of a non-proteolytic type F has been reported to occur at 4 C (Walls, 1967). Ohye and Scott (1953) reported no growth from spore inocula of types A and B at a temperature of 45 C and above, while they showed that inhibition of vegetative inocula required 50 C or above. The upper temperature levels for growth of type E spore and vegetative inocula are approximately 5 degrees less than those cited above for types A and B.

Segner et al. (1966) have shown that the limiting concentration of sodium chloride for spore outgrowth of type E is a function of the incubation temperature. At the optimal growth temperature (30 C) more sodium chloride (up to 5%) was needed to completely inhibit outgrowth than at lower temperatures. Lechowich (1968) summarized the results of his experiments and others (Segner et al., 1966; Baird-Parker and Freame, 1967; Ohye and Christian, 1967; Emodi and Lechowich, 1968a; Emodi and Lechowich, 1968b) and indicated a range of 4.9 - 5.8% sodium chloride to

completely inhibit outgrowth of type E spores (See Table 1). He also summarized the levels required for sucrose inhibition (Table 1). Emodi and Lechowich (1968b) reported 38.5% sucrose for complete inhibition of type E. Segner et al. (1966) found that inhibition of outgrowth of type E spores by pH is affected by the incubation temperature and the level of inoculum. Outgrowth occurred at pH levels as low as 5.03 in Trypticase peptone glucose medium at 30 C if a large spore inoculum (20 million) was used. At 8 C outgrowth did not occur at pH 5.7. Baird-Parker and Freame (1967) observed that by lowering both the pH and water activity (a_w) of the medium (Reinforced clostridial medium) the minumum growth levels of each of these factors was increased. This synergistic inhibition applied to both germination and outgrowth of C. botulinum types A and E. It is generally agreed that the limiting pH for outgrowth of C. botulinum type A and B spores is pH 4.8 - 5.0 (Ingram and Robinson, 1951)(See Table 2). However, recently botulism outbreaks have been traced to high acid (pH less than or equal to 4.6) home-canned food (USPHS, 1974b). Dowell and Merson (1974) have reported on five cases of botulism from acid foods during 1967-1974. A synergistic growth effect has been reported between clostridia and lactic acid bacteria (Benjamin et al., 1956). Meyer and Gunnison (1929) observed the growth and toxin production of C. botulinum at pH 4.0 in the presence of yeasts. These yeasts may produce growth factors, lower the Eh of the medium, or raise the pH prior to clostridial growth (Jay, 1970). Vacuum packing of fish has been demonstrated to enhance toxin production when compared to the non-vacuum packaged product (Kautter, 1964; Abrahamsson et al., 1965).

Table 1. Inhibitory levels of NaCl and sucrose required to inhibit *Clostridium botulinum* under optimal conditions.*

Type of *C. botulinum*	Inhibitory substance	Inhibitory concentration percent (w/w)	Inhibits
A and B	NaCl	8.5 - 10.5	Growth of vegetative and spore inocula.
A and B	NaCl	6.25 in aqueous phase of ham.	Proteolysis and putrefaction--not toxin production.
A and B	NaCl	9.0	Toxin production
A and B	NaCl	8.2	Growth of vegetative inocula.
E	NaCl	5.0	Spore outgrowth.
E	NaCl	5.8	Growth of vegetative cells.
E	NaCl	6.5 (4.8)	Growth of vegetative cells.
E	NaCl	4.9	Spore outgrowth.
A and B	Sucrose	50-55	Growth of vegetative cells.
B	Sucrose	30	Growth of vegetative cells.
B	Sucrose	31	Spore germination.
E	Sucrose	38.5	Spore outgrowth.

*From Lechowich (1968).

Table 2. pH limits for outgrowth of *Clostridium botulinum*.*

Type of *C. botulinum*	Inoculum	Lowest pH permitting Germination	Lowest pH permitting Outgrowth
A and B	Spores		4.6 - 5.0
A	Spores	5.3	5.3
A	Vegetative cells		5.0
B	Spores (30 C)	5.0	5.0
B	Spores (20 C)	5.0	no growth
B	Vegetative cells		5.3
E	Spores	5.0	5.3
E	Vegetative cells		5.3
E	Spores in TPG (30 C)		5.03
E	Spores in TPG (8 C)		5.90
E	Spores in liver broth	5.01	5.22
E	Spores at 15.6 C		5.2
E	Spores at 10.0 C		5.4
E	Spores at 7.2 C		5.6
E	Spores at 5.0 C		6.0

*From Lechowich (1968)

This is probably due in part to a restriction of competitive aerobes.

Reports on the requirement of bicarbonate for germination and outgrowth of C. botulinum spores have reflected a variety of results. Ward and Carroll (1966) found that neither carbon dioxide nor bicarbonate seems to be necessary for spore germination and vegetative growth of C. botulinum type E in their synthetic media. Holland et al. (1970) concluded that the germination of C. bifermentans and C. sporogenes spores was enhanced by the addition of carbon dioxide or bicarbonate. Their studies, however, showed no absolute requirement for such components. An absolute necessity for bicarbonate in the medium for the germination of C. botulinum type A spores was demonstrated by Treadwell et al. (1958). King and Gould (1971) suggested that carbon dioxide, rather than bicarbonate, was the active stimulant in the germination of C. sporogenes. They based this finding on their observance that the bicarbonate stimulation was greater at low pH.

Barker and Wolf (1971) found that thioglycolate showed varied effects on germination in clostridia depending on strain and conditions. Thioglycolate generally seemed to stimulate germination in media lacking carbon dioxide or bicarbonate. They, however, showed that at levels of 0.05% and 0.1% thioglycolate was inhibitory to vegetative growth in all three strains studied.

Nassaralla (1971) used anaerobic prereduced media to study the affect of reducing agents on the vegetative growth of C. botulinum type E. He found that Cleland's reagent, cysteine hydrochloride and sodium formaldehyde sulfoxylate allowed the best growth.

Sodium nitrate and nitrite have been shown to be an effective inhibitor of C. botulinum. They have been used to inhibit clostridial growth in processed meat products and cheese. Formation of nitric oxide is considered the major inhibitive mechanism. Inhibition in cheese by nitrate was probably the result of an increased redox potential (Jay, 1970).

C. Oxidation-Reduction Potential

1. Theory and Methods

The reduction potential of a metal ion, nonmetal or compound is a measure of the tendency of the substance to accept an electron from an electron donor. The higher the potential the greater the affinity of the acceptor for the electron. Oxidation is the loss of electrons from a substance. Analogously, oxidation potential (used primarily by physical chemists) is a measure of the tendency to donate electrons. These oxidation and reduction potentials are related to chemical equilibria by the Nertz formula:

$$Eh = Eo + \frac{RT}{nF} \ln \frac{[Ox.]}{[Red.]}$$

where Eh = reduction potential of the reaction with reference to the standard hydrogen electrode, R = molar gas constant, T = absolute temperature, F = Faraday constant, n = number of moles of electrons specified by either of the half-reactions that comprise the balanced net reaction, Eo = standard oxidation-reduction potential of the reaction, [Ox.] and [Red.] = molar concentration (more exactly, the activities)

of the oxidized and reduced forms in the system. It is evident from this equation that the oxidation-reduction potential, Eh, is dependent on Eo (a constant for the system), the ratio of oxidized and reduced forms in the system, and the temperature.

The potential of a system is not only a function of the inherent reducing or oxidizing tendency but also of the hydrogen ion concentration as follows:

$$Eh = Eo + \frac{RT}{nF} \ln \frac{[Ox.]}{[Red.]} + \frac{RT}{nF} \ln \frac{Ka}{[H^+] + Ka}$$

where Ka is the equilibrium constant for the acid dissociation of the reductant, i.e.

$$\frac{[Red.]\ [H^+]}{[H\ Red.]} = Ka$$

Leistner and Mirna (1959) used the equation:

$$Eh7 = E_{observed} + E_{reference} + 2.303\ (RT/F)\ (pH\ X\ -7.0)$$

to determine the Eh adjusted to pH 7.0 (Eh7) in order to compare Eh measurements determined at different pH values. By definition, the Eh is the potential referred to the normal hydrogen electrode which is arbitrarily given the value of 0.0 mv. By coupling a redox half-reaction of known potential (e.g. hydrogen electrode) to one of unknown potential, the potential of the unknown system can be determined. Indeed, empirically determined values for Eo exist for most common oxidation-reduction reactions.

Since the hydrogen half-cell is not a practical possibility, any convenient, reproducible standard half-cell of a known potential with reference to the hydrogen electrode may be used (e.g. calomel, quinhydrone or silver-silver chloride). The other half-cell requires simply an inert surface (e.g. platinum or gold) in contact with the system to be measured through which electrons can be donated or accepted. The direction and flow of electrons is determined through a system incorporating a potentiometer and galvanometer. As a result, any difference in potential between the half-cells can be quantitated.

Oxidation-reduction potentials of organic and biochemical systems are thoroughly discussed by Clark (1960) and Hewitt (1950).

2. Media

The redox potentials of bacteriological growth media have been measured by several investigators including Hirsch and Grinsted (1954) and Futter and Richardson (1970). Mossel (1971) proposed that the redox potential of most foods is strongly poised and resists any changes brought about by atmospheric oxygen. Fresh foods have been shown to contain many redox poising compounds such as sulfhydryl amino acids and peptides, reducing sugars, and ascorbic acid (Mossel and Ingram, 1955; Wirth and Leistner, 1970).

3. Germination and growth

Pasteur (1861, 1863) reported the existence of microorganisms which could not only live in the absence of free oxygen but, in fact, could not survive in its presence. He postulated that oxygen <u>per se</u> was toxic to anaerobes. This concept has been supported by many

researchers to the present time. Dack and Willison (1929) have shown that the viability of young C. botulinum cells is reduced considerably when they are exposed to oxygen. Liefson (1931) found that growth and sporulation of C. botulinum was inhibited by greater oxygen levels. Knaysi and Dutky (1936) inhibited the growth of a clostridial organism more effectively in an oxygen poised system than in a potassium ferricyanide system poised at a similar high potential. O'Brien and Morris (1971) attempted to determine whether dissolved oxygen or elevated Eh caused the cessation of growth of C. acetobutylicum under aerobic conditions. They used artificial electron acceptors (potassium ferricyanide, dithiothreitol, diamide and metronidazole) and oxygen to poise each growth medium at the desired level. They concluded that the free oxygen in the medium and not the Eh per se was the determining factor in causing growth inhibition. Holland et al. (1969) stated that it appeared likely that in complex media oxygen rather than Eh was the factor inhibiting the germination of PA 3679 S2 spores. Wynne and Foster (1948a) reported a drastic fall in the number of C. botulinum 62A spores which germinated following momentary exposure to air during anaerobic incubation. They also found that the germination of mature spores was considerably delayed in the presence of air (Wynne and Foster, 1948b). The germination of C. botulinum and C. roseum spores was found by other investigators to be inhibited by oxygen (Hitzman et al., 1957). Hambleton and Rigby (1970) concluded that there is a distribution of sensitivity to molecular oxygen for both the germinative processes and the biochemical processes which lead to emergence.

McLeod and Gordon (1923a, 1923b) proposed the catalase theory of anaerobic growth based on their findings and others (Callow, 1923) that anaerobes were devoid of the enzyme catalase. Without this enzyme the hydrogen peroxide formed by the reductive processes of the bacteria in the presence of oxygen would accumulate and eventually kill organisms. However, organisms exist which are not sensitive to hydrogen peroxide and do not form catalase (streptococci). Also, if sodium sulfide is used as an inhibitor of catalase activity, the organisms producing catalase are not inhibited during aerobic growth. Sherman (1926) indicated that a group of propionic acid bacteria existed that could be classed as strict anaerobes which produced large amounts of catalase. Avery and Morgan (1924) showed that aerobic growth of anaerobes was enhanced by adding sterile plant extracts presumably because of the catalase and peroxidase activity contained within the extracts.

Recently, a compound more toxic than hydrogen peroxide has been found to be produced by most biological systems in the presence of oxygen -- the reactive intermediate superoxide (O_2^-) resulting from the univalent reduction of oxygen (McCord and Fridovich, 1969). All organisms capable of aerobic growth have been found to contain the enzyme superoxide dismutase which catalyzes the reaction of the superoxide radical as follows:

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

Strict anaerobes do not produce the enzyme; while, it is found in all aerotolerant organisms except Lactobacillus plantarum which does not produce superoxide radicals when grown in air (McCord et al., 1971).

Direct oxygen toxicity of anaerobes has not been reported by all investigators. Quastel and Stephenson (1926) were able to grow anaerobes in the presence of air if the potential of the medium was sufficiently lowered by addition of 0.1% cysteine, glutathione or thioglycolic acid as reducing agents. Aubel and Houget (1945) found that Clostridium saccharobutyricum cells were neither killed nor seriously injured when exposed to oxygen. Laskin (1956) observed that oxygen had no direct toxicity for C. botulinum except in the case of newly germinated cells. He found that in the presence of oxygen germination could occur, but the resulting young cells might be destroyed. Hanke and Katz (1943) felt that the limiting factor in the growth of C. sporogenes and Bacteriodes vulgatus was the Eh level of the medium and not oxygen tension. Reed and Orr (1943) conducted their experiments utilizing Clostridium welchii and determined that a favorable O/R potential was the essential requirement for optimal growth. Fildes (1929) and Knight and Fildes (1930) found that there existed a positive upper limit of O/R potential above which germination of Bacillus tetani will not take place. Clark (1924) suggested that the term "anaerobe" should be applied to a microorganism which requires for growth an adequate reducing intensity in its surroundings. Dubos (1929a, 1929b) concluded from his work that the stimulatory effects on anaerobic growth could not be explained solely on the basis of the removal of oxygen from the broth. He suggested that a low oxidation-reduction potential per se must be established prior to growth.

It has been suggested that upper Eh limits exist above which many anaerobes will not grow. Vennesland and Hanke (1940) found that

the growth of Bacteriodes was prevented in media exhibiting a potential higher than +150 mv at pH 6.6. C. sporogenes seems to have an upper limit of about 150 mv, while for C. histolyticum it is 90 mv, C. tetani, 110 mv and B. vulgatus, 140 mv (Smith and Holdeman, 1968). Variations in these growth limiting values exist among members of the same species. Hanke and Bailey (1945) reported that C. perfringens had a growth limiting Eh of about +105 mv at pH 6.0; while Barnes and Ingram (1956) showed for the same species a limit of 250 mv at pH 6.0. Chang (1946) studied the protozoa, Entamoeba histolytica, and related its growth to the Eh of the medium. He showed that the protozoa grew best in mixed culture with bacteria and suggested that the bacteria lowered the Eh of the medium to optimal levels. Smith and Hungate (1958) used suspensions of pasteurized Escherichia coli to lower the Eh by reducing any dissolved oxygen in the medium and inactivating any organic peroxides present in the medium.

Inhibition by certain organic peroxides is suggested to be a major factor in the growth of anaerobes (Smith and Holdeman, 1968). The exact nature of such compounds is not known precisely. These substances may be the oxidation products of amino acids or fatty acids, or they may result from other factors. Proom et al. (1950) observed that agar from different batches varied in its ability to inhibit anaerobes after exposure of the autoclaved medium to air. C. perfringens seems to be little affected by such organic peroxides, and other pathogenic clostridia are only slightly affected (Smith and Holdeman, 1968). On the other hand, Harmon (1973) has reported significantly increased counts of C. perfringens, C. botulinum, C. sporogenes, C. tetani, C. histolyticum, and

C. butyricum on media to which catalase has been added. Presumably the catalase destroyed peroxides present in the media and consequently promoted growth.

Wynne and Foster (1948) and Foster and Wynne (1948) showed that the germination of Clostridium botulinum spores was inhibited by traces of unsaturated fatty acids. Their vegetative cells remained uninhibited even at much higher concentrations.

Nassralla (1971) found that an excess of air was not as lethal as limited aeration of cells of C. botulinum. He suggested that peroxides could be the possible lethal compounds present and may be completely oxidized under conditions of high aeration.

Smith and Holdeman (1968) have suggested that the growth promoting effects of a combination of chopped meat with pea-sized balls of steel wool is a result of the slow evolution of hydrogen. They believe that the hydrogen not only promotes a low redox potential but also prevents the autocatalytic formation of organic peroxides.

Kligler and Guggenheim (1938) observed that vitamin C enhanced the growth of C. welchii. They suggested that the significant function of vitamin C was to lower the reduction potential of the medium. From their studies they concluded that favorable conditions for the growth of anaerobes are determined by the Eh of the medium rather than by other factors such as oxygen tension. They proposed that the favorable effect of plant extracts added to aerobic cultures of anaerobes was due to the reducing substances present and not to the catalase or peroxidases as suggested by Avery and Morgan (1924). Holland et al.

(1969) found that when spores of the anaerobe PA 3679 S2 were held in suboptimal conditions of oxygen concentration, thioglycolate acted as a powerful stimulant of germination. Ando and Inoue (1957) in studying the growth of C. botulinum type E in different types of fish homogenates observed a correlation between growth and oxidation-reduction potential of the fish medium. In squid medium, growth was inhibited due (as they suggested) to a positive potential resulting from the oxidation of sulfhydryl groups by trimethylamine oxide extracted from squid.

The ability of sulfhydryl compounds to promote growth of anaerobes has been noted by many investigators. Hewitt (1950) has compiled a list of almost fifty enzyme systems which probably require activation by sulfhydryl groups. Dickens (1946) found that pyruvate oxidase was not activated in the presence of oxygen probably as a result of the oxidation of a sulfhydryl group required for activation. A similar mechanism was postulated for the oxygen sensitivity of choline oxidase, fructose-6-phosphate phosphorylase, triose phosphate dehydrogenase, succinic dehydrogenase and phosphoglucomutase. Wimpenny (1969) found that establishing different levels of dissolved oxygen in the culture medium caused variations in cell regulation by different groups of enzymes. Quastel and Stephenson (1926) suggested that the enhanced growth of Bacillus sporogenes from a very large inoculum was a result of introducing enough sulfhydryl compounds into the fresh medium to provide a lower Eh. A rapid initiation of growth has been correlated to large inoculum size by many observers (Gillespie, 1913; Laskin, 1956;

Fredette et al., 1967; Massralla, 1971). Reed and Orr (1943) showed that C. welchii would grow at higher redox potentials from larger inocula than from small inocula.

The development of reducing conditions during bacterial growth was probably first reported by Potter (1911). He attributed the drop in oxidation-reduction potential to bacterial decomposition of components of the medium. Coulter and Isaacs (1929) proposed that the reduction in Eh during growth was due initially to the exhaustion of oxygen by the bacteria and then to the lysis of cells resulting in the liberation of reducing substances. It was observed that different types of organisms manifest different degrees of potential change during growth. Cannan et al. (1926) suggested that this was due to differences in metabolic acitivty of the various bacterial types. The relationship of Eh to metabolic activity was shown by others (Clifton, 1933; Clifton and Cleary, 1934; Clifton et al., 1934). Others used culture potential as a criterion of species classification and identification (Gillespie, 1920; Hewitt, 1930a, 1930b, 1930c; Tuttle and Huddleson, 1934; Burrows and Jordan, 1936; Gillespie and Porter, 1938; Gillespie and Rettger, 1938a, 1938b; Seal and Mitra, 1939; Burrows and Jordan, 1941; Burrows and Jordan, 1943). Boyd and Reed (1931a, 1931b) studied the effect of various gases on Eh and found that hydrogen was capable of depressing the potential by as much as 250 mv. They concluded that the drop in Eh was due to the evolution of hydrogen gas produced by the organism and by an actively reducing metabolism. Jacob (1970) found that a polished platinum electrode was more sensitive than an unpolished one in detecting

Eh changes during the active growth of bacteria. Douglas and Rigby (1974) monitored the Eh during germination and outgrowth of C. butyricum spores. They found that the Eh drop as measured with a polished electrode occurred during emergence and not during germination or outgrowth. They found that adding hydrogen gas to the sterile medium lowered the Eh to the characteristic level occurring during growth. They considered that the low Eh was produced by the hydrogen gas of metabolism. Similarly, O'Brien and Morris (1971) found that they could raise the Eh of an actively growing culture of C. acetobutylicum by sparging with argon and presumably removing the metabolic hydrogen from the medium by reducing its partial pressure. Douglas and Rigby (1974) suggested that non-volatile metabolites had little effect on the redox potential.

Futter and Richardson (1970) studied the effects of gas environment and redox potential of the media on the recovery of several species of clostridia spores. In using various reducing agents and hydrogen, oxygen and methane, they found that the unjured organisms were clearly insensitive to change in environmental conditions of either redox potential or gas.

4. Chemical Inhibition of Growth and Injury

Relatively few investigators have studied the effect of the Eh of the medium on the inhibition of growth by chemical or physical agents. Hanke and Bailey (1945) showed that there was a marked effect of pH on the limiting potential for growth of C. welchii, C. sporogenes and C. histolyticum. Mead (1969) presented data showing that the oxidation-reduction potential threshold for growth of C. perfringens is influenced

by the salt concentration of the medium. In his experiments the redox potentials were poised and adjusted by various levels of oxygen. In the medium containing 5% sodium chloride, growth did not occur at Eh levels of +195 and +92 mv. However, when the Eh of the same medium was lowered to +66 mv growth occurred. Spencer (1966) observed that the growth of C. botulinum from small inocula did not occur in media containing limiting concentrations of sodium chloride. It was suggested that the growth failures from the small inocula resulted from the need for lower Eh conditions to initiate growth. Bowen and Deibel (1974) found that ascorbate added to bacon decreased the efficacy of nitrite to inhibit the growth of C. botulinum. They felt that this was a significant result and should be considered in bacon formulations. They suggested that the added ascorbate may have reduced the redox potential of the bacon and thereby enhanced the growth of the anaerobe.

Olsen and Scott (1950) found that heated spores of C. botulinum, as compared to unheated spores, showed significantly higher counts on media containing reducing agents such as thioglycolate even when incubated under anaerobic conditions. Payne (1973) showed that maximum heat injury of C. perfringens occurred at a higher temperature under anaerobic conditions as compared to aerobic conditions. Maximum injury at the high temperature also took a longer time to develop under the anaerobic conditions. Futter and Richardson (1970) observed the effects of redox potential and gas atmosphere on the recovery of clostridia after injury by heat, γ-rays, and ethylene oxide. They found that the concentration of cysteine hydrochloride in the medium had little effect on the recovery

of C. welchii and C. histolyticum; while C. septicum and C. sporogenes exhibited marked optimal recovery at 0.08% and 0.175% respectively. The percent of hydrogen gas in nitrogen proportionately decreased the recovery of ethylene oxide treated and γ-irradiated spores of C. welchii. Heat treated C. welchii showed decreased recoveries in the range of 25 - 80% hydrogen in nitrogen.

MATERIALS AND METHODS

A. Maintenance and Identification of the Test Organism

A lyophilized culture of Clostridium botulinum, type E, (Torry 4249 Tenno E) was obtained from the V.P.I. & S.U. Anaerobe Laboratory, Blacksburg, Virginia. This organism was used throughout the study. The general anaerobic methods of Holdeman and Moore (1972) for culture identification and maintenance and media preparation were followed. The lyophilized culture was transferred to chopped meat glucose medium and incubated at 30 C for 24 hours. Streaks were made in roll tubes of tryptose soytone agar (TSA) which consisted of the following:

Bacto-tryptose (Difco)	7.0 g
Bacto-soytone (Difco)	1.75 g
Bacto-yeast extract (Difco)	1.75 g
agar	15.0 g
resazurin	0.00035 g
cysteine hydrochloride	0.175 g
distilled water	1000.0 ml

An isolated colony was picked and inoculated to chopped meat glucose medium. After 24 hours incubation at 30 C the organism was transferred to chopped meat medium and maintained as the stock culture at room temperature. Transfers to fresh chopped meat medium were made every month during the experiments.

The results of biochemical tests, gas chromatographic analysis of volatile fermentation products and toxicity tests as outlined by Holdeman and Moore (1972) were used to confirm the identity of the test organism.

B. Preparation of Media

The basal media used in all growth experiments and the majority of Eh7 measurements were Bacto-brain heart infusion broth (Difco) and Trypticase soy broth (BBL). Dehydrated media were taken from the same control lot to insure maximum consistancy of Eh, pH and growth. The media to be used for growth curves in size 18 x 24 mm anaerobic culture tubes (Bellco Glass Co., Vineland, N.J.) were prepared and dispensed as described by Holdeman and Moore (1972). All tubes were adjusted to pH 7.0 ± 0.1 with 5 N hydrochloric acid and 8 N sodium hydroxide prior to autoclaving. The various media which were used for growth curves and Eh7 measurements in the electrode vessel were prepared as described below. A 1000 ml round bottom boiling flask (Corning Glass Works, Corning, N.Y.) was immersed in 3 N hydrochloric acid overnight and rinsed with distilled water to insure the removal of any adsorbed detergents which could cause variations in Eh7 readings. To this flask was added the basal medium which was rehydrated to 750 ml with distilled water. A silicone antifoaming agent, "Siliclad" (Becton, Dickinson and Co., Parsippany, N.J.), was added to the medium to a total concentration of 300 ppm in order to prevent foaming during sparging and growth. At this point, if sodium chloride or sucrose inhibition was desired, these compounds were added to the medium in the specified amounts. The medium was boiled and refluxed for 15 minutes in a special chimney fitted to the top of the flask in order to eliminate dissolved gases (i.e. oxygen) (Holdeman and Moore, 1972). The anaerobic medium was removed from the heat, placed in an ice water cooling bath and

immediately sparged with prepurified nitrogen. To further insure an oxygen-free gas, the prepurified nitrogen was passed through an oxygen-removing, copper-filled furnace (Sargent-Welch, Skokie, Ill.). The various reducing agents were added to the cooled medium in the amounts specified, and the pH was adjusted (if necessary) to 7.0 ± 0.2 with 5 N hydrochloric acid or 8 N sodium hydroxide. These oxygen-free media were stoppered and autoclaved at 121 C for 15 minutes in a clamp press (Bellco Glass Co., Vineland, N.J.) to prevent the stopper from being blown off as a result of the pressure in the flask during heating. The media were allowed to cool at room temperature or immersed in a cold water bath. For the growth studies in which oxidized media were utilized, compressed air was used to sparge the cooling medium prior to autoclaving rather than the prepurified nitrogen. As a result, the medium was autoclaved under an atmosphere of air instead of oxygen-free nitrogen. After autoclaving, the oxidation of the medium components was further promoted by agitating the flask and mixing the head space air with the medium on a shaker (150 RPM) during cooling at room temperature. For media in which a high hydrogen ion concentration was desired for inhibition of growth, concentrated hydrochloric acid was added to lower the pH to approximately 5.4 prior to autoclaving. After sterilizing and dispensing in the electrode vessel, the final pH of this high acid medium was adjusted to the desired value with 5 N hydrochloric acid added aseptically.

During the preparation of Brewer thioglycolate medium (Difco), Fluid thioglycolate medium (BBL), Fluid thioglycolate medium (Difco) and

Cooked meat phytone medium (BBL), an effort was made to duplicate conditions under which these media are conventionally prepared according to instruction on the containers. Therefore, these media were autoclaved under air but were not shaken after sterilization. Instead of heating and sealing the medium with vaspar just prior to use, it was sparged with prepurified nitrogen until the Eh stabilized (all dissolved oxygen was removed). The equilibrium Eh7 readings were then recorded.

C. Eh7 Measurements

1. Electrode Vessel

A general diagram of the electrode vessel is illustrated in Figure 1. This apparatus was used not only as a bacterial growth chamber but also as an equilibrating chamber for the various anaerobic media. The apparatus was designed in order to constantly monitor Eh and pH and to allow for the convenient addition of certain gases. The medium was kept agitated by a magnetic stirring bar rotated by a submersible magnetic stirrer (Cole-Parmer, Chicago, Ill.). The speed was adjusted until a visible vortex appeared on the surface of the medium. The electrode vessel consisted of a 1000 ml 4-neck distilling flask (Corning No. 400764; Corning Glass Works, Corning, N.Y.). Rubber stoppers were bored in order to accomodate a platinum inlay electrode (Corning No. 476060) and a combination pH electrode (Corning No. 476051) in two of the necks. The third neck was similarly arranged with a cotton-plugged, fritted glass gas dispersion tube through which the various gases entered the flask and were mixed in the medium and a glass tube which led to a

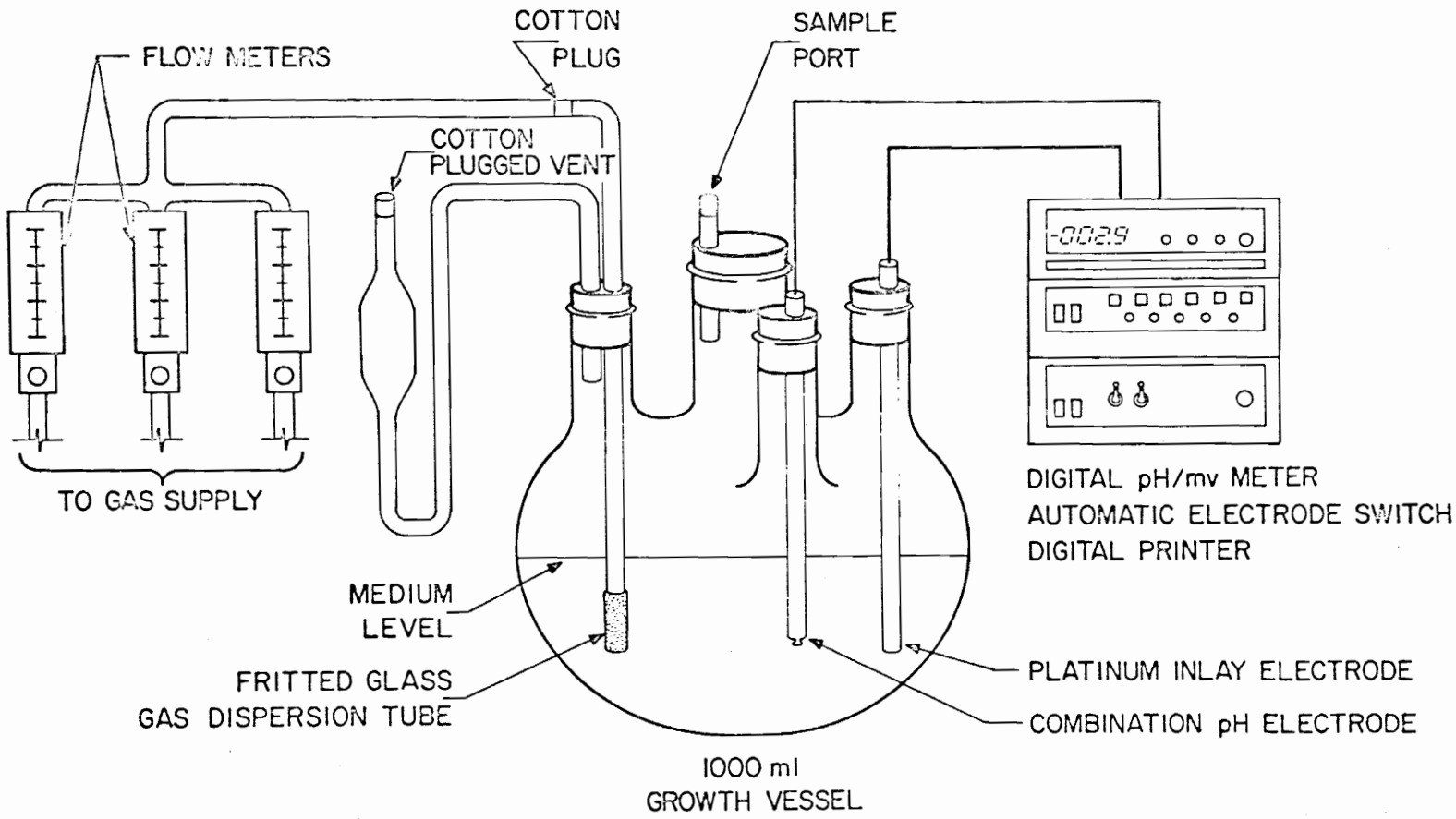

Fig. 1. Electrode vessel.

cotton-plugged vent serving as the gas exit. All tubing was constructed of glass and connected by joints of thick walled, pure gum rubber tubing. The fourth neck contained an air tight screw cap tube through which the medium was dispensed into the vessel and samples were taken. Gases were mixed and controlled by a series of flow meters (Air Products and Chemicals, Inc., Allentown, Pa.; Code No. E29-M-150MM1). All gases were obtained from Industrial Gas and Supply, Bluefield, W.Va. The following types and grades were used:

Nitrogen	prepurified grade
Carbon Dioxide	anaerobic grade
Hydrogen	standard grade
0.1% Oxygen in Nitrogen	specialty gas

All oxygen-free gases (i.e. nitrogen, carbon dioxide, hydrogen) were directed through the copper-filled furnace described previously to remove any traces of oxygen before entering the medium.

Potentials in the platinum and pH electrodes were displayed and automatically recorded with a Model 801 Digital pH/mv Meter (Orion Research Inc., Cambridge, Mass.), a Model 855 Automatic Electrode Switch (Orion) and a Model 851 Digital Printer (Orion). The electrode vessel was placed in a water bath to the level of the medium and the temperature of the bath (30 C) was maintained by a Haake Model E52 constant temperature circulator (Haake Instruments, Inc., Rochelle Park, N.J.).

Preceding every experiment in which Eh was measured the platinum electrode was polished with a fine polishing jeweler's rouge (Barnes Engineering Co., Stamford, Conn.) to insure maximum sensitivity.

Sterilization of the entire electrode vessel was performed prior to each growth experiment. The 4-neck distilling flask containing a stir bar, attached sample port and gas tubing (including the cotton plugs) was sterilized by autoclaving at 121 C for 15 minutes. The vessel was removed from the autoclave and immediately placed under a bacteriological hood equipped with a germicidal ultraviolet lamp. The electrodes were sterilized separately by immersion in a solution of 0.05% sodium hypochlorite for 15 minutes, followed by rinsing three times in sterile distilled water and drying with sterile cheesecloth prior to attachment to the electrode vessel. Only after the apparatus was completely assembled was it removed from the hood and placed in the water bath with gas connections made.

2. Eh and pH Calculations

The pH readings were measured in millivolts rather than pH units in order to avoid resetting the pH meter after each Eh reading. A standard curve of pH versus millivolts was prepared at 30 C for calculation of pH. A potassium phthalate buffer, pH 4.0, saturated with quinhydrone, was used to standardize the platinum electrode. This system has a known Eh of +456 mv at 30 C. The potential with reference to the Ag-AgCl half cell ($E_{Ag-AgCl}$) of the combination pH electrode was calculated by using: $+456 = E_{obs} + E_{Ag-AgCl}$. The integrity and constancy of the Eh measuring system was monitored by measuring the phthalate quinhydrone standard before each set of experiments. With this data the Eh of every experimental reading could be calculated from the E_{obs} by using: $Eh = E_{obs} + E_{Ag-AgCl}$. In order to compare different

experiments and to eliminate the effect of pH on the Eh of the system, all Eh values were adjusted to pH 7.0 by using the formula adapted by Leistner and Mirna (1959): $Eh7 = E_{obs} + E_{ref} + 2.303(RT/F)\ (pH \times -7.0)$ where E_{obs} = the measured potential, E_{ref} = the potential of the reference electrode used at the experimental temperature, 2.303(RT/F) = 60.1 at 30 C and (pH X -7.0) = pH correction term.

3. Anaerobic Media

After the media were prepared as outlined in the prior section, they were dispensed anaerobically into the electrode vessel. After the prereduced medium was autoclaved and cooled it was removed from the press, unstoppered and immediately placed under a stream of prepurified nitrogen during dispensing into the electrode vessel as illustrated in Figure 2. Before dispensing the media in this manner the electrode vessel was thoroughly purged of any air by allowing prepurified nitrogen to be flushed through the system for one hour at a rate of 100 ml/minute. All Eh and pH readings were recorded and all growth studies were initiated only after the system had reached equilibrium. The Eh7 of each system containing 2-mercaptoethanol (MER), cysteine hydrochloride (CYS), sodium thioglycolate (THIO), and ascorbic acid (ASC) was constant after equilibrium and reproducible within 2 to 5 mv; therefore, only one reading at each concentration was recorded. However, media containing sodium formaldehyde sulfoxylate (SFS) exhibited standard deviations ranging from 11.6 mv to 23.3 mv for each level of reducing agent. The average value for 5 measurements was used in calculations.

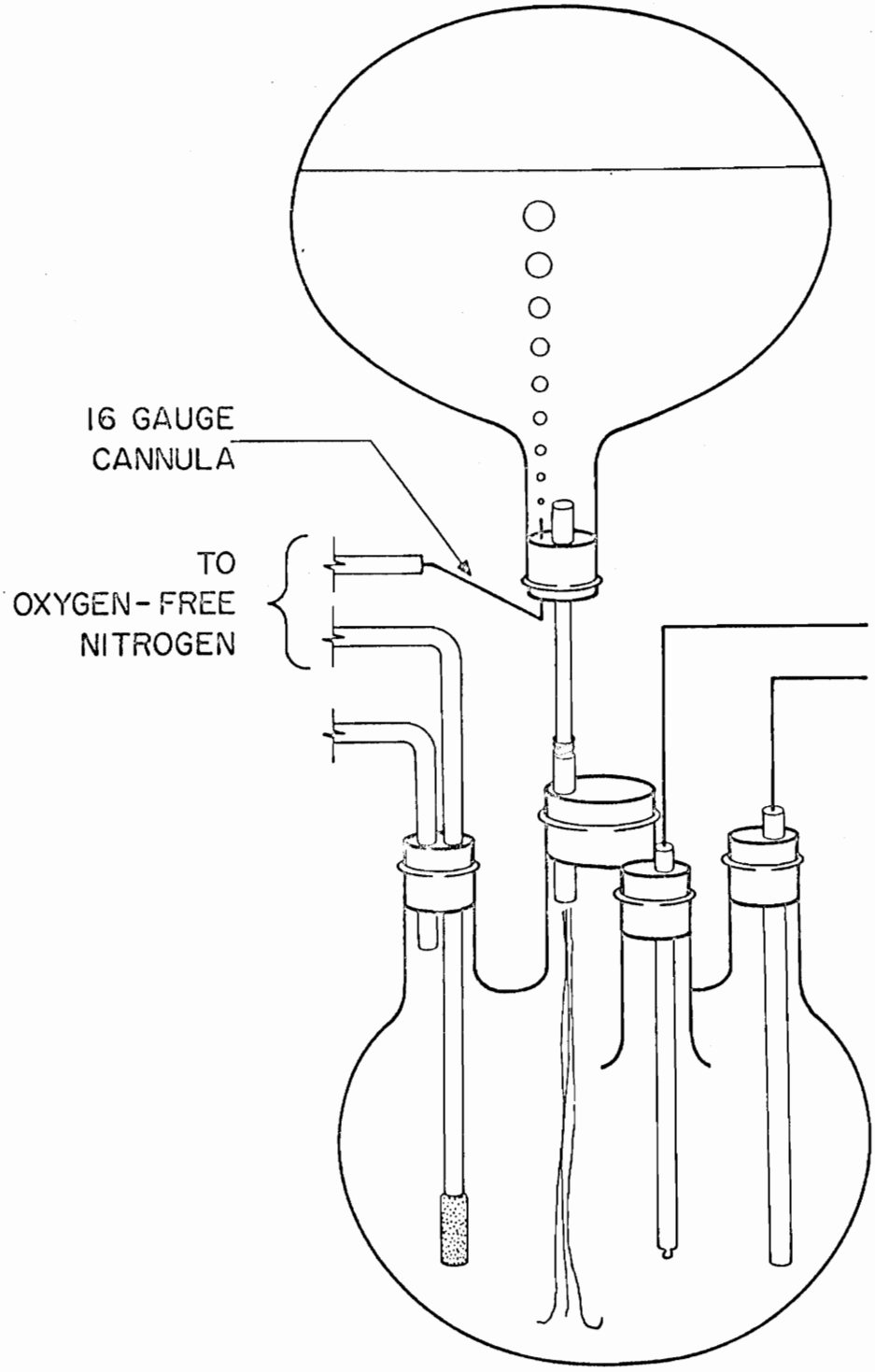

Fig. 2. Anaerobic dispensing of prereduced medium into the electrode vessel.

Equilibrium Eh7 values were measured for the following types of media:

A. Brain heart infusion broth (Difco) containing

1. no reducing agent
2. 0.03% sodium formaldehyde sulfoxylate
3. 0.05% cysteine hydrochloride
4. 0.01% sodium thioglycolate

B. Trypticase soy broth (BBL) containing

1. no reducing agent
2. 0.01% sodium formaldehyde sulfoxylate
3. 0.03% sodium formaldehyde sulfoxylate
4. 0.05% sodium formaldehyde sulfoxylate
5. 0.06% sodium formaldehyde sulfoxylate
6. 0.1% sodium formaldehyde sulfoxylate
7. 0.2% sodium formaldehyde sulfoxylate
8. 0.005% 2-mercaptoethanol
9. 0.01% 2-mercaptoethanol
10. 0.05% 2-mercaptoethanol
11. 0.1% 2-mercaptoethanol
12. 0.2% 2-mercaptoethanol
13. 0.025% cysteine hydrochloride
14. 0.03% cysteine hydrochloride
15. 0.05% cysteine hydrochloride
16. 0.075% cysteine hydrochloride
17. 0.1% cysteine hydrochloride
18. 0.2% cysteine hydrochloride
19. 0.01% sodium thioglycolate
20. 0.05% sodium thioglycolate
21. 0.1% sodium thioglycolate
22. 0.2% sodium thioglycolate
23. 0.005% ascorbic acid
24. 0.01% ascorbic acid
25. 0.05% ascorbic acid
26. 0.1% ascorbic acid
27. 0.2% ascorbic acid

C. Brewer thioglycolate medium (Difco)

D. Fluid thioglycolate medium (BBL)

E. Fluid thioglycolate medium (Difco)

F. Cooked meat phytone medium (BBL)

4. Canned Foods

All canned foods used in this study were commercial products purchased on the retail market. No attempt was made to determine whether the cans were commercially sterile except to note that there were no swollen containers or obvious defects. All Eh measurements of canned foods were undertaken in a sealed anaerobic glove box in order to prevent atmospheric oxygen from contaminating the can contents upon opening. Such oxygen would result in artificially high Eh values which would not be an accurate indication of the potential of the food system within the hermetically sealed container. Preceding the measurement of Eh values in canned foods, all sealed cans to be measured, pH/mv meter and attached electrodes, distilled water and other equipment were placed inside the glove box. The cans were not shaken, disturbed or inverted for approximately one week prior to the Eh measurements in order to prevent mixing of the headspace gases with the food contents. The glove box was sealed and flushed with prepurified nitrogen for 24 hours. A Bunsen valve on the glove box provided a one-way exit for the nitrogen. Prereduced 0.05% cysteine 0.1% peptone buffer containing resazurin was used to indicate oxygen-free conditions within the box. The cans were then opened and the combination pH electrode and platinum electrode were placed in the food and pushed to the bottom of the can. After the Eh and pH values stabilized (usually 30 minutes), the measurements were recorded. One sample of each food was measured. The electrodes were cleaned with oxygen-free distilled water between each sample measurement. Oxygen-free prepurified nitrogen was allowed to bubble through

the distilled water and flush the glove box during the entire period of measurements.

D. Dissolved Oxygen Measurements

Dissolved oxygen readings were made with a Model 57 Oxygen Meter (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The oxygen probe utilized high-sensitive Clark-type membranes and entered the electrode vessel through an air tight hole near the sample port. The probe was sterilized as described in the previous section for the electrodes. The probe was suspended in the medium directly over the stirring bar to insure adequate circulation at the surface of the membrane.

E. Hydrogen Gas Measurements

Metabolic hydrogen gas which evolved during the growth of the test organism was detected and quantitated by utilizing a Model 29 Fisher-Hamilton Gas Partitioner connected directly to the cotton-plugged vent of the electrode vessel. A 6½ foot stainless steel column (O.D. 3/16 in.) containing 42-60 mesh Molecular Sieve-13X packing (Linde) was used. The carrier gas was argon at a rate of 40 ml/minute. Samples of the gas were taken directly through the sampling valve located on the gas partitioner. Quantitation was calculated by comparing peak heights to a standard gas mixture. This procedure was also used to confirm the 0.1% oxygen in nitrogen gas mixture used in some of the growth studies.

F. Growth Studies

1. Preparation of Inocula

All inocula consisted of spore preparations of the test organism grown and harvested as described by Bruch et al. (1968). The spore crops were harvested by centrifugation and washed 5 times with sterile distilled water. Each crop was subdivided into 3 ml portions and stored frozen at -20 C in glass screw cap tubes. Sporulation was estimated to be at least 95% in all preparations by phase microscopy.

2. Determination of Growth in Culture Tubes

The growth of the test organism in culture tubes was determined by absorbance (600 nm) readings on a Bausch and Lomb Spectronic 20 Spectrophotometer. Size 18 X 142 mm anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.) containing 10 ml of the various media were inoculated anaerobically by the methods of Holdeman and Moore (1972). Spore inocula were prepared by dispensing 0.1 ml of the thawed spore stock suspension into 9.9 ml of prereduced 0.05% cysteine 0.1% peptone buffer (pH 7.0). After heat shocking for 13 minutes at 60 C, 0.1 ml of the suspension was inoculated into each tube of the different media and then incubated at 30 C.

All inocula used in these growth studies were prepared from the same spore crops and consisted of essentially identical numbers of viable spores (approximately 2.64×10^9 spores/ml) in the frozen stock. These counts were determined by roll tube numbers using TSA incubated at 30 C for 48 hours. Absorbance readings were made directly through the culture tube every hour. Duplicate tubes were used for each growth curve. Growth of the test organism was measured in culture tubes for

the following types of media adjusted to pH 7.0 ± 0.2:

A. Brain heart infusion broth (Difco) containing

1. no reducing agent
2. 0.03% sodium formaldehyde sulfoxylate
3. 0.05% sodium formaldehyde sulfoxylate
4. 0.05% cysteine hydrochloride
5. 0.1% cysteine hydrochloride
6. 0.05% sodium thioglycolate
7. 0.1% sodium thioglycolate

B. Trypticase soy broth (BBL) containing

1. no reducing agent
2. 0.01% sodium formaldehyde sulfoxylate
3. 0.03% sodium formaldehyde sulfoxylate
4. 0.05% sodium formaldehyde sulfoxylate
5. 0.1% sodium formaldehyde sulfoxylate
6. 0.385% (0.025 M) sodium formaldehyde sulfoxylate
7. 0.01% 2-mercaptoethanol
8. 0.05% 2-mercaptoethanol
9. 0.1% 2-mercaptoethanol
10. 0.200% (0.025 M) 2-mercaptoethanol
11. 0.025% cysteine hydrochloride
12. 0.05% cysteine hydrochloride
13. 0.075% cysteine hydrochloride
14. 0.1% cysteine hydrochloride
15. 0.2% cysteine hydrochloride
16. 0.439% (0.025 M) cysteine hydrochloride
17. 0.01% sodium thioglycolate
18. 0.05% sodium thioglycolate
19. 0.1% sodium thioglycolate
20. 0.285% (0.025 M) sodium thioglycolate
21. 0.01% ascorbic acid
22. 0.05% ascorbic acid
23. 0.1% ascorbic acid
24. 0.440% (0.025 M) ascorbic acid

In a similar manner, growth of the test organism was determined in prereduced TSB containing 0.03% sodium formaldehyde sulfoxylate and various concentrations of inhibitory substances. The growth tubes were repeated in triplicate. The inhibitory agents and their respective concentrations were prepared as follows:

a. Sodium chloride -- 0.5%, 2.5%, 3.5%, 4.0%, and 4.5% (includes 0.5% present in TSB).

b. Hydrogen ion -- pH 7.15, 6.32, 5.64, 5.46, 5.38, 5.24, 5.15, 5.06, 5.02, and 4.85.

c. Sucrose -- 0.0%, 10%, 20%, 30%, 35%, 36%, 38%, and 40%.

Minimum hibitory concentrations (MICs) for rifampin and penicillin G were determined by a broth dilution method in anaerobic prereduced TSB containing 0.05% cysteine hydrochloride. Growth was determined in duplicate tubes at each concentration by an increase in tubidity. The lowest concentration which completely inhibited growth was the MIC.

3. Determination of Growth and Eh7 in the Electrode Vessel

Spore inocula were prepared and utilized exactly as described in the preceding section; however, one ml of the heat shocked spore suspension was inoculated into the flask. Spore outgrowth was monitored by periodic sampling and quantitating by either TSA roll tube counts incubated at 30 C for 48 hours or by absorbance (600 nm) readings of 3 ml samples. Spore inocula for the growth experiments in which hydrogen gas was added to media containing 30% sucrose were prepared from a second spore crop containing 8.5×10^7 viable spores/ml in the final frozen stock.

During the entire equilibration and growth periods prepurified nitrogen was passed through the copper catalyst furnace and sparged the medium.

During all growth studies in the electrode vessel, it was

necessary to add carbon dioxide gas to the medium to germinate the spores. Carbon dioxide was continuously added to the medium to lower the pH by 0.3 units. This amount of carbon dioxide was mixed with the nitrogen and other gases entering the medium and regulated precisely so that a constant pH (0.3 pH units less than the system containing no carbon dioxide) was maintained before inoculation of the flask.

Growth and Eh7 were monitored in the following types of media:

A. Prereduced trypticase soy broth (BBL) with 0.05% cysteine containing:

1. carbon dioxide lowering the pH a total of 0.6 units
2. no additional components
3. 0.1% oxygen in the sparging gas
4. 3% added sodium chloride
5. 3.5% added sodium chloride
6. hydrochloric acid adjusting pH to 5.11
7. hydrochloric acid adjusting pH to 5.05
8. 30% sucrose
9. 35% sucrose
10. 30% sucrose and 0.1% oxygen in the sparging gas
11. 30% sucrose and hydrogen (4 ml/min) in sparging gas
12. 30% sucrose, 0.1% oxygen and hydrogen (4 ml/min) in the sparging gas
13. 0.002 µg/ml rifampin (Minimum Inhibitory Concentration)
14. 20 µg/ml penicillin G (1585 units/mg) (Minimum Inhibitory Concentration)

B. Oxidized trypticase soy broth (BBL) with 0.05% cysteine containing:

1. no additional components
2. 0.1% oxygen in the sparging gas
3. 3% added sodium chloride
4. 3.5% added sodium chloride
5. hydrochloric acid adjusting pH to 5.11
6. hydrochloric acid adjusting pH to 5.05
7. 30% sucrose
8. 35% sucrose
9. 30% sucrose and prereduced 0.5% cysteine water lowering the equilibrium Eh7 95 mv
10. 30% sucrose and hydrogen gas (4 ml/min) in the sparging gas

Minimum inhibitory concentrations for rifampin and pencillin G were determined by the broth dilution method described previously.

G. Comparisons of Growth, Reducing Agent Concentration and Eh7

Absorbance (600 nm) readings made 10 hours after inoculation were chosen for the criteria of growth in order to take into account both the generation rate and the lag time. These growth responses in each type of media were compared in graphical form to the variables of reducing agent concentration and Eh7.

RESULTS AND DISCUSSION

A. Identification of the Test Organism

1. Biochemical Tests

The results of the biochemical tests which were performed to identify the test organism are presented in Table 3. All of the results support the confirmation of the test organism as Clostridium botulinum type E (Holdeman and Moore, 1972).

2. Gas Chromatography

The volatile fatty acid end products of fermentation were primarily butyric acid with a slight amount of acetic acid as shown in Figure 3. These products are typical of C. botulinum type E (Holdeman and Moore, 1972).

3. Toxin Test

The test organism was positive for type E botulinum toxin.

B. Determination of Reference Half-Cell Potential

The reference half-cell potential was determined to have a value of +180 ± 3.0 mv at 30 C. This was based on the average of 22 measurements throughout the course of this study.

C. Eh7 Measurements of Uninoculated Media and Food

1. Uninoculated Media

The Eh7 values for the Trypticase soy broth (TSB) containing various reducing agents are presented in Figure 4. Generally 30-60

Table 3. Growth and biochemical tests performed to verify the identity of the test organism (*Clostridium botulinum* type E).

Test Performed	Results
Gram reaction	Gram + rods
Spore stain	Subterminal spores
Starch hydrolysis	+
Starch pH	acid
Meat digestion	-
Indol production	-
Milk	curd, gas, no digestion
Gelatin	+
Carbohydrate fermentation	
xylose	-
sucrose	+
sorbitol	+
melibiose	-
mannose	+
mannitol	-
maltose	+
lactose	-
glucose	+
fructose	+

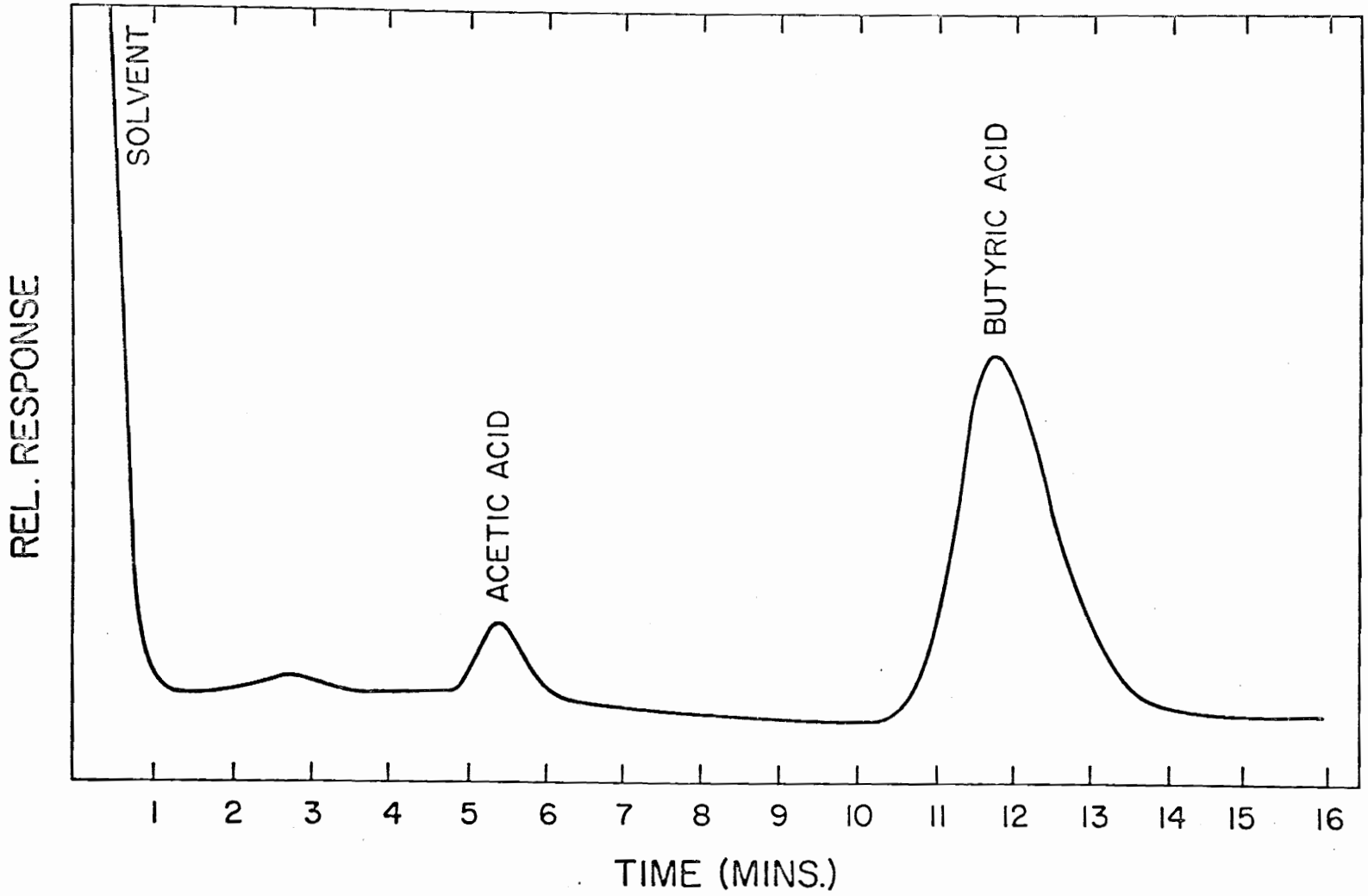

Fig. 3. Gas chromatographic profile of ether extracted volatile fatty acid products of C. botulinum type E.

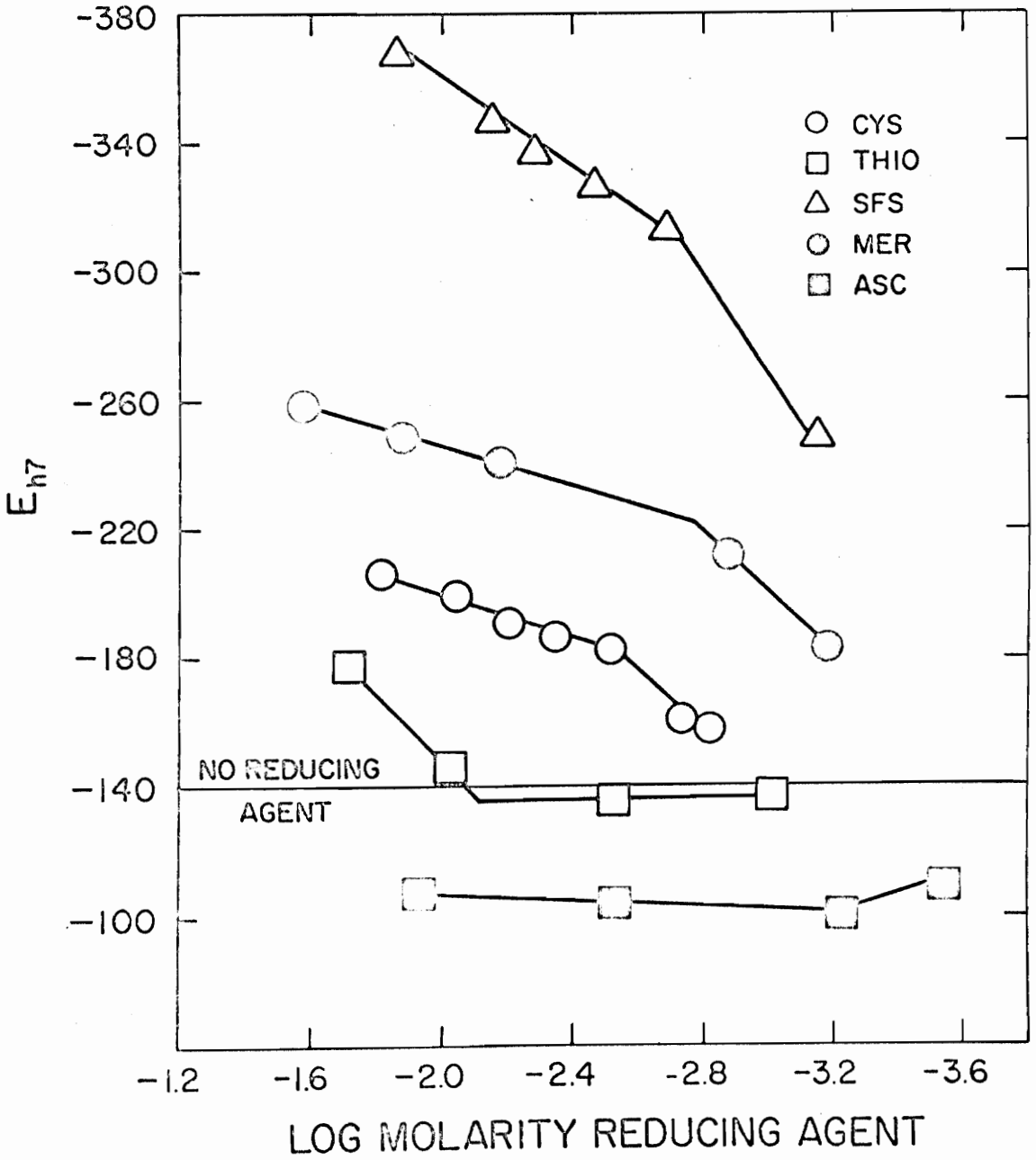

Fig. 4. Effect of cysteine hydrochloride (CYS), sodium thioglycolate (THIO), sodium formaldehyde sulfoxylate (SFS), 2-mercaptoethanol (MER), and ascorbic acid (ASC) at different concentrations on the equilibrium Eh7 of uninoculated prereduced anaerobic trypticase soy broth.

minutes were required for each system to reach equilibrium. During this period the volatile components affecting the redox potential (e.g. H_2S, H_2, O_2, SO_2) were removed by sparging with nitrogen gas. An absence of such components in the system may accurately reproduce the Eh7 during normal growing conditions. Typically, media or food are heated or allowed to stand and lose such volatiles to the head space or environment. The initial evolution of metabolic gas (H_2, CO_2, etc.) during emergence and outgrowth of the spores establishes a high partial pressure which would also remove dissolved volatiles.

TSB containing no added reducing agents exhibited an Eh7 value of -141 mv. Surprisingly ascorbic acid (ASC) at all levels and sodium thioglycolate (THIO) at 0.01% and 0.05% when added to TSB produced an Eh7 higher than -141 mv. At the higher levels of THIO the Eh7 became progressively lower with concentration. These reducing agents in low amounts apparently affected the natural reducing systems of TSB in such a way as to lower their reducing power resulting in a higher Eh7. This initial negative effect on the Eh7 seems to have been reversed by the inherent reducing power of THIO at the higher concentrations. Conventionally prepared anaerobic media are typically rehydrated, mixed and autoclaved in the presence of oxygen. Any added reducing agents would interact with the oxygen and oxidized components of the system to lower the Eh7 below what it would normally have been without the added reducing agent.

The addition of cysteine hydrochloride (CYS), 2-mercaptoethanol (MER), and sodium formaldehyde sulfoxylate (SFS) to TSB resulted in a

reduction of Eh7 compared to the system without added reducing agents (i.e. less than -141 mv). This reduction was more effective at the lower levels of reducing agent (up to about 0.03%). At higher concentrations the drop in the Eh7 was less pronounced. Apparently at low levels of these reducing agents there is a synergistic reducing effect until about the 0.03% concentration (See Fig. 4).

The variability in the Eh7 observed for media containing MER, CYS, THIO, and ASC was only slight with duplicate preparations differing by only two or three mv. Media containing SFS, on the other hand, showed marked variation between identical preparations of the same composition. These differences cannot be explained here.

The equilibrium Eh7 values for other types of anaerobic media are outlined in Table 4. When the Brain heart infusion broth (BHIB) media are compared to TSB media containing the same reducing agents at identical concentrations, in every case the BHIB media possessed an Eh7 of about 100 mv less than the TSB media. The lower Eh7 must have been the result of the greater reducing intensity of the reducing agents present in BHIB as compared to TSB. The Eh7 of TSB has been reported by Oblinger and Kraft (1973) to be more sensitive to Eh changes and therefore was utilized in the majority of the experiments in this study. Tabatabai and Walker (1970) reported the Eh7 of TSB after equilibrium to be 40 mv compared to -141 mv in this study. The difference may be attributed to the prereduction procedures carried out in media preparation--refluxing, cooling and autoclaving under nitrogen. The conventionally prepared (cooled and autoclaved under air) anaerobic media produced

Table 4. Equilibrium Eh7 values for some prereduced and conventionally prepared anaerobic media.

Prereduced anaerobic media	Eh	pH	Eh7
Brain heart infusion broth with no additions	-263.0	6.91	-268.5
Brain heart infusion broth with 0.03% sodium formaldehyde sulfoxylate	-381.0	6.78	-394.3
Brain heart infusion broth with 0.05% cysteine hydrochloride	-256.7	6.79	-269.7
Brain heart infusion broth with 0.01% sodium thioglycolate	-225.5	6.97	-229.4
Conventionally prepared anaerobic media			
Brewer thioglycolate medium (Difco)	-189.9	6.98	-193.2
Fluid thioglycolate medium (Difco)	-200.1	6.77	-213.8
Fluid thioglycolate medium (BBL)	-146.3	6.77	-160.0
Cooked meat phytone medium (BBL)	- 53.4	6.49	- 83.8

a wide range of Eh7 values even among similar types of media as shown in Table 4.

Futter and Richardson (1970) measured the potentials of uninoculated Reinforced clostridial medium (RCM) and RCM + 0.05% cysteine hydrochloride under nitrogen and recorded values of +150 mv and +155 mv respectively. Hirsh and Grinsted (1954) reported Eh values of from +40 to +279 mv for the various media they measured. Thioglycolate and cooked meat media showed the lowest potentials. The potential readings were generally much higher than those obtained here for similar media. It can only be assumed that the media or electrode surfaces contained atmospheric oxygen which would tend to raise the potential correspondingly.

Reed and Orr (1943) found a potential of -175 to -200 mv for their peptone-thioglycolate medium. The same media without thioglycolate measured +225 to +250 mv. It would seem from these results that the thioglycolate was effectively reducing the molecular oxygen present in the medium. Reed and Orr (1943) also reported that their peptone medium with cysteine and ascorbic acid gave low Eh values similar to the thioglycolate. Sodium formaldehyde sulfoxylate gave slightly more negative levels. This is in agreement with the findings of this study, i.e. SFS produced the lowest potential. Laskin (1956) found similar results when he added various reducing agents to Difco Brain heart infusion medium. He reported that ascorbic acid gave the highest potential--approximately +100 mv. Apparently the oxygen present in his medium was not reduced by the comparatively weak reducing agent, ascorbic

acid. His Brain heart infusion medium containing thioglycolate resulted in a potential of about -75 mv. Sodium formaldehyde sulfoxylate produced the lowest potential, about -85 mv.

It is extremely difficult to compare the final potentials obtained here with those of the previous reports. Strict anaerobic conditions and prereduced media were utilized throughout this study. Potentials of other types of bacteriological media have never been reported to have been measured under such controlled conditions. The ability of these reducing agents to establish a low potential in media prepared conventionally under aerobic conditions was not determined here. In fact, it is conceivable that in the presence of molecular oxygen a different scale of reducing intensities would be revealed for the same reducing agents.

2. Canned Foods

The Eh7 values for the canned foods which were measured showed a considerable range--from an Eh7 of -441.5 mv for sliced beets to -33.4 mv for pimentos (Table 5). The higher Eh7 values for items such as whole pimentos and whole salad peppers are probably the result of oxygen within the large pieces of food which could oxidize the natural reducing substances within the can during processing or may directly influence the Eh7 during measurement.

It is of interest to note some of the general characteristics of Table 5. The food systems producing the very low Eh7 values (less than -400 mv) are all low acid vegetable products. Presumably these foods contain very strong reducing substances. However, the measured

Table 5. Eh7 values for several canned foods

Very low Eh7	Eh	pH	Eh7
Sliced beets	-335.1	5.23	-441.5
Yellow sliced squash	-326.4	5.30	-428.6
Green lima beans	-354.6	5.94	-418.3
Sliced carrots	-282.0	4.99	-402.8
Low Eh7			
Tomato sauce	-226.0	4.12	-399.1
Applesauce	-170.8	3.40	-387.2
Orange juice	-189.8	3.74	-385.7
Blackberries	-174.5	3.65	-375.8
Salad greens	-227.0	4.86	-355.6
Cherries	-124.2	3.69	-323.1
Sauerkraut	-114.2	3.58	-319.7
Vegetable with beef stock soup	-195.4	5.01	-315.0
Tomato juice	-141.6	4.21	-309.3
Cut asparagus	-208.5	5.43	-302.8
Peeled tomatoes	-121.4	4.10	-295.7
Sliced green beans	-164.6	5.08	-280.0
Water chestnuts	-201.1	5.76	-273.6
Tomato sauce with bits	- 94.4	4.06	-271.1
Vegetable and beef baby food	-173.5	5.43	-267.8
Red Salmon	-210.2	6.22	-257.1
Salmon	-208.5	6.23	-254.8
Mushroom soup	-141.8	5.18	-251.2
Medium Eh7			
Cheddar cheese soup	-169.6	5.78	-242.9
Vichyssoise soup	-197.0	6.27	-240.2
Yellow sweet corn	-188.7	6.19	-237.4
Strained pears baby food	- 39.8	3.78	-233.3
Mushrooms	-181.0	6.21	-228.5
Cream of potato soup	-146.3	5.64	-228.0
Bartlett pears	- 50.2	4.12	-223.3
Peas	-153.5	5.92	-218.4
Chili	-133.0	5.62	-215.9
Split pea soup	-154.3	6.04	-212.0
Herring roe	-151.5	6.00	-211.6
Cut green beans	-106.1	5.36	-204.7
Tuna	-169.0	6.41	-204.5
Cream of mushroom soup	-139.5	6.06	-195.9
Tomato bisque soup	- 19.2	4.10	-193.5
Pumpkin	- 68.8	4.95	-192.0
Chunk bonito	-121.6	5.85	-190.7

Table 5. (Cont.). Eh7 values for several canned foods.

Medium Eh7	Eh	pH	Eh7
Fish roe	-144.8	6.24	-190.5
Cream of potato soup	- 11.5	5.81	-137.0
Yams	- 80.5	5.30	-182.7
Beef stew	-107.5	5.77	-181.4
Navy beans	-118.4	6.01	-177.9
Chili con carne	- 73.6	5.31	-175.2
Clam chowder	-123.5	6.14	-175.2
Blackeye peas	-113.5	6.05	-170.6
White potatoes	- 67.3	5.30	-169.5
Cream of celery soup	-104.3	5.97	-166.2
Minced clams	-135.1	6.50	-165.2
Pepper pot soup	- 85.3	5.82	-156.2
Cream corn	-108.9	6.22	-155.8
Mixed vegetables	- 59.7	5.43	-154.1
Pitted olives	-156.4	7.08	-151.6
Shrimp	-116.0	6.42	-150.9
White corn	- 94.7	6.08	-150.0
High Eh7			
Bean with bacon soup	- 71.8	5.73	-148.1
Spinach	- 24.3	5.22	-131.3
Mixed vegetables	- 22.8	5.62	-130.8
Mixed vegetables	- 20.5	5.51	-110.0
Onion soup	- 11.1	5.50	-101.0
Strained prunes baby foods	+ 88.4	3.89	- 98.5
Very high Eh7			
Salad peppers	+203.4	2.98	- 38.2
Oyster stew	- 4.5	6.49	- 35.2
Pimentos	+132.5	4.24	- 33.4

potentials are so low that it may reflect the presence of hydrogen gas within the system. Such gas may be the result of a detinning action and subsequent chemical production of hydrogen. Hydrogen in the system would result in an artificially low Eh measurement as a result of localized ionization of hydrogen at the platinum tip of the electrode. This effect could mask the true reducing intensity of the food.

The majority of the high acid foods are categorized as having low Eh7 values (-250 mv to -400 mv). Reducing agents such as ascorbic acid and reducing sugars may help to achieve these low levels. It must be recalled that the Eh7 values are not influenced by pH variations since they are all based on a neutral pH.

The Medium Eh7 category contains most of the animal products (i.e. fish and meat) with salmon meat being at the very high end of the Low Eh7 category. The sulfhydryl compounds characteristic of meat and fish products afford considerable reducing power.

D. Growth Responses of the Test Organism in Culture Tubes

1. Effect of Reducing Agents

The growth curves of the test organism in prereduced BHIB containing various levels of SFS, CYS, and THIO are presented in Figures 5, 6 and 7 respectively. Growth was enhanced in the media containing both levels of SFS (0.03% and 0.05%) compared to growth in the control BHIB containing the low level of CYS (0.05%) but was retarded in the higher level (0.1%). Growth was retarded at all levels of THIO (0.05% and 0.1%) over that of the control. When the growth rates in BHIB are compared to

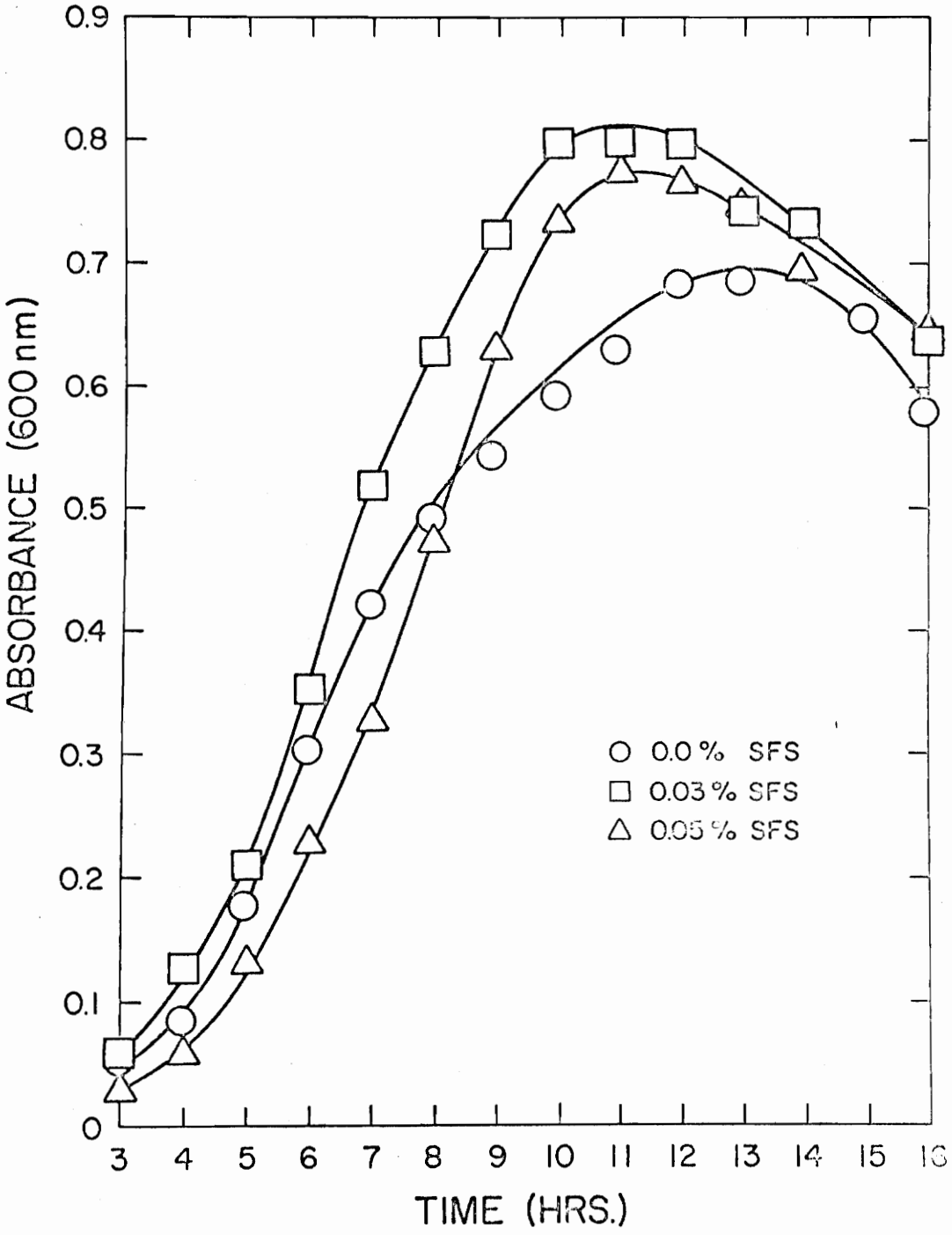

Fig. 5. Growth of C. botulinum type E in prereduced anaerobic brain heart infusion broth containing varying concentrations of sodium formaldehyde sulfoxylate.

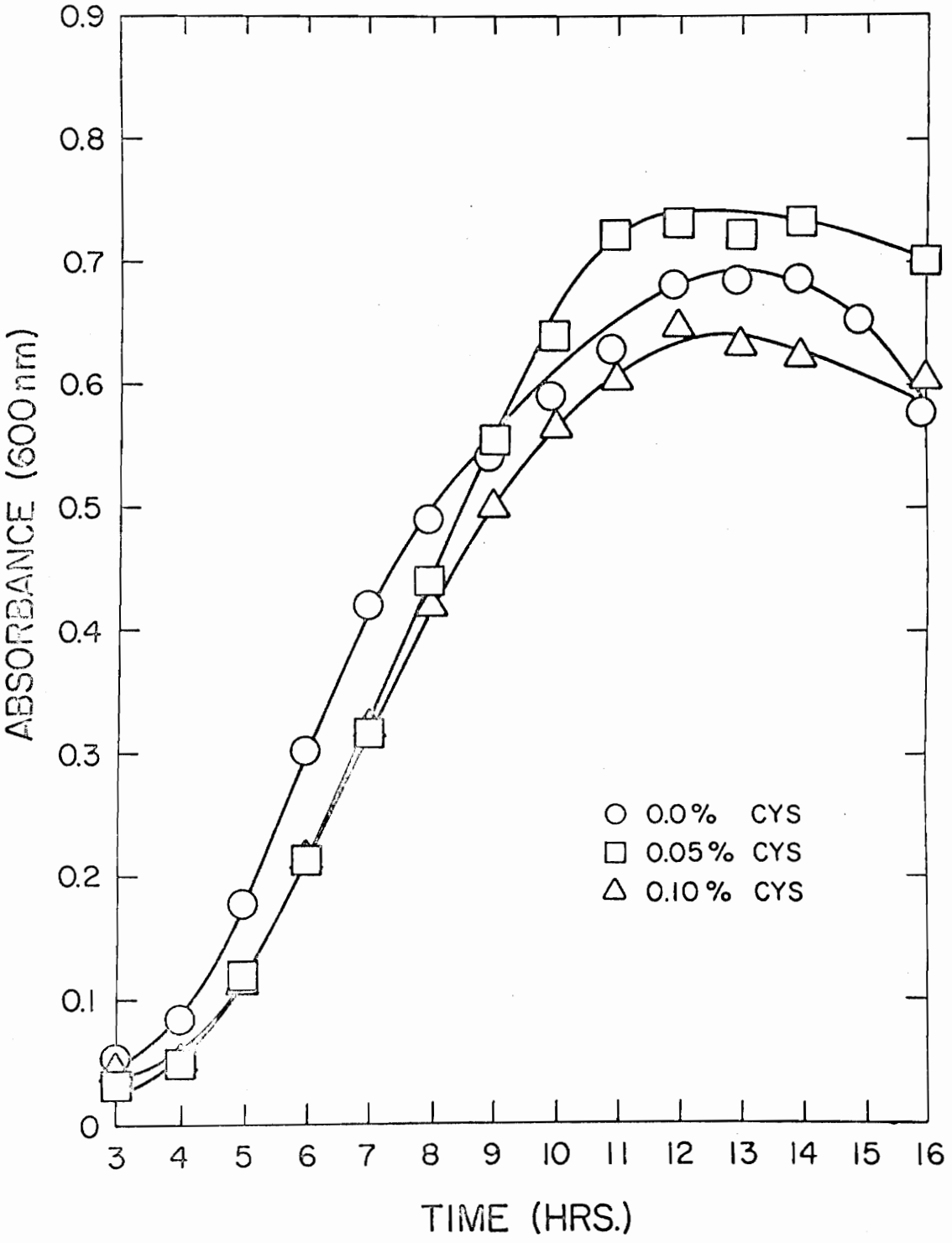

Fig. 6. Growth of C. botulinum type E in prereduced anaerobic brain heart infusion broth containing varying concentrations of cysteine hydrochloride.

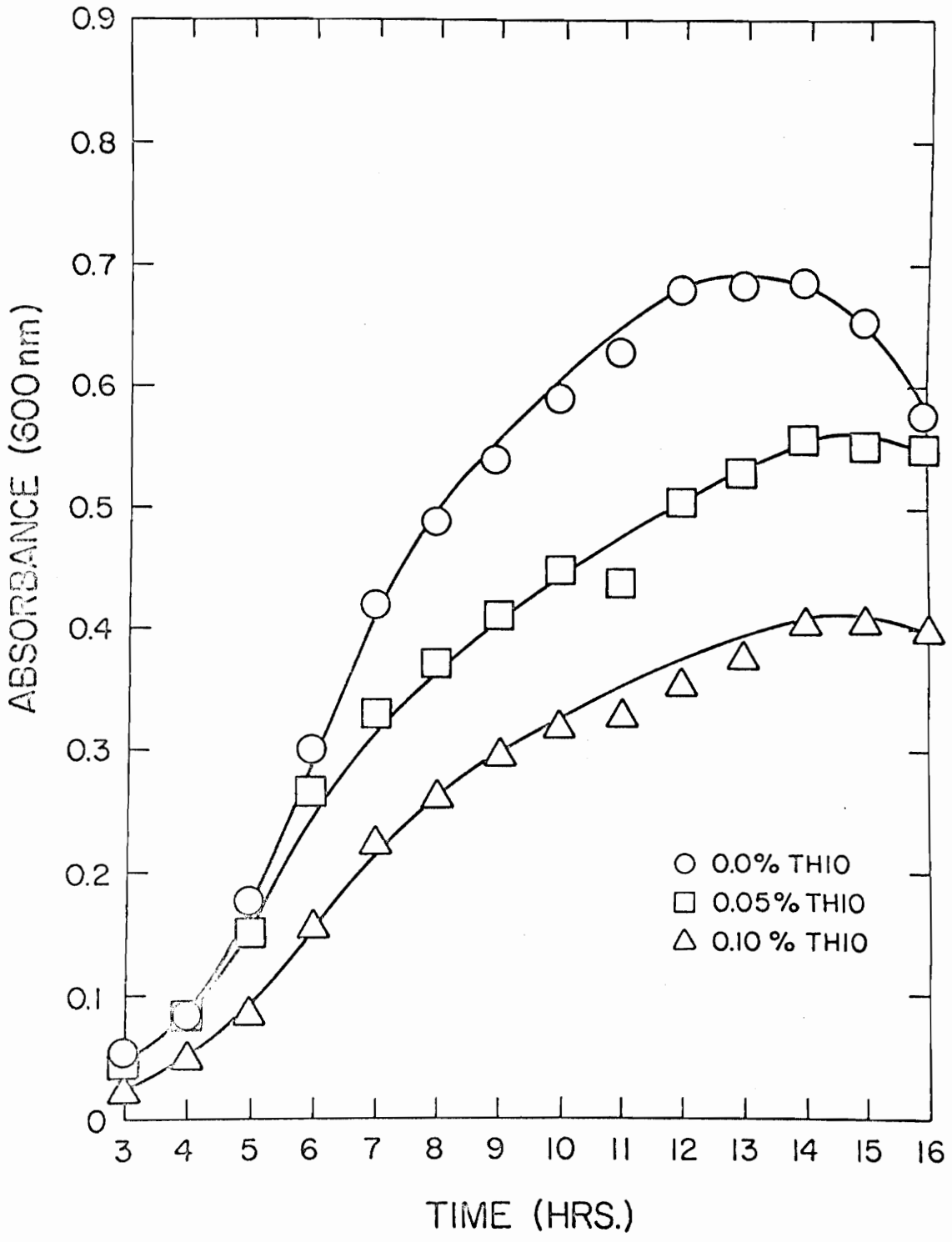

Fig. 7. Growth of C. botulinum type E in prereduced anaerobic brain heart infusion broth containing varying concentrations of sodium thioglycolate.

TSB containing the same levels and types of reducing agents, it can be seen that the rate in BHIB is greater in every case. This may be correlated to the lower Eh7 values of the BHIB system as compared to TSB (See Section E of Results and Discussion). Absorbance values in BHIB approximately 10-12 hours after inoculation exhibited progressively and drastically reduced levels. These lower absorbance values during the late log stage of growth may have been due to accumulation of inhibitory substances resulting in death and lysis of cells.

The test organism exhibited enhanced growth rates and growth maxima in TSB containing low levels of SFS (0.01%, 0.03% and 0.05%) as compared to TSB without added SFS; however, at the higher levels (0.1% and 0.385%) the growth rate and extent of growth was inhibited (Fig. 8). A higher growth rate and maximum absorbance were obtained in TSB containing 0.01% MER compared with TSB without any added reducing agent. At the higher levels (0.05%, 0.1% and 0.2%) this reducing agent appeared to be inhibitory (Fig. 9). Enhancement of growth at the lower levels (0.025% and 0.05%) of CYS was also found. The higher concentrations (0.075%, 0.1%, 0.2% and 0.439%) resulted in inhibited growth compared to TSB without added reducing agents (Fig. 10).

Growth of the test organism in TSB containing all levels of THIO (0.01%, 0.05%, 0.1% and 0.285%) and ASC (0.01%, 0.05%, 0.1% and 0.440%) was inhibited both in rate and maximum extent compared to TSB containing no added reducing agents (Figs. 11 and 12).

Initiation of growth in TSB containing the very high concentrations of each reducing agent was considerably delayed, especially at the

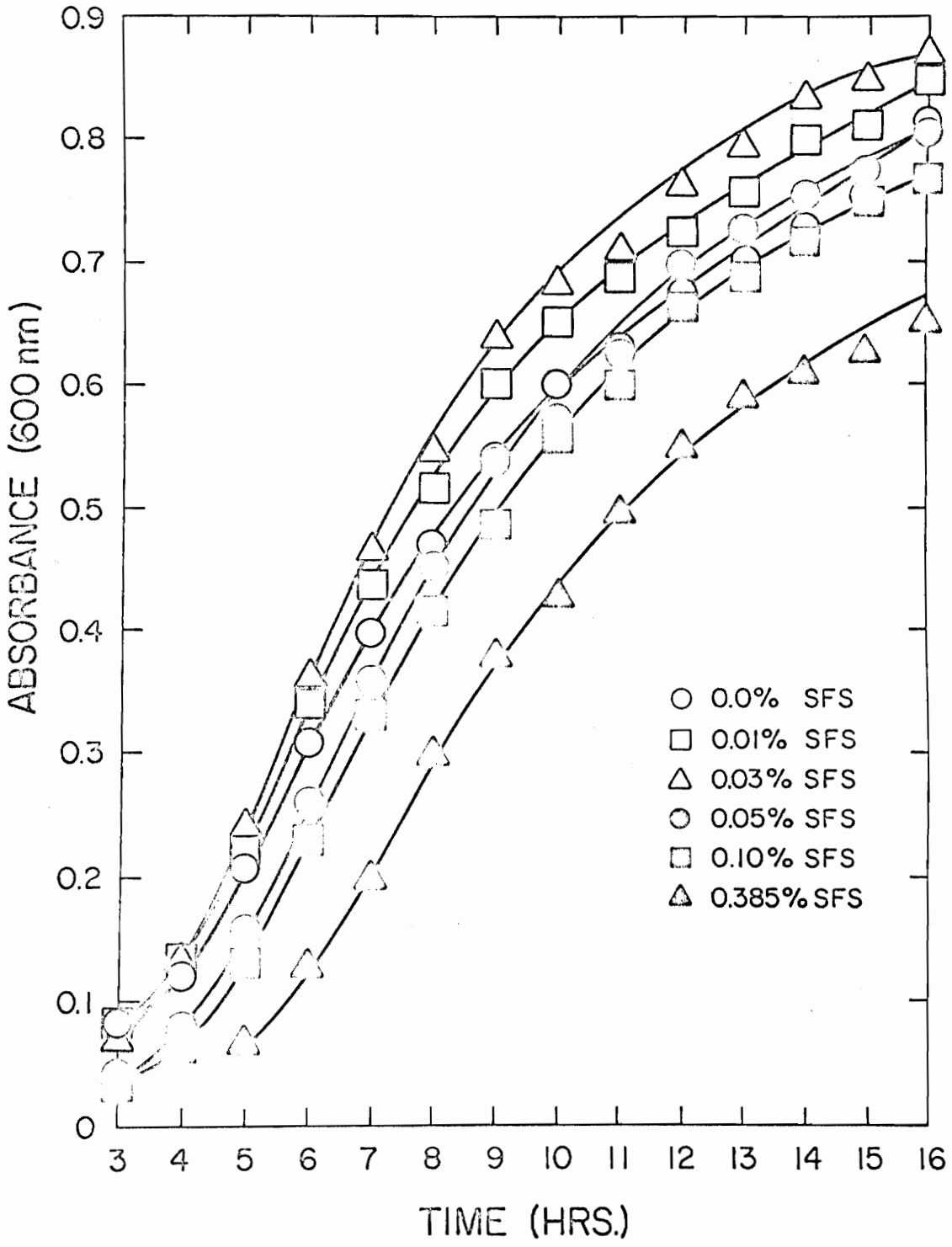

Fig. 8. Growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing varying concentrations of sodium formaldehyde sulfoxylate.

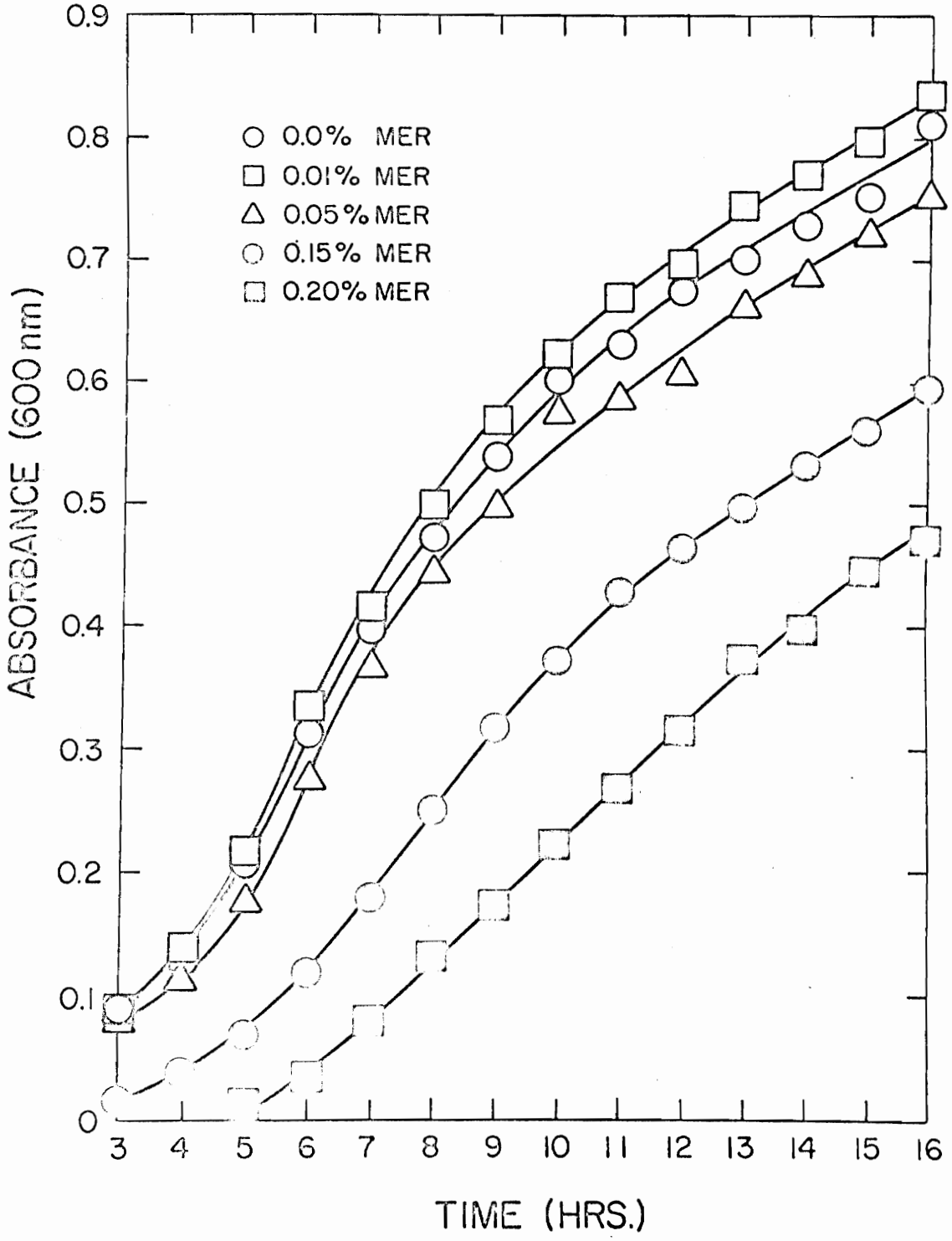

Fig. 9. Growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing varying concentrations of 2-mercaptoethanol.

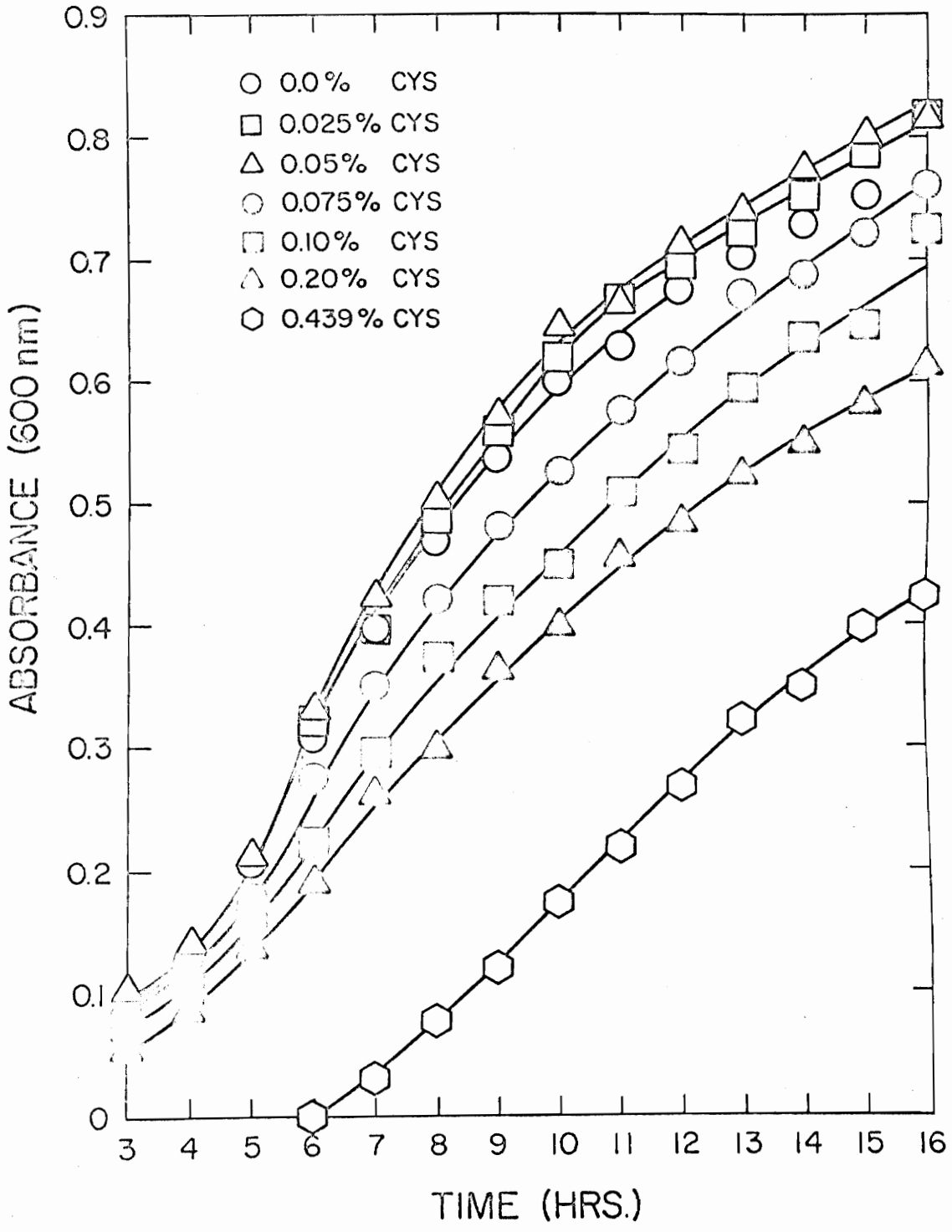

Fig. 10. Growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing varying concentrations of cysteine hydrochloride.

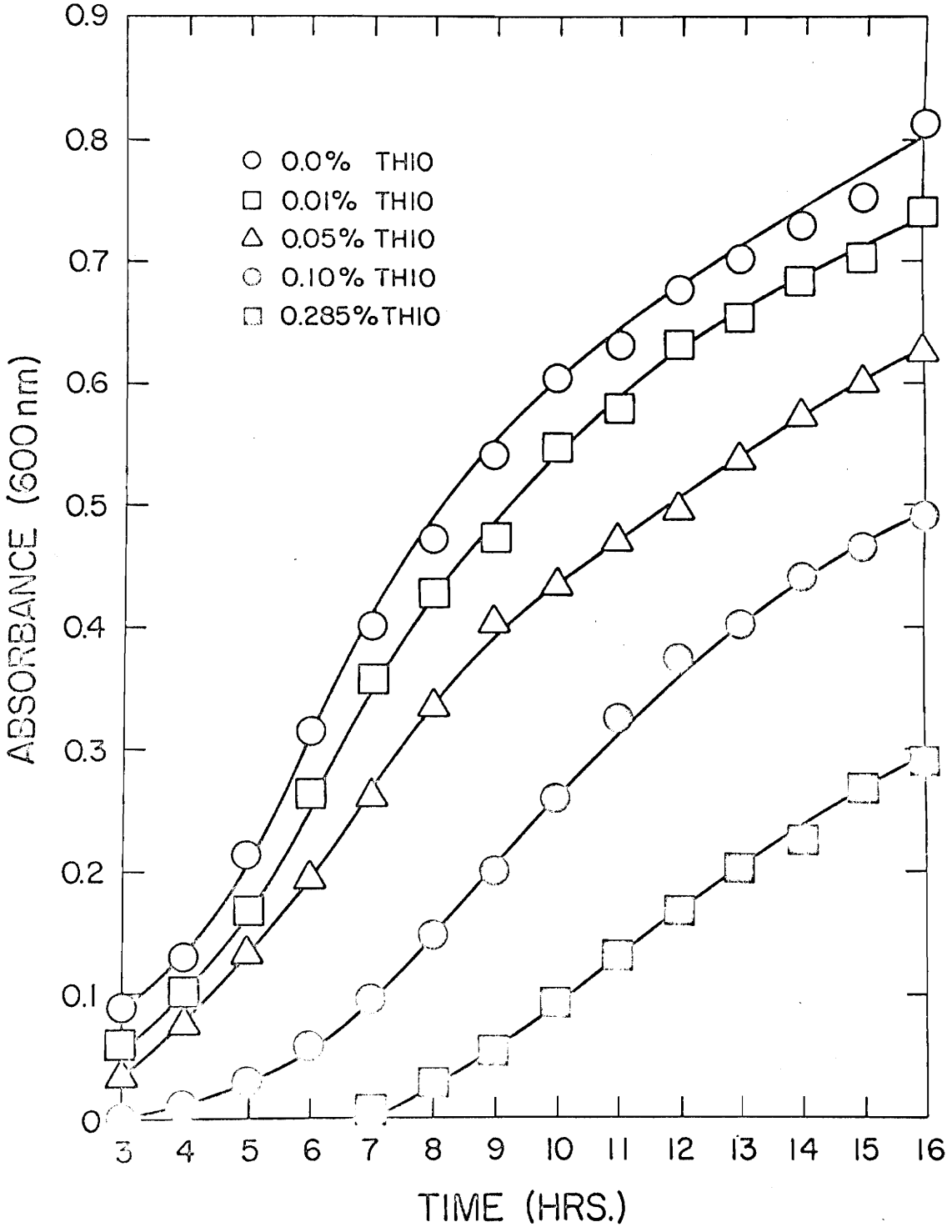

Fig. 11. Growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing varying concentrations of sodium thioglycolate.

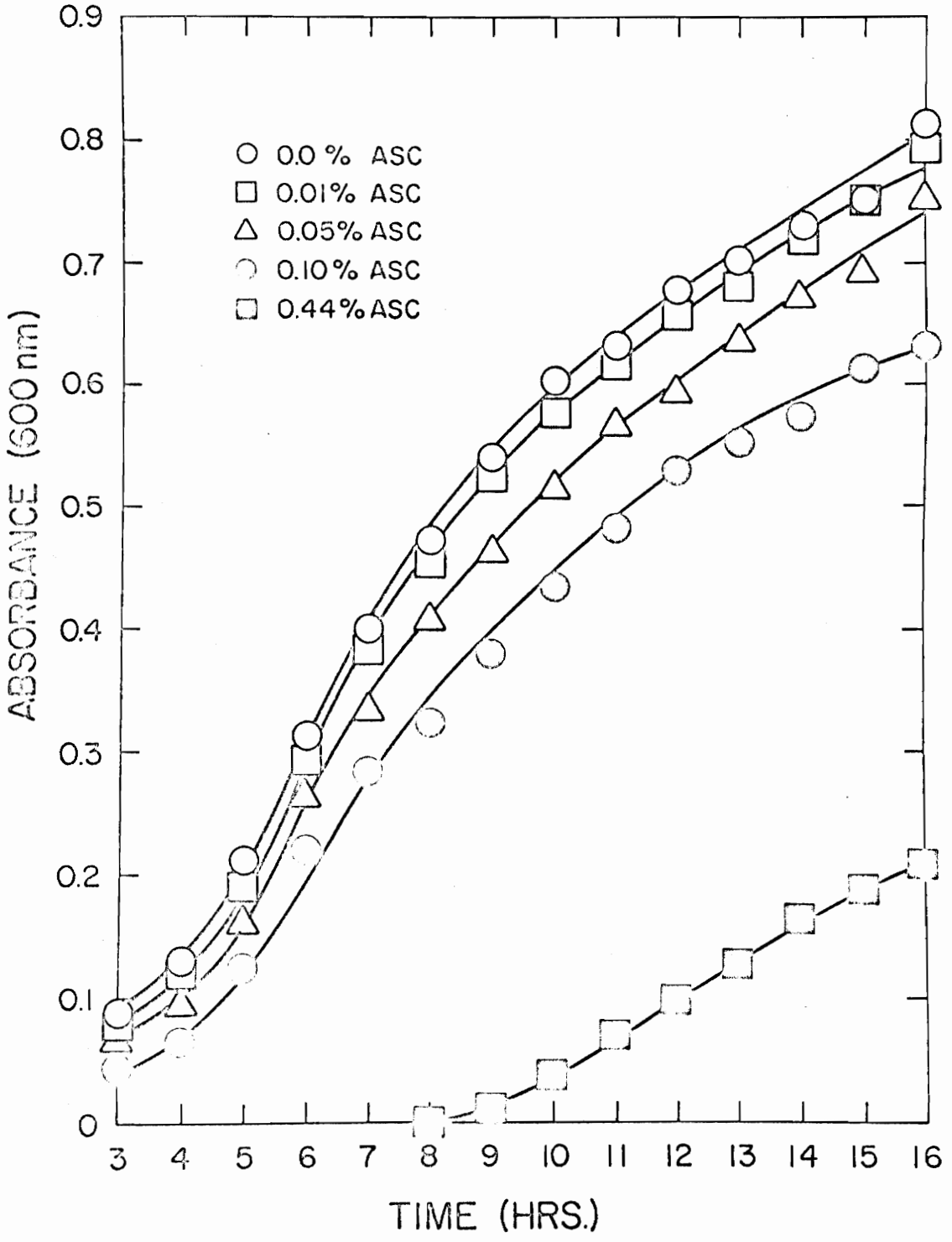

Fig. 12. Growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing varying concentrations of ascorbic acid.

0.1% level of THIO and the 0.025 M concentrations of all the reducing agents.

In similar prereduced anaerobic media, Nassaralla (1971) also found that C. botulinum was inhibited at 0.1% ascorbic acid. He, however, reported no inhibition by thioglycolate at concentrations of 0.01% and 0.1%. He also observed that cysteine and sulfoxylate provided the best growing conditions. Barker and Wolf (1971) showed that thioglycolate at concentrations of 0.05% and 0.1% inhibited vegetative growth of their strains of C. sporogenes and C. bifermentans.

2. Effect of Sodium Chloride, Hydrogen Ion, and Sucrose

The mechanism of sodium chloride inhibition can be considered one of moisture limitation--the sodium chloride restricting the amount of water which is biologically available for the growth of the microorganism (i.e. lowers the water activity) (Jay, 1970). In addition, salt may have specific toxic effects (Riemann, 1968). Table 6 outlines the growth responses (as absorbance changes) of the test organism in 0.03% SFS prereduced TSB containing various concentrations of sodium chloride. Five percent salt was the minimum concentration for complete inhibition of the test organism for a period of 200 hours. This result compares well with the data of Segner et al. (1966) for type E spore outgrowth. They reported that up to 5.0% sodium chloride was required for inhibition at 30 C.

Inhibition by increased hydrogen ion concentration (low pH) may be the direct result of hydrogen ion inhibition on enzyme reaction, transport mechanism, etc., or a toxic effect caused by the undissociated

Table 6. Inhibition of C. botulinum type E spore outgrowth by sodium chloride as measured by absorbance changes in anaerobic prereduced trypticase soy broth containing 0.03% sodium formaldehyde sulfoxylate.

Total Sodium Chloride Concentration	Incubation Time (hours)	Change in Abs. at 600 nm. (average of 3 tubes)
0.5%	10	0.57
	45	1.05
	90	1.15
2.5%	10	0.17
	45	0.97
	90	1.15
3.5%	10	0.17
	45	0.26
	90	0.28
4.0%	10	0.002
	45	0.08
	90	0.14
4.5%	10	0.012
	45	0.023
	90	0.065
5.0%	10	0
	45	0
	90	0

acid moiety (Jay, 1970). Table 7 presents the absorbance changes in 0.03% SFS prereduced TSB at several pH levels. Minimum pH for growth was observed to be 5.06. At pH 4.98 no significant absorbance increases were found in any of the culture tubes. However, in one tube at pH 4.85, growth was recorded. The lowest pH reported for the outgrowth of type E spores is 5.03 in a TPG medium (Segner et al., 1966).

The inhibitory effect of increased sucrose concentration is the result of lowering the water activity (Jay, 1970). Sucrose inhibition data are presented in Table 8. Possible growth was observed even at the highest concentration studied (40%). The limits of the methods used to indicate growth must be considered when comparing any of the results obtained here to previous studies. Emodi and Lechowich (1968b) reported 38.5% sucrose to be the minimum level for complete inhibition of C. botulinum type E spores.

Variation in the pH, sucrose, and salt inhibition levels observed in this study and the others mentioned above may be the result of differences in strains, media or level of inocula. The use of prereduced media may not only affect the growth of the organism but also the lag phase prior to growth. Assuming that the inoculum requires an environment of low redox potential before growth can be initiated, prereduced media may help establish such a low potential. Conventional media may exhibit a prolonged lag period since the inoculum could require an extended time during which the endogenous metabolism of the cells functions to lower the potential and allow for outgrowth. In prereduced media the potential may be sufficiently low to allow for immediate outgrowth.

Table 7. Inhibition of C. botulinum type E spore outgrowth by pH as measured by absorbance changes in anaerobic prereduced tryptic-case soy broth containing 0.03% sodium formaldehyde sulfoxylate.

pH level	Incubation time (hours)	Change in Abs. at 600 nm. (average of 3 tubes)
7.15	10	0.54
	45	1.0
	109	1.2
6.32	10	0.35
	45	1.1
	109	1.3
5.64	10	0.005
	45	0.90
	109	1.15
5.46	10	0
	45	0.74
	109	0.95
5.38	10	0
	45	0.63
	109	0.80
5.24	10	0
	45	0.27
	109	0.71
5.15	10	0
	45	0.20
	109	0.52
5.06	10	0
	45	0.01
	109	0.28
5.02	10	0
	45	0.004
	109	0.008
4.85	10	0
	45	0
	109	0.023 (possible growth in one tube)

Table 8. Inhibition of C. botulinum type E spore outgrowth by sucrose as measured by absorbance changes in anaerobic prereduced trypticase soy broth containing 0.03% sodium formaldehyde sulfoxylate.

Total Sucrose Concentration	Incubation time (hours)	Change in Abs. at 600 nm. (average of 3 tubes)
0.0%	10	0.54
	45	1.15
	90	1.2
10%	10	0.28
	45	0.77
	90	1.0
20%	10	0.052
	45	0.45
	90	0.60
30%	10	0.007
	45	0.245
	90	0.38
35%	10	0.004
	45	0.120
	90	0.125
36%	10	0.001
	45	0.09
	90	0.10
37%	10	0
	45	0.07
	90	0.04
38%	10	0
	45	0.06
	90	0.036
39%	10	0
	45	0.04
	90	0.034
40%	10	0
	45	0.02
	90	0.034

E. Comparisons of Growth, Eh7 and Reducing Agent Concentration

Absorbance readings after 10 hours were selected as the growth criteria (See Figs. 8, 9, 10, 11 and 12). When these growth readings were compared to the Eh7 measurements in Figure 4, it was found that optimal growth occurred in TSB containing no added reducing agents for those types of agents which raise the Eh7 of the media (i.e. THIO and ASC). For the TSB containing reducing agents which lower the Eh7 at low concentrations (SFS, MER and CYS), the optimal growth occurred at an intermediate level. This level appeared to be at the point were the ratio of -Eh7 to concentration of reducing agent was a maximum. When growth is plotted on the ordinate and -Eh7 / (-log M reducing agent)$^{-1}$ or -Eh7 X -log M reducing agent is plotted on the abscissa, a direct relationship is evident (Fig. 13).

These data suggest that growth of the test organism depends on a low concentration of reducing agent and a low Eh7 level in the medium. O'Brien and Morris (1971) felt that the growth rate of C. acetobutylicum depends on the free oxygen in the medium and not culture Eh. They effectively poised the Eh at different levels with artificial electron acceptors and varied the oxygen concentration. Their data indicated that oxygen has an inhibiting effect on the organism that cannot be simply explained by a raised Eh of the medium. Knaysi and Dutky (1936) found a similar result with their clostridial organism. However, when poising the system at a high Eh with an artificial electron acceptor such as potassium ferricyanide, the additional influence of the ferricyanide must be determined. It cannot be assumed that ferricyanide simply raises

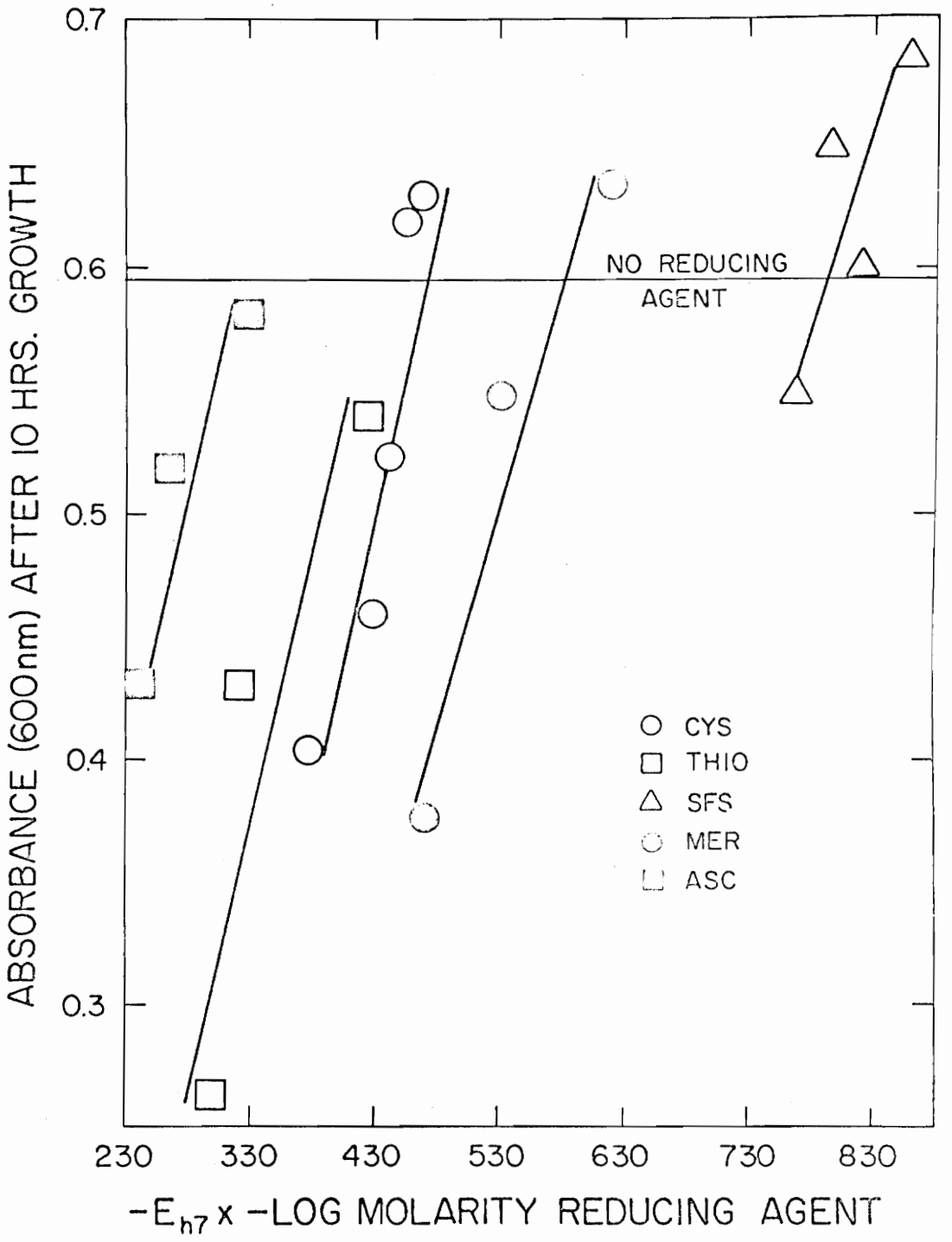

Fig. 13. The effect of Eh7 and concentration of reducing agent on the growth of C. botulinum type E in prereduced anaerobic trypticase soy broth containing different reducing agents at several concentrations.

the culture Eh and does not influence growth in any other way. In fact, the ferricyanide may enhance growth in some way, thereby offsetting any inhibition due to elevated Eh. The oxygen in the medium may have inhibitive qualities other than an elevated Eh. Indeed, oxygen may be the crucial inhibitor in the medium, but the Eh value cannot be overlooked as an additional inhibiting influence.

The experiments summarized in Figure 13 were all performed in the complete absence of molecular oxygen. A low Eh7 consistantly produced optimal growth as long as the concentration of the reducing agent was not too high.

There exists the possibility that the data summarized in Figure 13 reflect an entirely opposite conclusion--that the growth is proportional to a high Eh level and a high concentration of reducing agent. Indeed, -Eh7 X -log M reducing agent simplies to Eh7 X log M. This possibility is rejected, however, when a comparison is made of growth in media containing different concentrations of reducing agents and approximately the same Eh7 value. For instance, TSB containing 0.01% and 0.05% THIO produced almost identical Eh7 values--approximately -136 mv. Growth in TSB with 0.01% and 0.05% THIO resulted in absorbances of 0.540 and 0.430 respectively at 600 nm. It can be seen that at the same Eh7 level growth was inhibited by the higher concentration of reducing agent. A similar finding is reflected in the case of TSB containing 0.05% and 0.1% ASC where each has an Eh7 of about -102 mv and absorbances are 0.518 and 0.435 respectively. These results bring back the original hypothesis that optimal growth of the test organism depends on a low Eh7 and a low

concentration of reducing agent. Figure 12 indicates a direct relationship between growth, low Eh7, and low reducing agent concentration for each reducing agent. Additional variables such as the relative growth-promoting or growth-inhibiting properties of each type of reducing agent must be considered if a comparison is to be made between the different reducing agents. By comparing the growth curves for 0.025 M concentrations of added reducing agent (Figs. 7, 8, 9, 10, and 11), it can be seen that the best growth occurred in SFS followed by MER, CYS, THIO and ASC. These variations in growth may not only be due to Eh7 levels but to other inherent growth-promoting or growth-inhibiting differences between the various reducing agents.

F. Growth and Eh7 in the Electrode Vessel

1. Carbon Dioxide Requirement for Germination and Outgrowth

Without the addition of carbon dioxide to the gas entering the prereduced TSB within the electrode vessel, the test spores would not germinate for up to 3 days following inoculation. During this period the medium was monitored by direct counts and heat shocked roll tube counts. No increases or decreases in numbers were observed. The spores continued to be phase bright and heat resistant. It was concluded that germination had not occurred. A carbon dioxide requirement or enhancement of germination has been shown for other clostridia (Treadwell et al., 1958; Holland et al., 1970; King and Gould, 1971).

Previous investigations of the carbon dioxide or bicarbonate requirement for germination in clostridia have not been very well

controlled to insure an absence of carbon dioxide in the prepared media. They have not utilized the methods of prereduction (i.e. refluxing and subsequent autoclaving under nitrogen) and strict anaerobic conditions (no chance for atmospheric carbon dioxide to enter the media). Treadwell et al. (1953) demonstrated an absolute necessity of bicarbonate for germination in C. botulinum; however, he did not incubate his spores for longer than 120 mins.

In order to determine the optimal amount of carbon dioxide for germination of the test organism, two levels were utilized as shown in Figure 14. A high level of carbon dioxide was obtained in the medium by controlled addition of carbon dioxide to the sparging gas resulting in a lowering of the pH by 0.6 units. A low level was obtained by lowering the pH by 0.3 units. Since the high level did not significantly increase outgrowth of the spores, the low level was used in the remainder of the growth experiments performed in this study. In the media of very low pH (5.05 - 5.11), difficulty was encountered in trying to lower the pH by addition of carbon dioxide. The high concentration of hydrogen ions shifted the equilibrium to prevent the dissociation of carbonic acid molecules. If bicarbonate is required for germination in this organism, it is interesting to speculate that the reason C. botulinum does not grow in foods or media of high acidity is because of the absence of dissociated carbonic acid or bicarbonate ions.

The fraction of undissociated carbonic acid, bicarbonate ion, and carbonate ion can be determined at various pH levels by using the necessary dissociation constants. At pH 4.0 the fraction as H_2CO_3

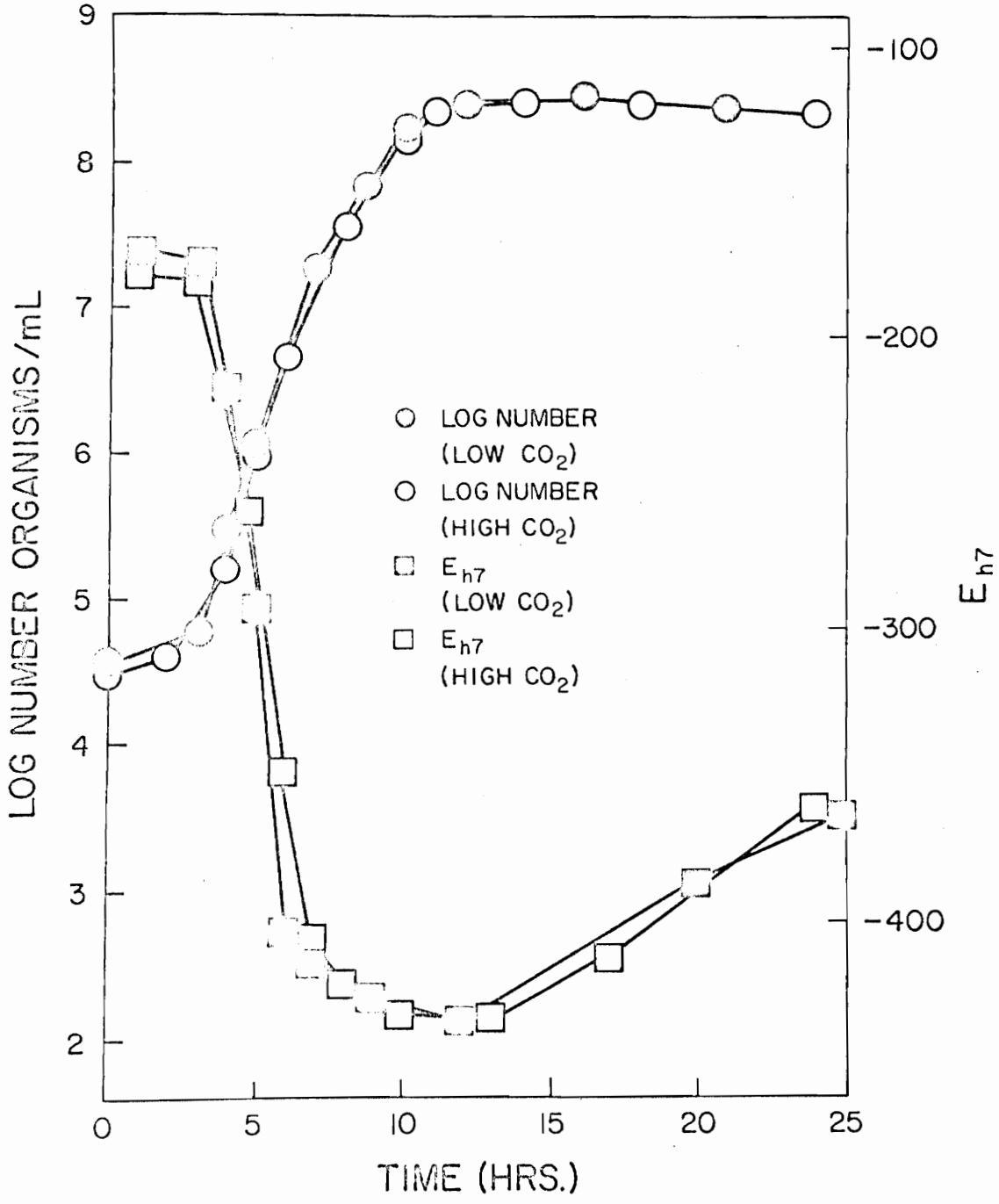

Fig. 14. Growth of C. botulinum type E and Eh7 changes in pre-reduced anaerobic trypticase soy broth plus 0.05% cysteine with high (0.6 pH units) and low (0.3 pH units) carbon dioxide levels.

would be 0.9957, the fraction as HCO_3^- would be 0.0043, and the fraction as $CO_3^=$ would be 2.0×10^{-9}. Bicarbonate ions do not become a principal species in solution until the pH is raised to approximately 5.0.

2. Uninhibited Growth in Oxidized and Reduced TSB

Oxidized TSB (cooled after refluxing and autoclaved under air) was purged of any dissolved air by sparging with nitrogen as explained in the Methods Section. Air was then added to the oxidized medium and the reduced medium. The Eh7 versus log parts per million dissolved oxygen was plotted for both types of media as shown in Fig. 15. The slopes of both curves are identical. From this it can be concluded that the difference between these two types of media is the result of a chemical oxidation of the components of the oxidized medium during preparation and autoclaving and is not due to any difference in dissolved oxygen levels.

The growth and Eh7 changes during growth are recorded in Figure 16 (growth as determined by roll tube counts) and in Figure 17 (growth as determined by absorbance). Figure 16 indicates that growth of the organism occurs simultaneously with the Eh7 drop. The growth begins earlier than any increase in absorbance in the medium. This is expected since absorbance changes typically are not detected until the concentration of bacteria reaches about 10^5 or 10^6 organisms/ml.

Increases in numbers of bacteria are not seen until approximately 3 hours following inoculation of the flask. This is consistent with previous studies showing that C. botulinum is characterized by a delayed germination (Treadwell et al., 1958).

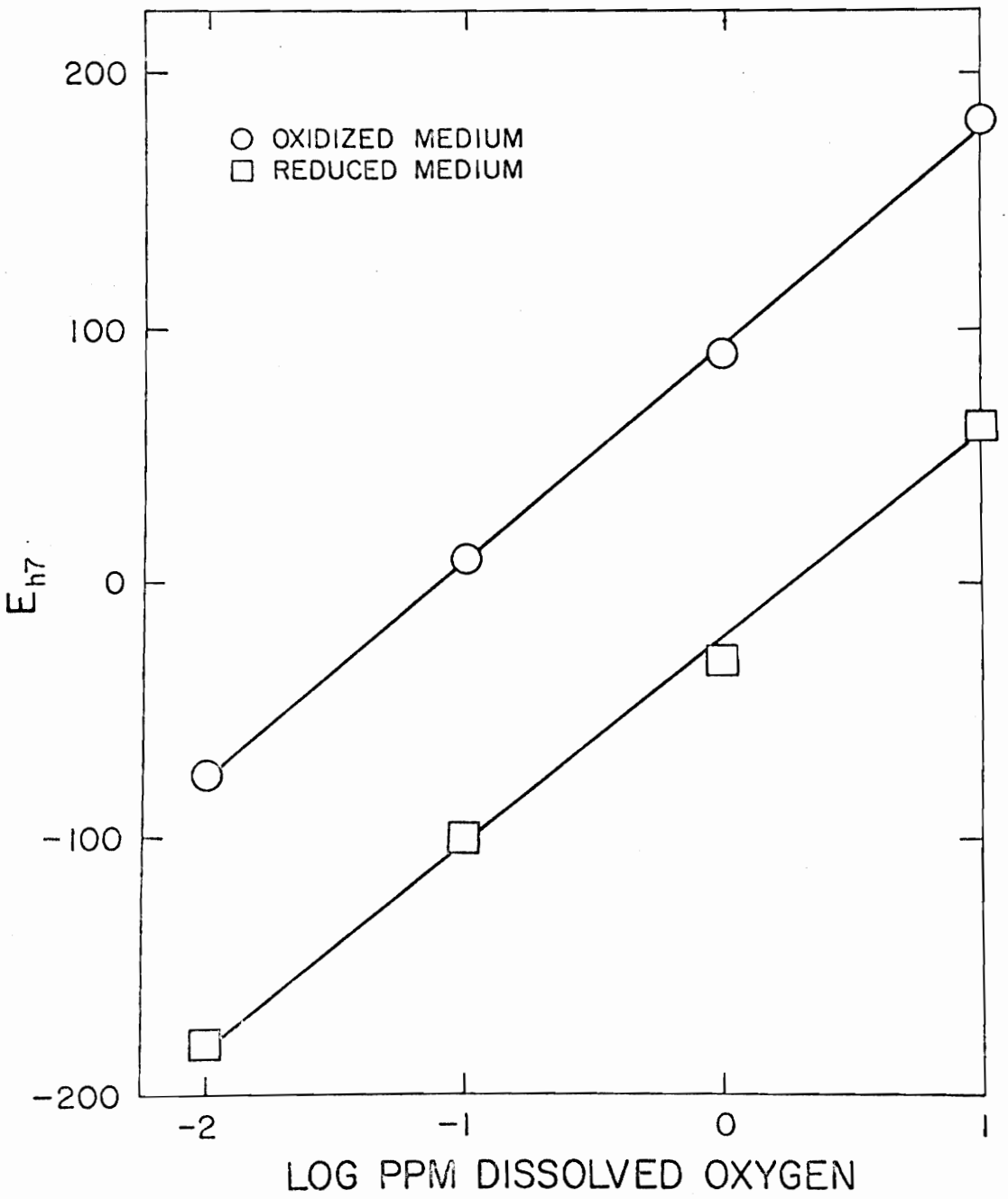

Fig. 15. The effect of dissolved oxygen on the Eh7 of oxidized and prereduced trypticase soy broth plus 0.05% cysteine.

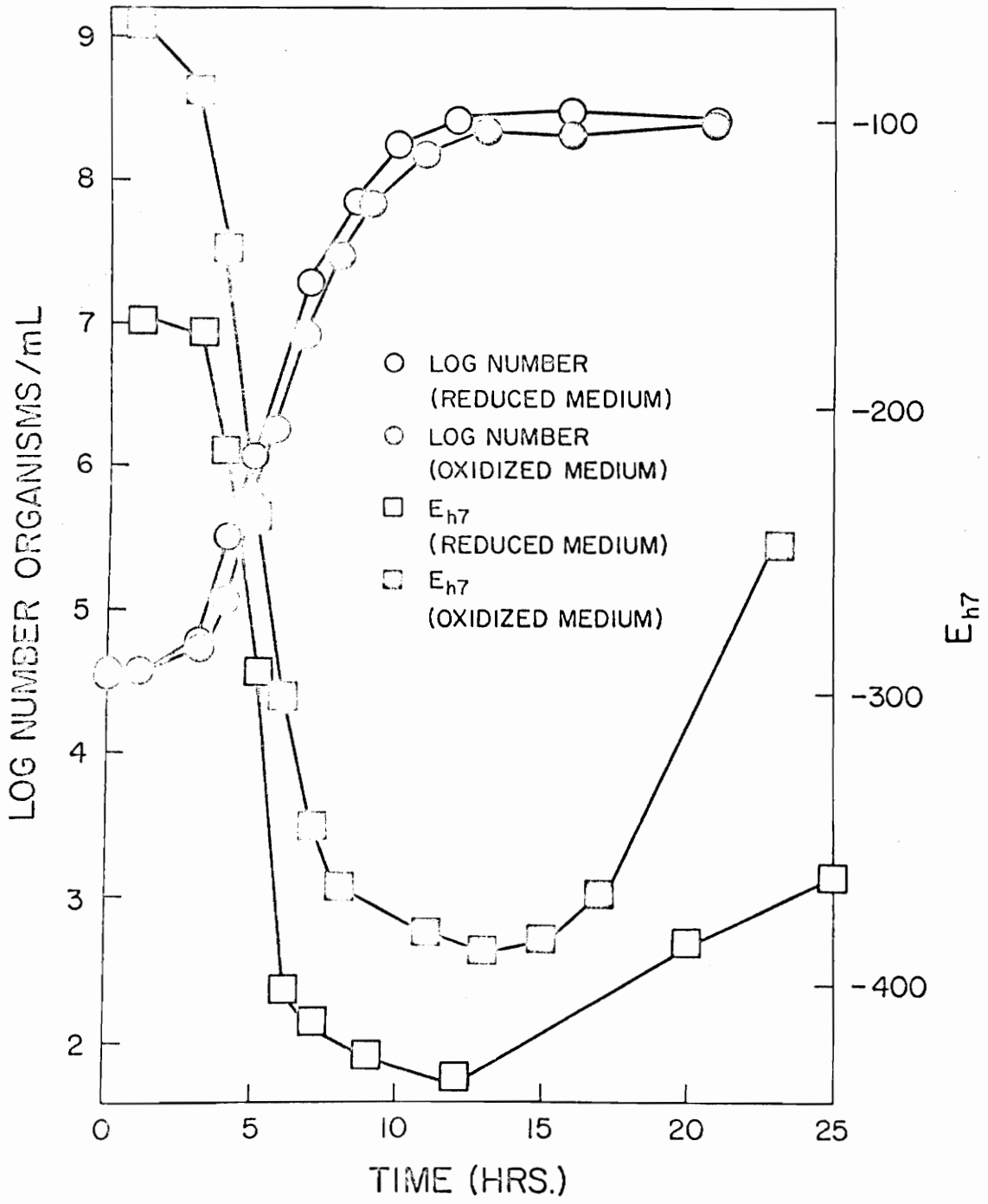

Fig. 16. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine.

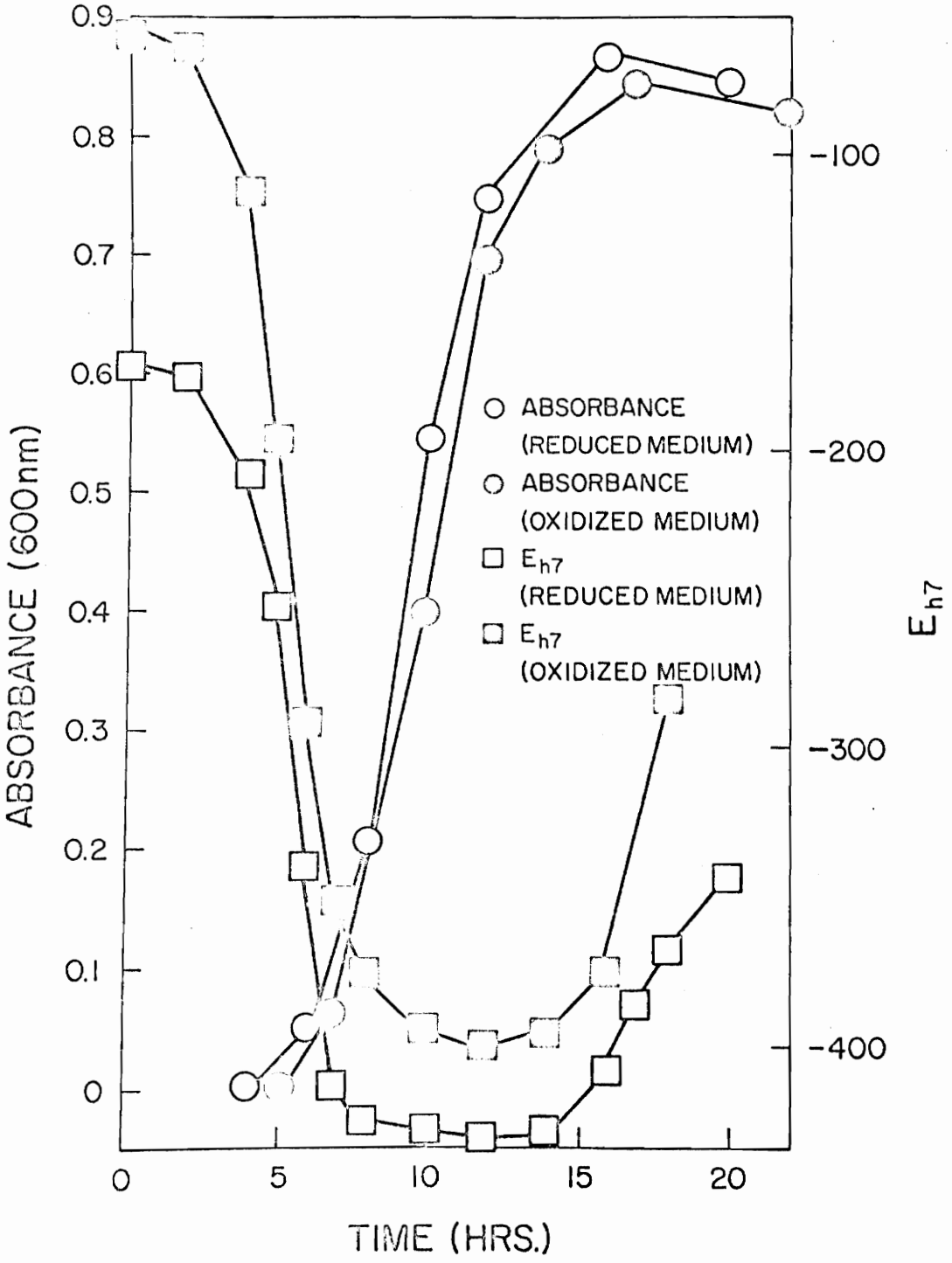

Fig. 17. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine.

Inhibition of the outgrowth by the oxidized medium is not significant either when measured by roll tube counts, absorbance or Eh7 lowering. The Eh7 in the oxidized medium did not attain the same low level as with growth in the reduced medium, but the total drop is as far or further than the reduced medium drop. The incubation time required for the Eh7 drop to begin is approximately the same in each type of medium.

The lack of any difference in the growth curves of both oxidized and reduced media (media differing by a total of 100 mv) supports a conclusion that the Eh7 of the medium does not have a substantial effect on anaerobic bacterial growth in the range studied--80 to -190 mv.

The initial Eh7 value for reduced TSB during sparging with nitrogen containing 0.1% oxygen (Fig. 18) was very similar to that of the oxidized medium (Fig. 17). Both were approximately 100 mv higher than the reduced anaerobic medium. Figure 11 shows that the Eh7 of the medium being sparged with 0.1% oxygen is poised at a high valve by the continuous exchange of oxygen in the system. As a result, in this medium the Eh7 drop was much slower and less precipitous than in the oxidized medium shown in Figure 17. However, growth rates in both media seem to be the same and not significantly different from growth rates in the reduced anaerobic medium.

Although C. botulinum is considered an obligate anaerobe, it can grow relatively uninhibited in media having a low level of oxygen as illustrated in Figure 18. The uninhibited growth in a medium containing oxygen may result from the well poised low Eh (-80 mv). Uninhibited growth of the test organism under conditions of partial aeration probably

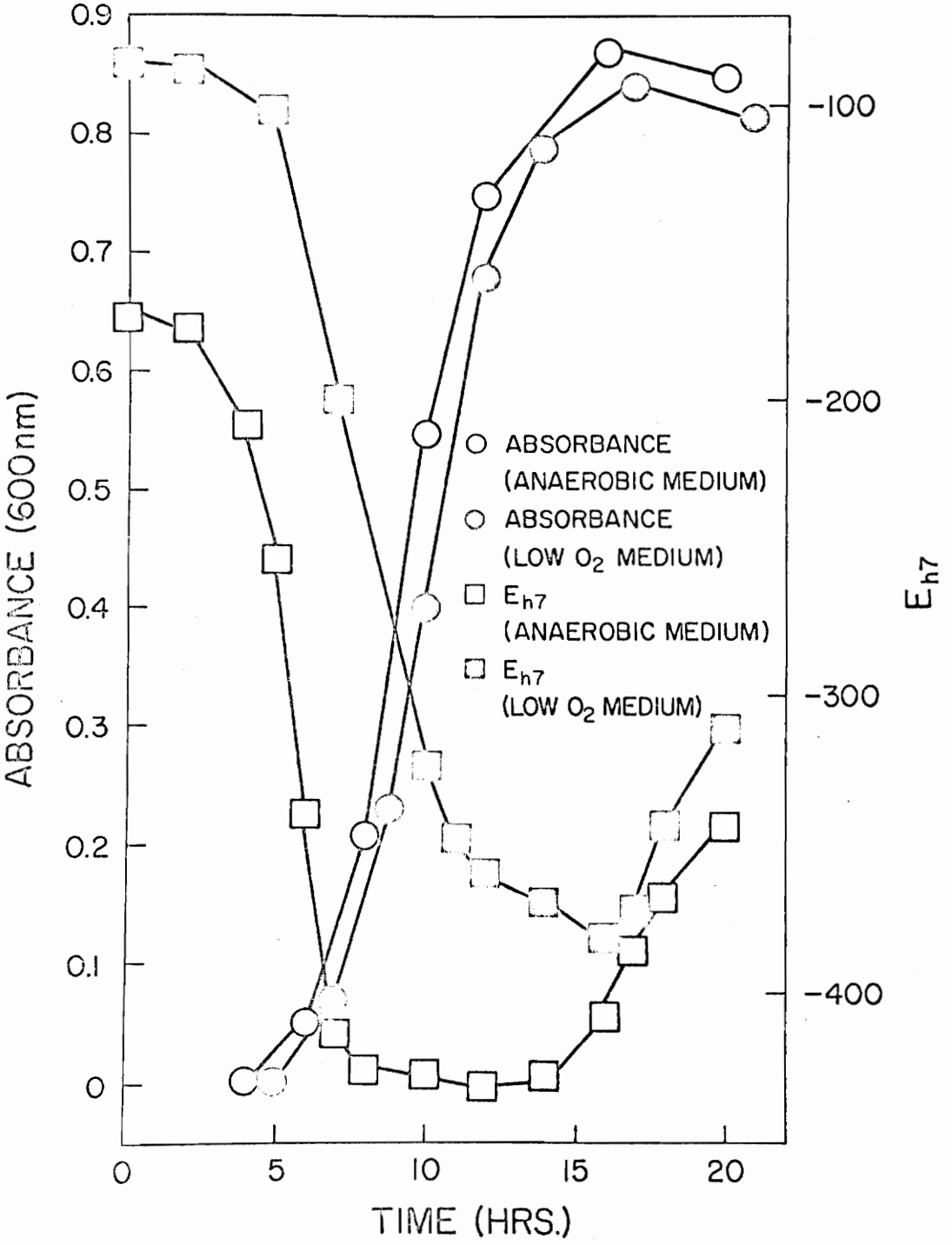

Fig. 18. Growth of C. botulinum type E and Eh7 changes in anaerobic and low oxygen (0.1%) prereduced trypticase soy broth plus 0.05% cysteine.

indicates that the organism can divert part of its reducing power to the reductive detoxification of the oxygen present. O'Brien and Morris (1971) have shown a similar finding with the limited aeration of C. acetobutylicum. They suggested that since the aeration provoked an increase in NADH oxidase, NADH must serve as a major reducing source to neutralize the oxygen effects. If the level of aeration was increased, the consumption of NADH would exceed the rate of supply at the expense of the essential reductive reactions of the cell and growth would cease. Douglas and Rigby (1974) found similar results with the lag period prior to growth being progressively lengthened by sparging the culture with increaseing levels of oxygen in nitrogen. With pure nitrogen the spores showed outgrowth in about 2 hours. Sparging with 0.5% oxygen in nitrogen resulted in outgrowth after 5 hours. Nitrogen containing 1.2% oxygen resulted in a spore outgrowth at 14 hours, and a lag of 20 hours was required before growth was initiated with a sparging gas containing 2.1% oxygen. No growth occurred at a level of 3.0% oxygen.

Although such experiments were not undertaken in the present study, the test organism would undoubtedly be inhibited completely at some high oxygen level.

Tally et al. (1975) varied the oxygen concentrations inside anaerobe jars to test the oxygen tolerance of 57 freshly isolated anaerobic strains from clinical specimens. They found 36 strict anaerobes (growth at <0.4% to 0.4% oxygen), 18 moderate anaerobes (0.8% to 2.5% oxygen), and 3 aerotolerant strains (7.5% to 10% oxygen). The clostridial strains were all moderate to aerotolerant anaerobes.

3. Inhibited Growth in Oxidized and Reduced TSB

a. Sodium chloride

Comparison of growth and Eh7 changes for oxidized and reduced TSB containing 3% added sodium chloride is shown in Figure 19. Growth and Eh7 drop in the reduced TSB were initiated at approximately 12-14 hours after inoculation of the flask. Growth and Eh7 reduction began about 10 hours later in the oxidized TSB (at 20-24 hours). The rate of bacterial growth in the oxidized TSB was slightly less than in the reduced medium. The extent of growth in the oxidized medium was less with the late log absorbance leveling at about one tenth absorbance unit less than the reduced TSB. These results indicate that in determining absolute levels for the inhibition of the test organism by sodium chloride, it must be considered that the Eh7 has a definite influence on the inhibition levels. Indeed, more sodium chloride may be necessary for complete inhibition in a reduced medium as compared to an oxidized medium. To test this hypothesis the level of sodium chloride was increased to 3.5% added to both reduced and oxidized TSB. The results are shown in Figure 20. In the reduced medium the reduction in Eh7 began at about 30 hours after inoculation with absorbance increasing about 8 hours later. Neither absorbance increase nor Eh7 decrease were indicated in the oxidized TSB containing the same level of salt for a period of 200 hours.

The above results should be taken into consideration when attempting to determine the minimum sodium chloride levels for total inhibition of C. botulinum. For example, the product processed under conditions of higher aeration may require less salt to completely inhibit

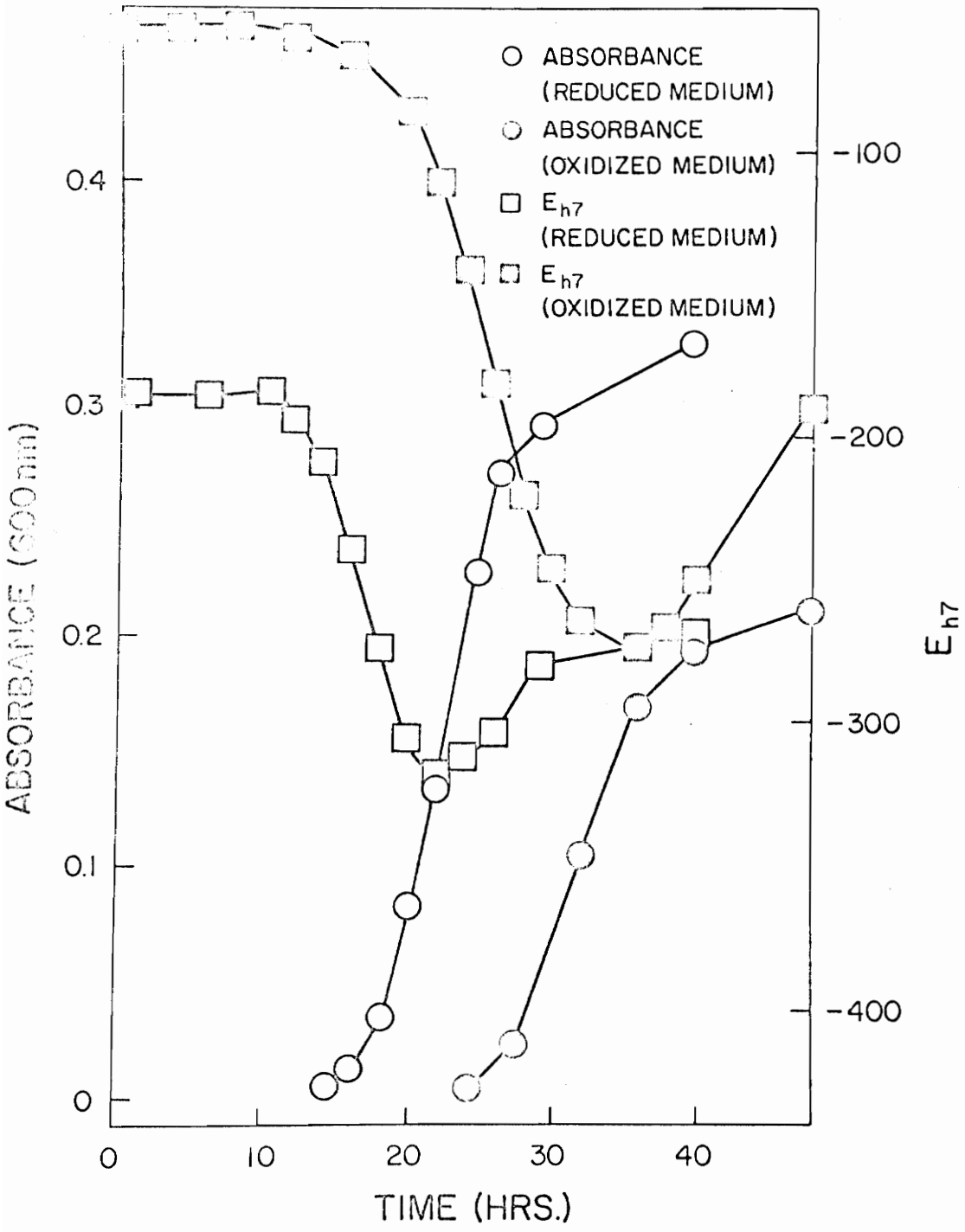

Fig. 19. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 3% added sodium chloride.

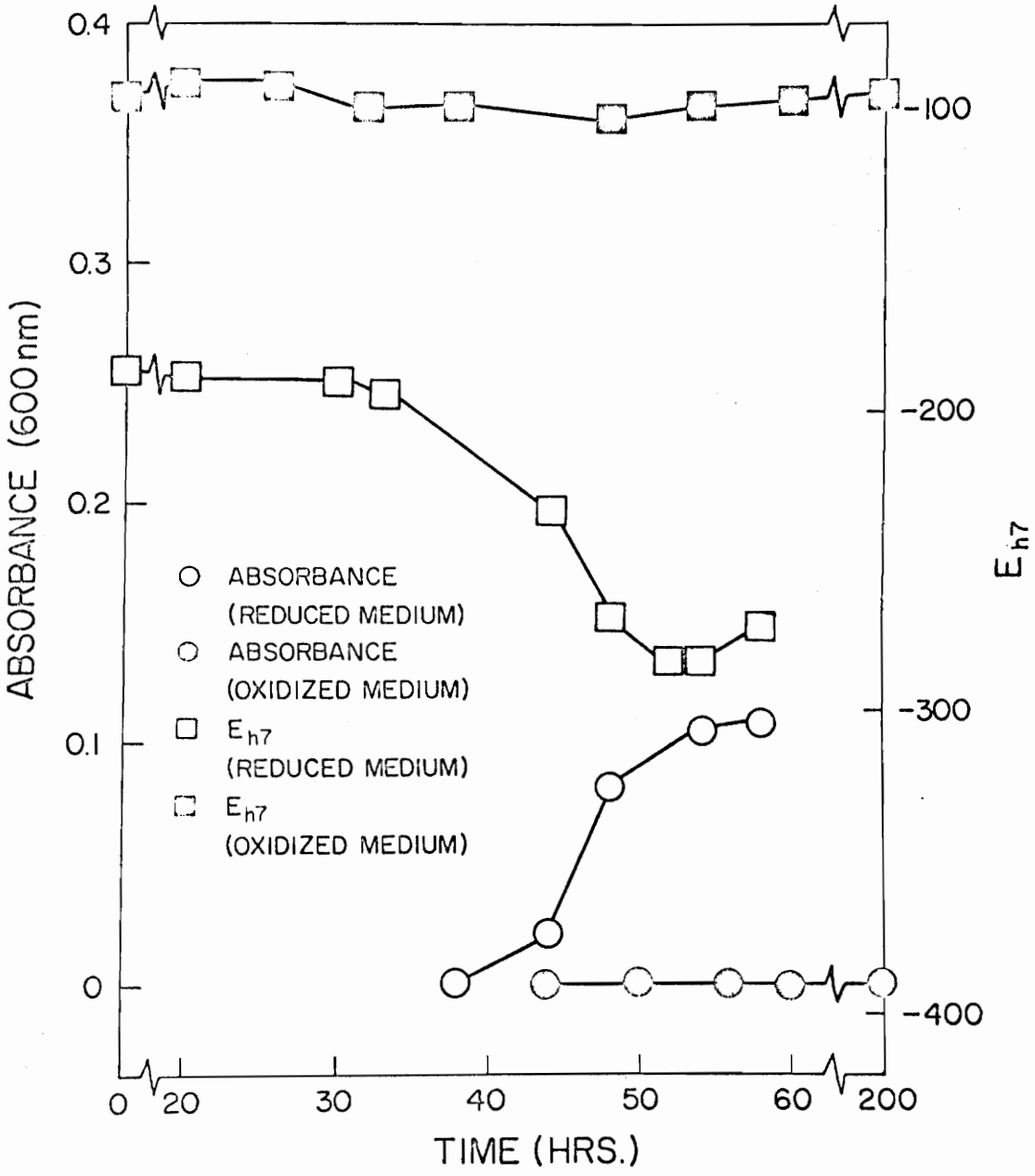

Fig. 20. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 3.5% added sodium chloride.

the organism than the identical product processed under more restricted aeration--even if the product is identically packaged and has exactly the same storage environment.

C. botulinum type E has been shown to require 4.9-5.8% sodium chloride for complete inhibition in various media (Lechowich, 1968). The oxidized TSB of the present study did not allow outgrowth for up to 200 hrs. when 4% salt was present (3.5% added). The oxidized state of the TSB was probably an added inhibition on the spores.

Similar results on the influence of redox potential on the growth limiting salt concentrations for C. perfringens have been reported by Mead (1969). He showed that at Eh levels of +195 and +92 mv growth was inhibited in media containing 5% salt. However, at +66 mv growth occurred. His media were poised by different levels of dissolved oxygen; and, therefore, it was not determined whether the Eh per se was the influencing variable or the molecular oxygen in the system.

b. Hydrogen ion

Figure 21 illustrates the absorbance and Eh7 changes in oxidized and reduced TSB at pH 5.11. In the reduced medium both the Eh7 drop and absorbance increase began at approximately 30 hours following inoculation of the flask. In the oxidized TSB at pH 5.11, an absorbance increase was not observed until 45 hours after inoculation. When TSB at a lower pH (5.05) was examined, the absolute lower level of hydrogen ion inhibition was also a function of the Eh7 of the system (See Fig. 22). Absorbance increases occurred at approximately 46 hours incubation in the reduced TSB of low Eh7. In the oxidized TSB at the same pH, absorbance increase

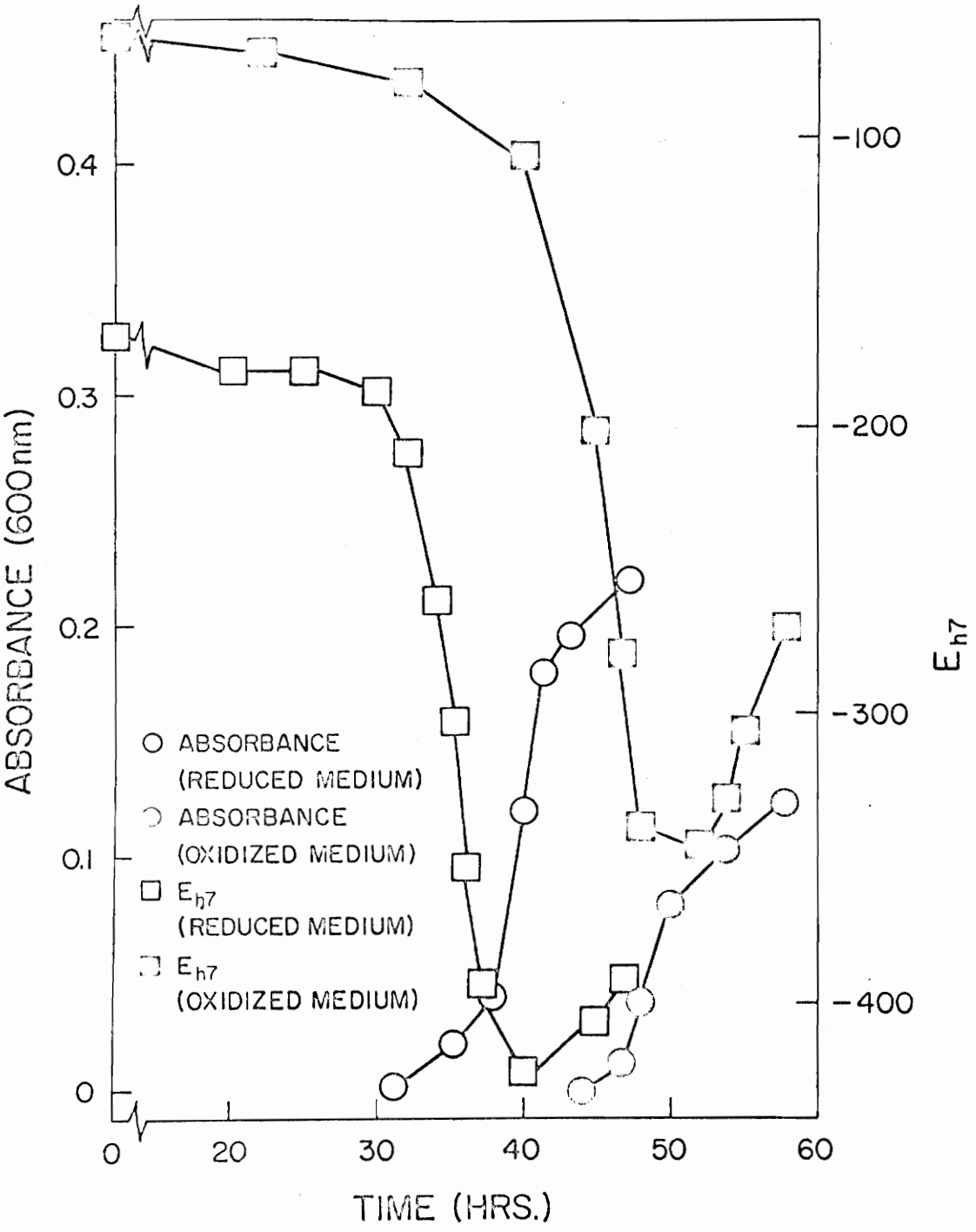

Fig. 21. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and adjusted to pH 5.11.

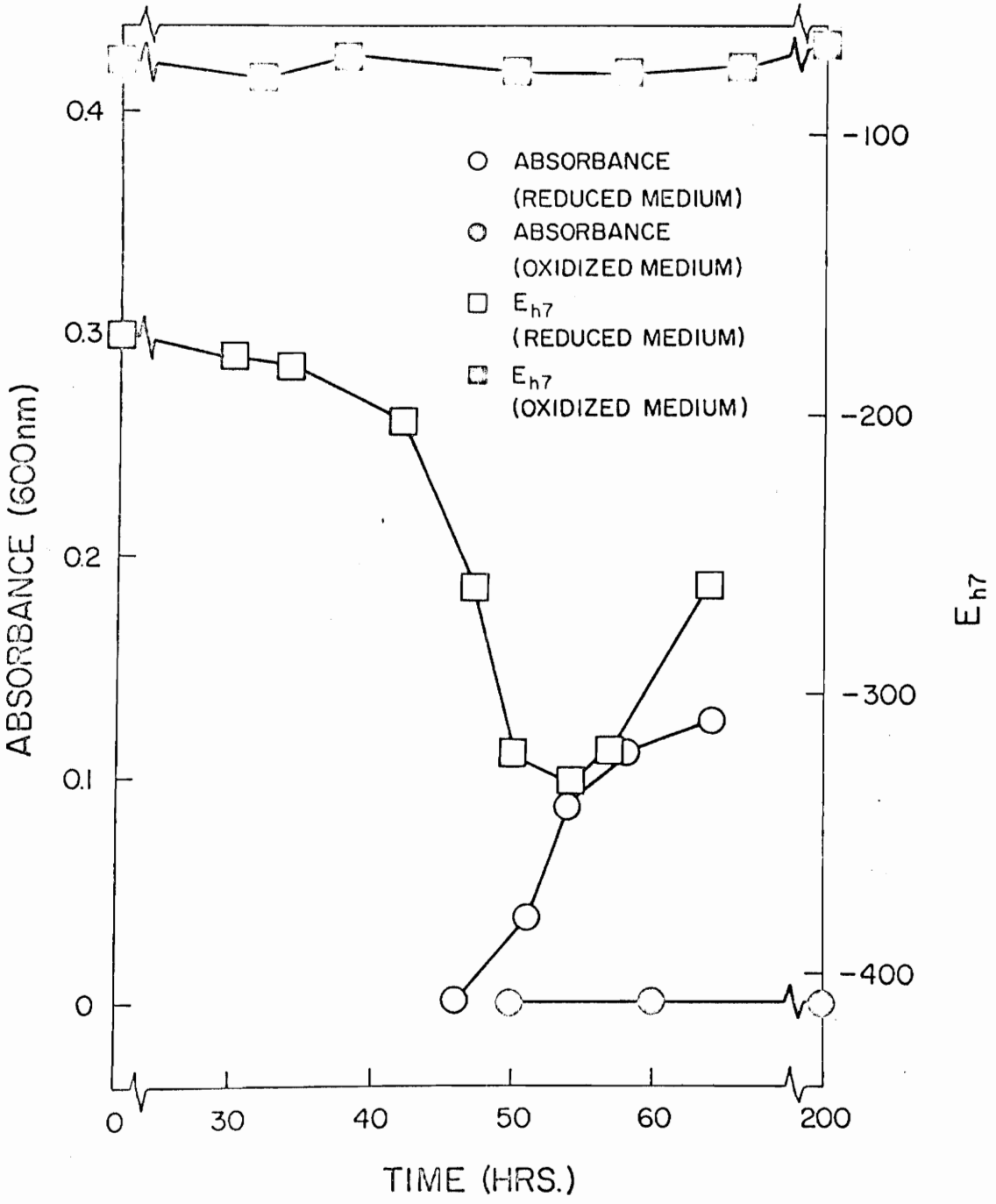

Fig. 22. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and adjusted to pH 5.05.

or Eh7 drop did not occur after 200 hours of incubation.

The direct relationship between Eh and pH was shown by Leistner and Mirna (1959). As the pH of a system is decreased the Eh is at the same time increased. Several workers have postulated an upper limit of Eh for growth of certain anaerobes (Vennesland and Hanke, 1940; Hanke and Bailey, 1945; Barnes and Ingram, 1956). As the lower pH limit is reached, both the hydrogen ion concentration and the resultant high Eh may be inhibitory factors determining whether or not growth occurs. Data presented in Figures 21 and 22 support this contention. Both oxidized and reduced media in Figure 22 contained the same hydrogen ion level (pH 5.05); the reduced medium, however, was the only one supporting growth. No growth occurred in the oxidized medium after 200 hours.

Hanke and Bailey (1945) summarized their experiments by reporting that there was a marked effect of pH on the limiting Eh for growth of C. histolyticum, C. welchii and C. sporogenes. Conversely, the results of the present study would indicate a marked effect of Eh on the limiting pH for growth of C. botulinum.

c. Sucrose

The effect of Eh7 and 30% sucrose on inhibition of the test organism is illustrated in Figure 23. The medium having a lower Eh7 (reduced TSB) exhibited a lag time of approximately 15 hours. On the other hand, the oxidized TSB showed a lag of about 24 hours prior to initiation of growth as measured by an absorbance increase. The extent of growth as measured by absorbance is clearly less in the oxidized medium.

The level of sucrose was increased to 35% and growth was

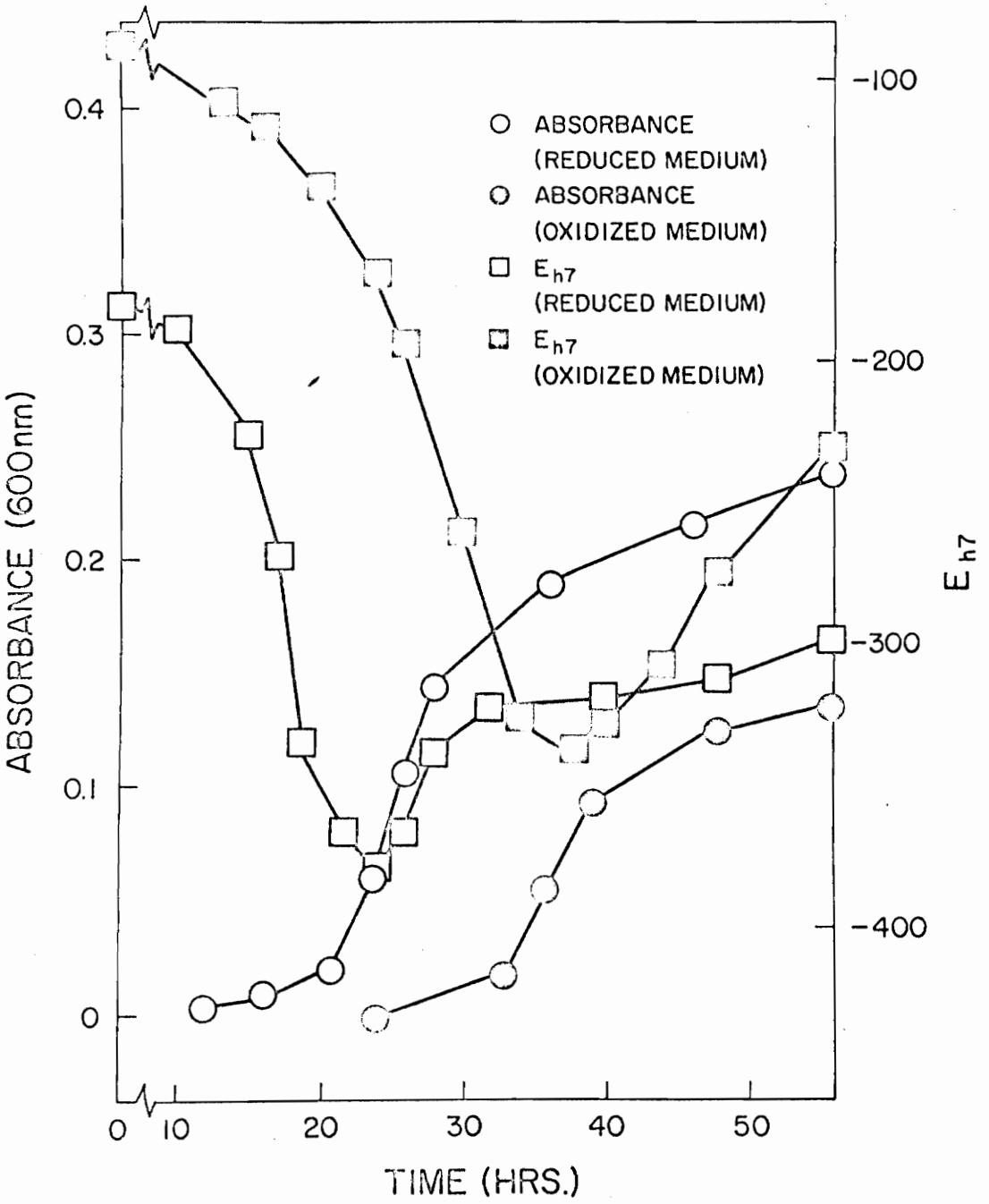

Fig. 23. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 30% sucrose.

compared (Fig. 24). The combination of high Eh7 and high sucrose level inhibited the growth totally in the oxidized medium for 200 hours. The same level of sucrose (35%) did not by itself completely inhibit growth if the Eh7 of the medium was very low as in the reduced medium (Fig. 24). Since both media contained the same amount of sucrose, the differences in growth responses were due to the variations in the media resulting from the two different methods of preparing the oxidized and reduced media. In order to determine whether any inhibitory substances (e.g. organic peroxides) are formed during the oxidation of the medium components, cysteine hydrochloride was added back to the oxidized medium after autoclaving and sparging the medium in the electrode vessel with prepurified nitrogen to remove dissolved oxygen. Growth in such an oxidized and subsequently reduced medium containing 30% sucrose is compared to growth in the standard reduced 30% sucrose medium (Fig. 25). The lack of any significant differences in absorbance increase or Eh7 decrease between both of these media indicate that no inhibitory substances were formed during the oxidation steps. It is possible that if such inhibitory substances were present in the oxidized medium, they may have been converted back to their reduced forms by the addition of the cysteine. It is unlikely that all of these oxidized products were reduced since the original oxidation was carried out at a high temperature (121 C), and it is known that in biological systems many oxidation reactions are irreversible. It is suggested that the added cysteine simply masks the high redox potential provided by the oxidation products by supplying additional reducing intensity to the medium.

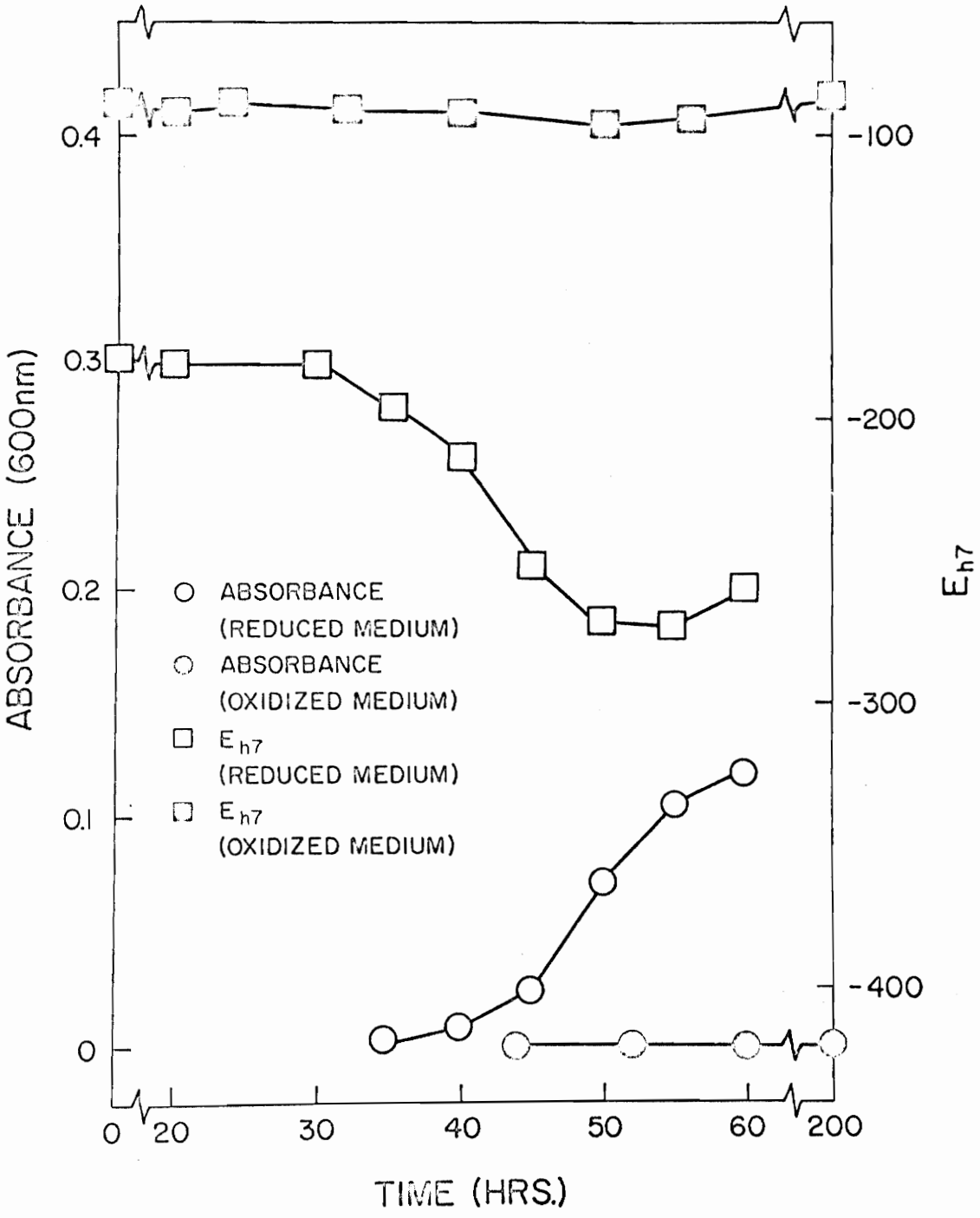

Fig. 24. Growth of C. botulinum type E and Eh7 changes in oxidized and prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 35% sucrose.

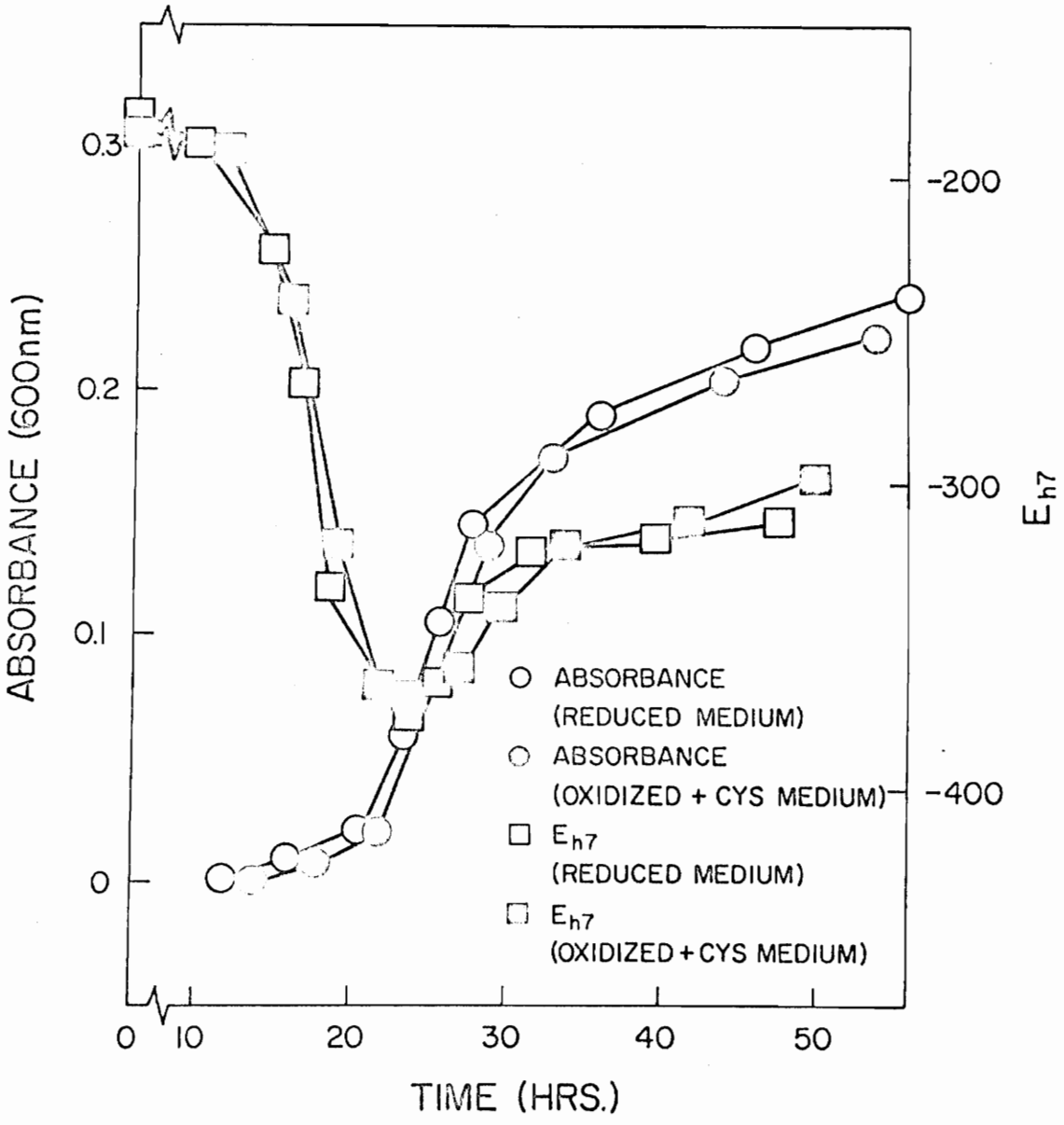

Fig. 25. Growth of C. botulinum type E and Eh7 changes in prereduced anaerobic medium and oxidized anaerobic medium which has been reduced by adding cysteine after autoclaving (both media are trypticase soy broth plus 0.05% cysteine and 30% sucrose).

The differences in growth between the oxidized TSB and reduced TSB are not apparent in the media not containing substances inhibitory to growth (Figs. 16, 17, 18). However, in the experiments using media containing substances which inhibit growth (Figs. 19, 20, 21, 22, 23, 24), the growth differences are quite evident. Concluding from the data outlined in Figure 25, it would appear that these growth differences are due to an increase in the concentration of cysteine hydrochloride or sulfhydryl groups *per se* or to a decrease in the general overall Eh7 of the growth system. The previous experiments summarized in Figure 13 indicate that the important factors contributing to maximum growth of the test organism are a low concentration of reducing agent and a low Eh7 value for the medium. These data would suggest that the growth differences shown for the oxidized and reduced media containing various levels of chemical inhibitors are a result of the differences in the Eh7 values between the oxidized and reduced systems. This does not mean to say that the Eh7 of the system *per se* is the dictating factor in growth. The Eh7 value may be (and certainly is) a reflection of the composition of the medium. In other words, the Eh7 value must be a result of such variables as the number of free sulfhydryl groups available for chemical and biological reaction or the number of free reducing radicals in the system.

The Eh7 of the reduced 30% sucrose TSB was poised at a high Eh7 value by sparging with 0.1% oxygen in nitrogen gas (Fig. 26). The final equilibrium Eh7 of this system was very similar to that attained in the oxidized TSB system (approximately -80 mv). Again, the medium

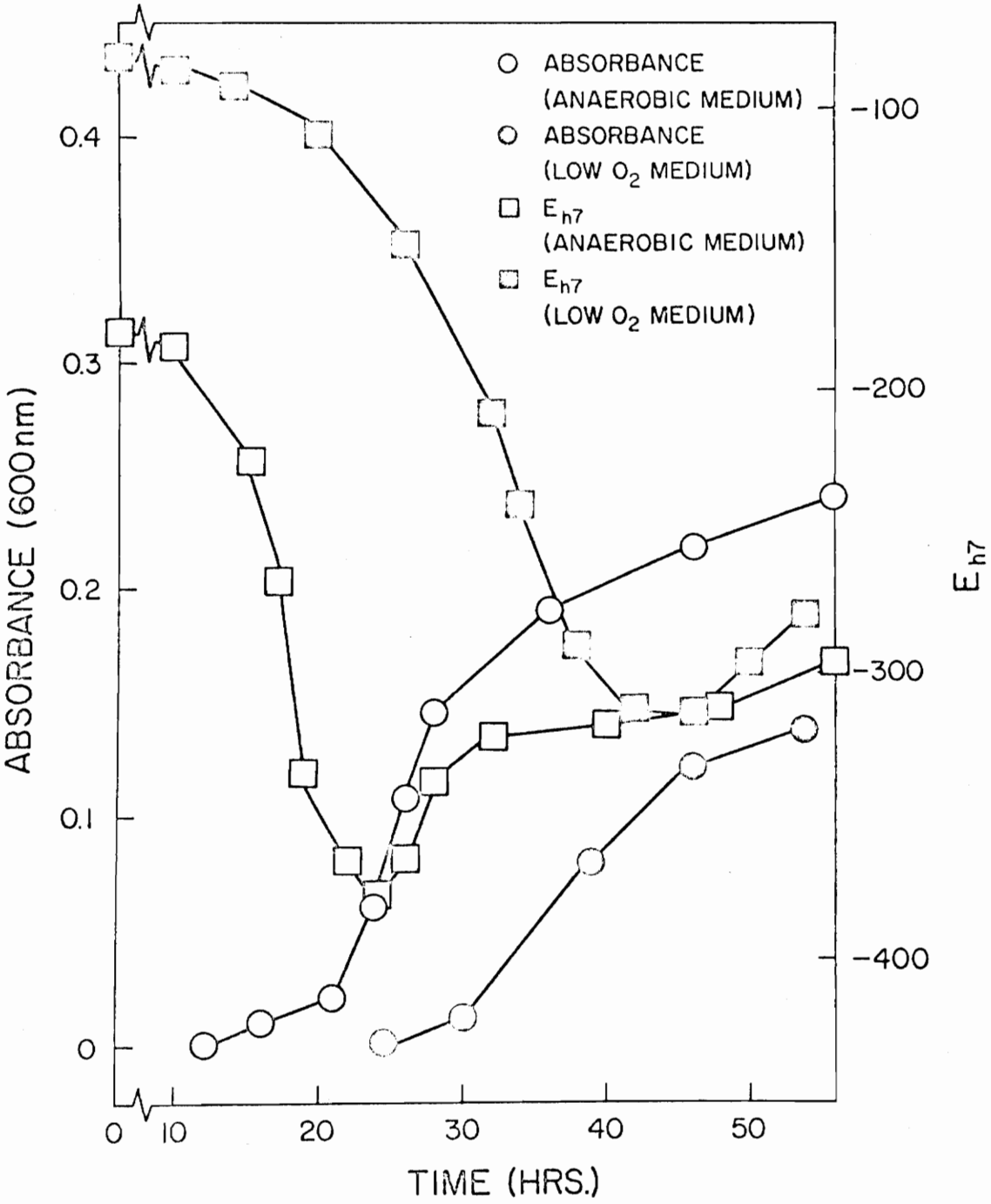

Fig. 26. Growth of C. botulinum type E and Eh7 changes in anaerobic and low oxygen (0.1%) prereduced trypticase soy broth plus 0.05% cysteine and 30% sucrose.

having the low Eh7 level initiated growth first and grew more as shown by the higher absorbance obtained. In contrast, growth of the test organism in the medium poised with a low level of oxygen was more inhibited.

There is very little difference between growth responses in the oxidized medium and the oxygen poised medium as shown by comparing Figures 23 and 26. Since the Eh7 values for both of these systems are similar, this further indicates that the Eh7 of the medium significantly influences the growth response of the test organism irrespective of the poising agent.

4. Effects of Hydrogen Gas

In an effort to determine the factors involved in lowering the Eh of the culture during growth of C. botulinum, Laskin (1956) speculated that the reduced conditions are a result of metabolic activity involved in the breakdown of complex components of the medium. He compared Eh to the amounts of cysteine, cystine, methionine, volatile mercaptans and hydrogen sulfide in the medium during different stages of growth. No correlation was found between the Eh of the system and amounts of these reducing components.

Since hydrogen is known to reduce the Eh of a system dramatically as is found during the growth of many anaerobes, this gas was considered a possible mechanism which could be responsible for lowering the Eh7 of the system during growth. When the hydrogen produced during the metabolic processes of growth is compared to the Eh7 drop during growth, a definite correlation is evident (Fig. 27). Eh7 values began to drop

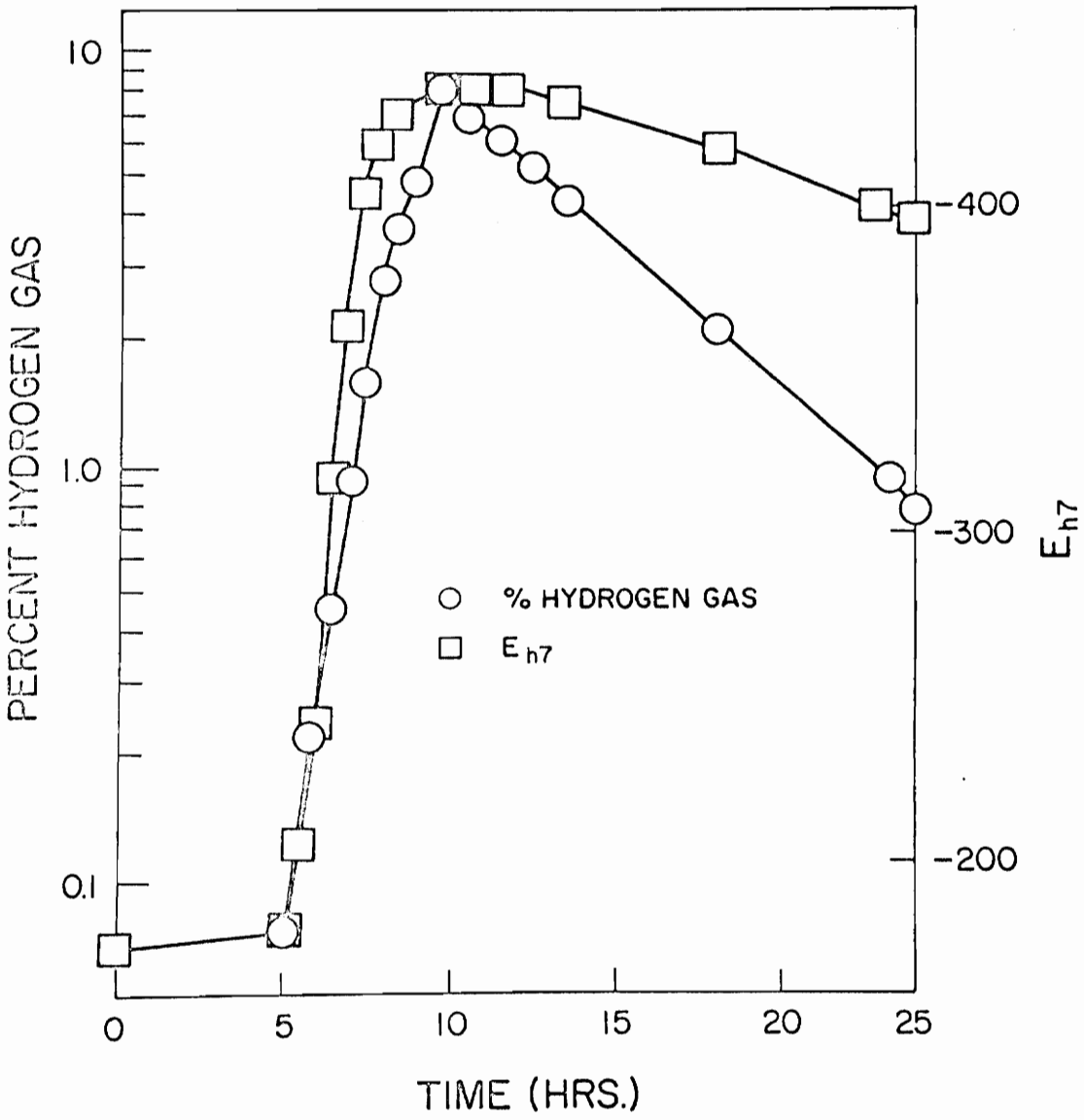

Fig. 27. Hydrogen gas evolution and Eh7 changes during growth of C. botulinum type E in prereduced anaerobic trypticase soy broth plus 0.05% cysteine.

as soon as hydrogen gas was detected and continued decreasing until the hydrogen production decreased. By comparing Figure 27 with the growth in the prereduced medium under uninhibited growth conditions (Fig. 16), it can be seen that three events occur after approximately 10 hours incubation: the growth enters late log phase, hydrogen is produced at a slower rate, and the Eh7 values level out and begin to become more positive. Whether or not these represent cause-and-effect relationships cannot be determined. However, if hydrogen gas is allowed to be sparged into the uninoculated sterile medium, the Eh7 reduction is quite similar; and the lowest level of Eh7 is practically identical. The very significant drop in Eh7 as a result of hydrogen gas in the system is probably a consequence of establishing what is essentially a hydrogen electrode at the platinum surface of the Eh electrode. Hydrogen also may have an effect on increasing the sulfhydryl groups present in the medium during growth, i.e. the equilibrium of the reaction:

$$2RSH \rightleftharpoons RSSR + H_2 \quad \text{(Hewitt, 1950)}$$

would shift to the left with increased hydrogen pressure. The latter effect is probably insignificant compared to the overwhelming influence of hydrogen gas on the potential. This effect may be largely artificial occurring primarily at the electrode tip where the platinum would act to catalyze the ionization of the molecular hydrogen and loss of electrons.

The large and rapid reduction of Eh in growing cultures has been suggested by others to be due to hydrogen gas evolution. O'Brien and Morris (1971) proposed such a mechanism for C. acetobutylicum, and Douglas and Rigby (1974) suggested it for C. butylicum.

Since hydrogen gas production (reflected by a sudden Eh7 drop) appears to be one of the first events detectable after inoculation of the medium with spores, it was of interest to see if growth of the test organism in inhibitory media was enhanced by the presence of hydrogen. Other observers have found that incubation of anaerobes in an environment of hydrogen resulted in no increased numbers of bacteria except when the bacteria have been injured prior to incubation (Futter and Richardson, 1970). No studies relating to the effect of hydrogen on the growth rate of anaerobes in a medium containing growth inhibitors have been reported. Such growth comparisons were performed and the results are presented in Figures 28, 29, and 30.

Figure 28 compares the growth of the test organism in reduced TSB containing 30% sucrose with and without added hydrogen gas. The presence of hydrogen in the medium lowered the Eh7 significantly (here again this reduction may be artificial--see above discussion) and resulted in a slight decrease in the lag time and an increase in the extent of growth. When oxidized 30% sucrose TSB was utilized for growth, the decrease in lag time in the medium containing hydrogen was much more significant (Fig. 29). The difference in the initial Eh7 of the oxidized medium (Fig. 29) is greater than in the reduced medium (Fig. 28). Based on a theory of enhanced growth of the test organism at reduced Eh7 levels, it would be expected that the growth response differences are as accentuated as they appear. As stated above, the Eh7 level for the systems containing hydrogen gas may not be representative of the entire medium and may be the result of a localized platinum catalytic effect. Nevertheless,

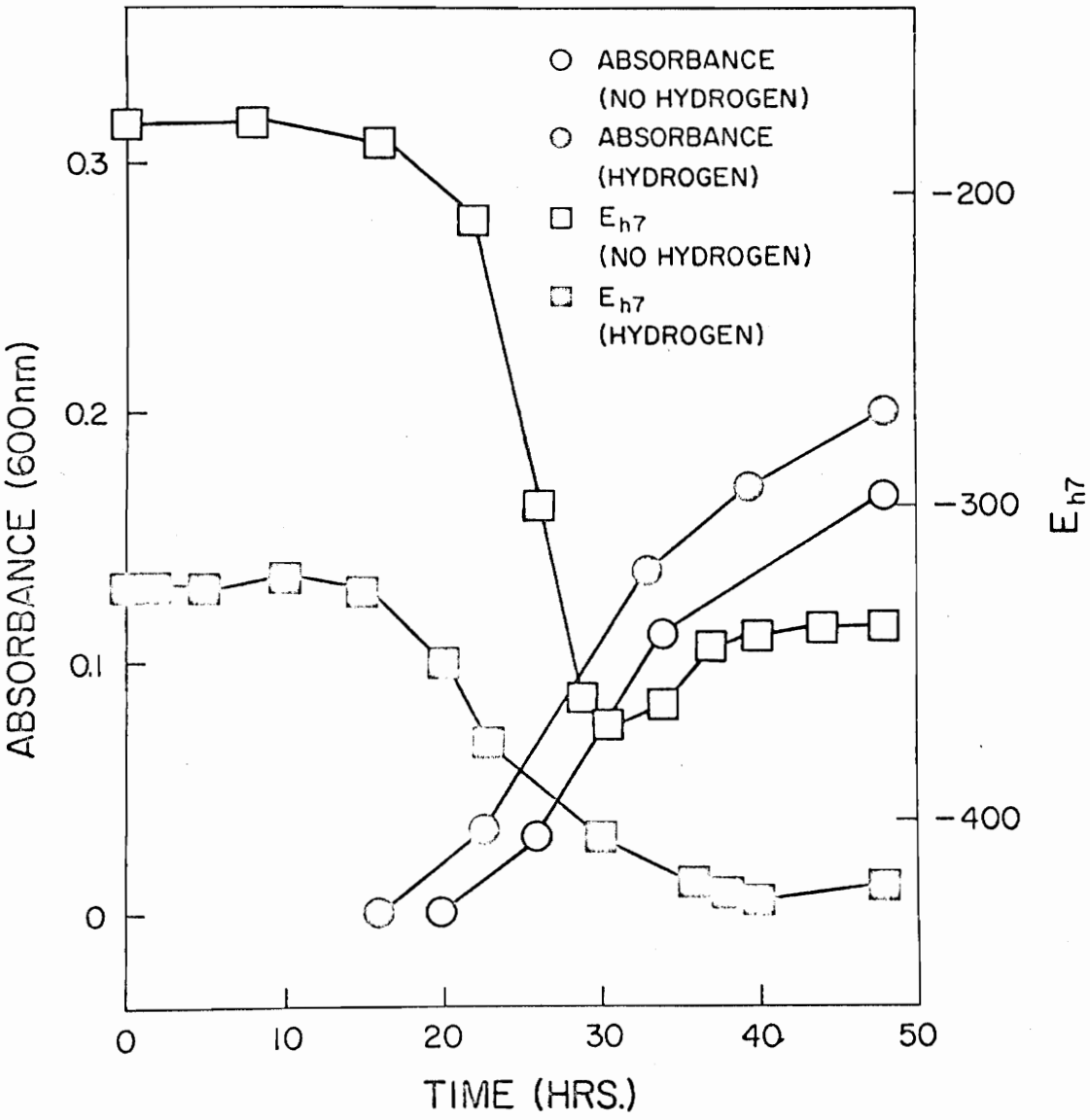

Fig. 28. Growth of C. botulinum type E and Eh7 changes in prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 30% sucrose with and without added hydrogen gas.

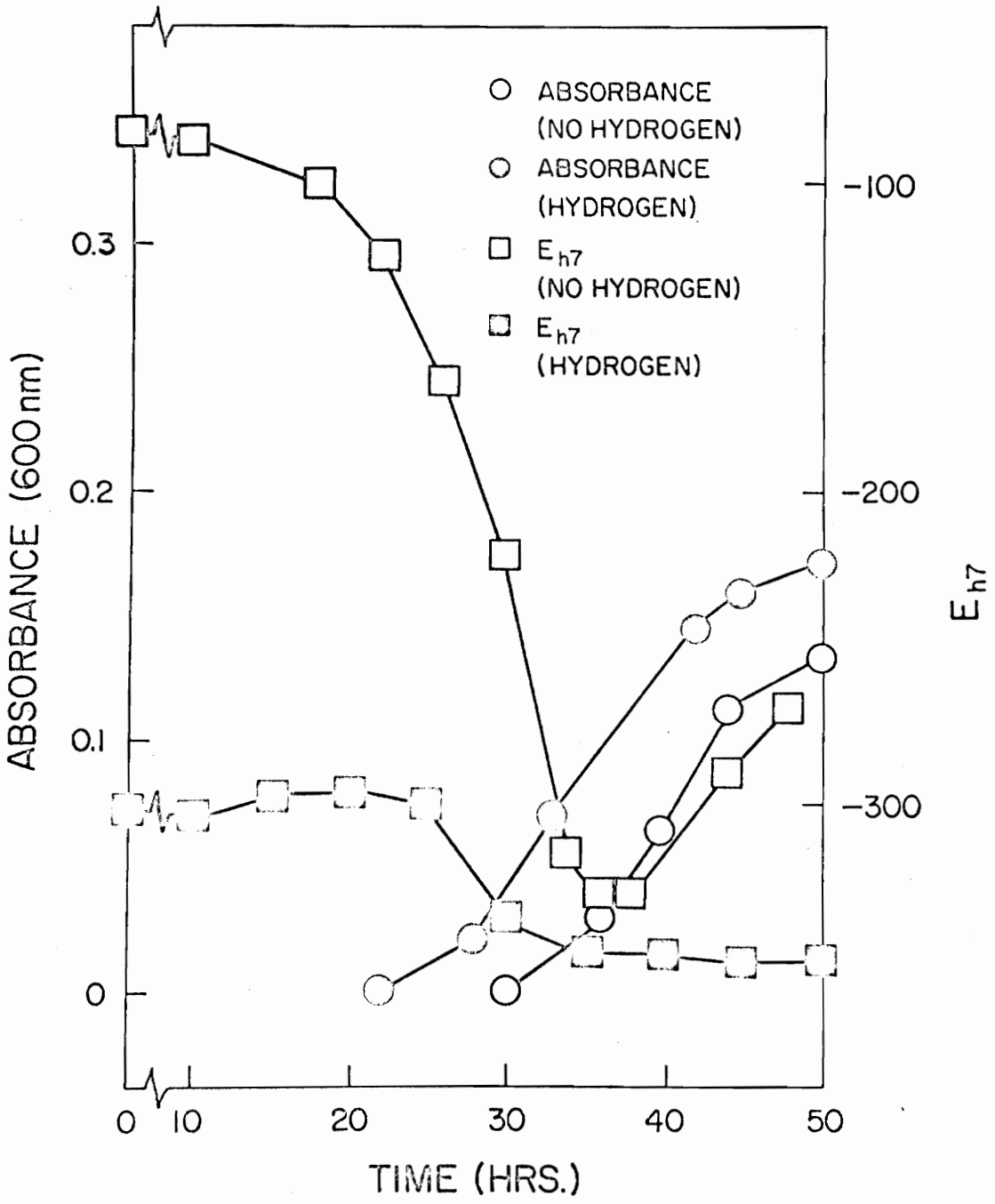

Fig. 29. Growth of C. botulinum type E and Eh7 changes in oxidized anaerobic trypticase soy broth plus 0.05% cysteine and 30% sucrose with and without added hydrogen gas.

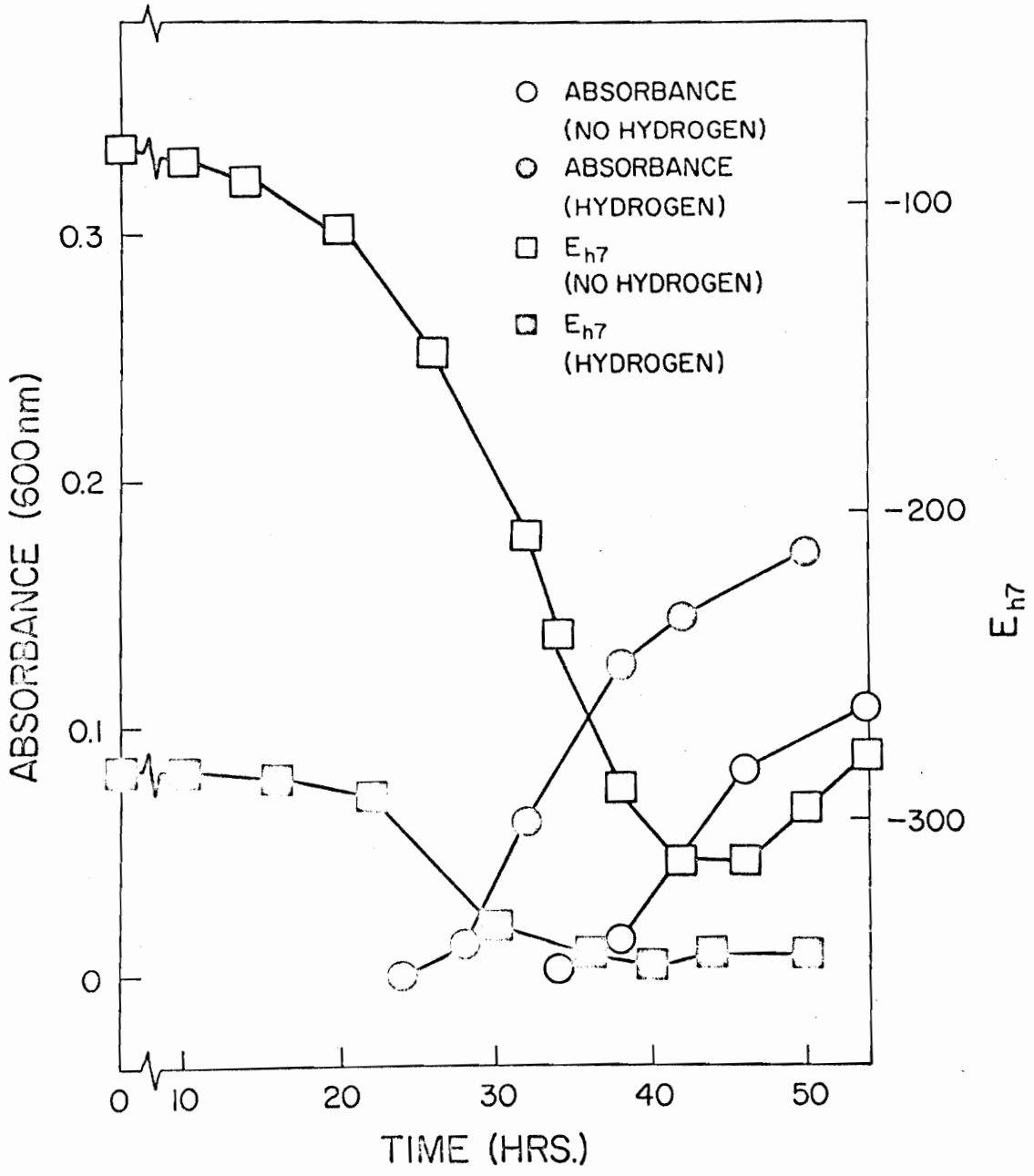

Fig. 30. Growth of C. botulinum type E and Eh7 changes in low oxygen (0.1%) prereduced trypticase soy broth plus 0.05% cysteine and 30% sucrose with and without added hydrogen gas.

the hydrogen may serve to provide more reductive radicals in the system, and thereby explain the enhanced growth in the media containing hydrogen.

In the medium in which the Eh7 was poised at a high value with dissolved oxygen (0.1% oxygen in the sparging gas), a similar decrease in lag time and increase in growth was obtained by introducing hydrogen into the medium (Fig. 30). Apparently the presence of hydrogen gas in this case serves the function of both lowering the Eh7 of the system and reducing the partial pressure of the oxygen in the medium, thereby making the environment more suitable for the anaerobic growth. It can be concluded that the hydrogen gas evolved during the growth of the test organism not only functions as a metabolic end product (convenient electron acceptor) but also as a mechanism to reduce the oxygen tension within the growth medium and, thereby, indirectly reduce the Eh of the system. There is also the possibility of a direct reducing effect on the medium by the hydrogen as discussed above.

An effort was made to determine at which stage of germination or vegetative growth the Eh7 drop (and presumably the hydrogen gas production) occurs. According to Vinter (1970) the first changes to occur after germination involve RNA synthesis. Therefore, if RNA synthesis is inhibited emergence and outgrowth will be prevented. In addition, if cell wall synthesis is inhibited outgrowth will not occur (Vinter, 1970).

Rifampin has been shown to irreversibly bind to DNA-dependent RNA polymerase. In this way it serves as a potent inhibitor of RNA synthesis. At the same time it does not appear to interact with DNA

(Frontale and Tecce, 1967; Sippel and Hartman, 1968; Umezawa et al., 1968). The inhibition by penicillin G is known to be the result of the blocking of the cross-linking of linear peptidopolysaccharide chains into the complex peptiodoglycan (Strominger et al., 1967).

Spores were inoculated into prereduced TSB containing 0.002 µg/ml rifampin (MIC for the test organism). As illustrated in Figure 31 the counts rapidly decreased. More importantly, the Eh7 values did not significantly change during this period. On the other hand, if the spores are allowed to emerge (form a single viable vegetative cell) and outgrowth is prevented by penicillin, the Eh7 is significantly reduced (Fig. 32). Presumably the emerged cells produced hydrogen gas to lower the Eh7. The rapid decrease in counts as a consequence of the bacteriocidal effects of rifampin can be compared to the slower lowering of counts as a result of the bacteriostatic effects of penicillin. A reduction in the Eh level of the culture during emergence of C. butyricum was reported by Douglas and Rigby (1974).

5. Effects of Level of Inocula

In order to determine the effect of the number of spores inoculated into the medium and the subsequent growth lags, two levels of inocula were utilized--10^4 spores/ml and 10^2 spores/ml. The high inoculum resulted in a shorter lag period in reduced TSB containing 30% sucrose compared to the low level of inoculum in the same medium (Fig. 33). The greater number of viable cells probably produced more hydrogen, lowered the Eh7 and increased in number (raised absorbance) more rapidly. Presumably in the low inoculum there existed fewer spores which could adapt

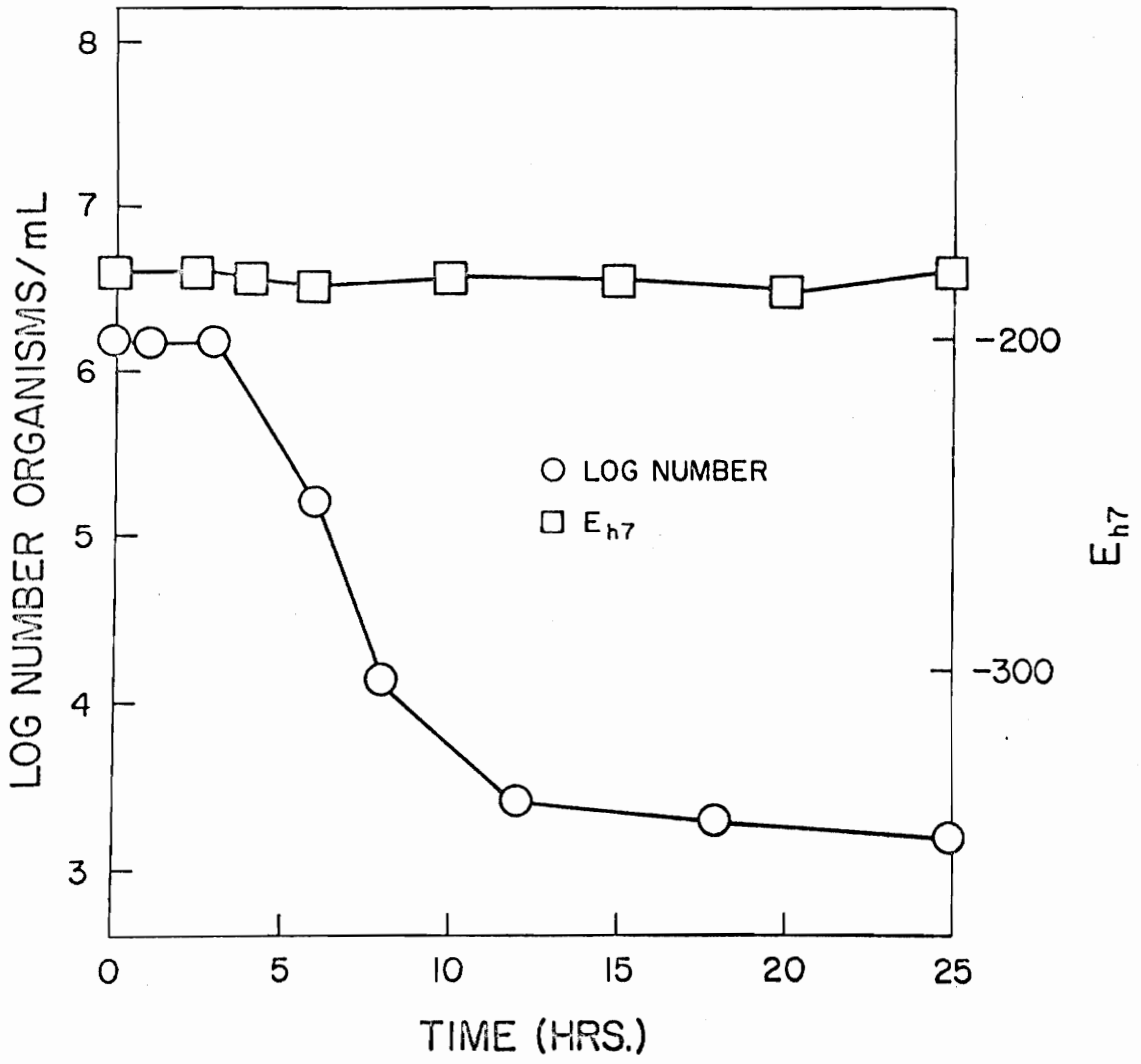

Fig. 31. Growth of C. botulinum type E and Eh7 changes in prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 0.002 µg/ml rifampin.

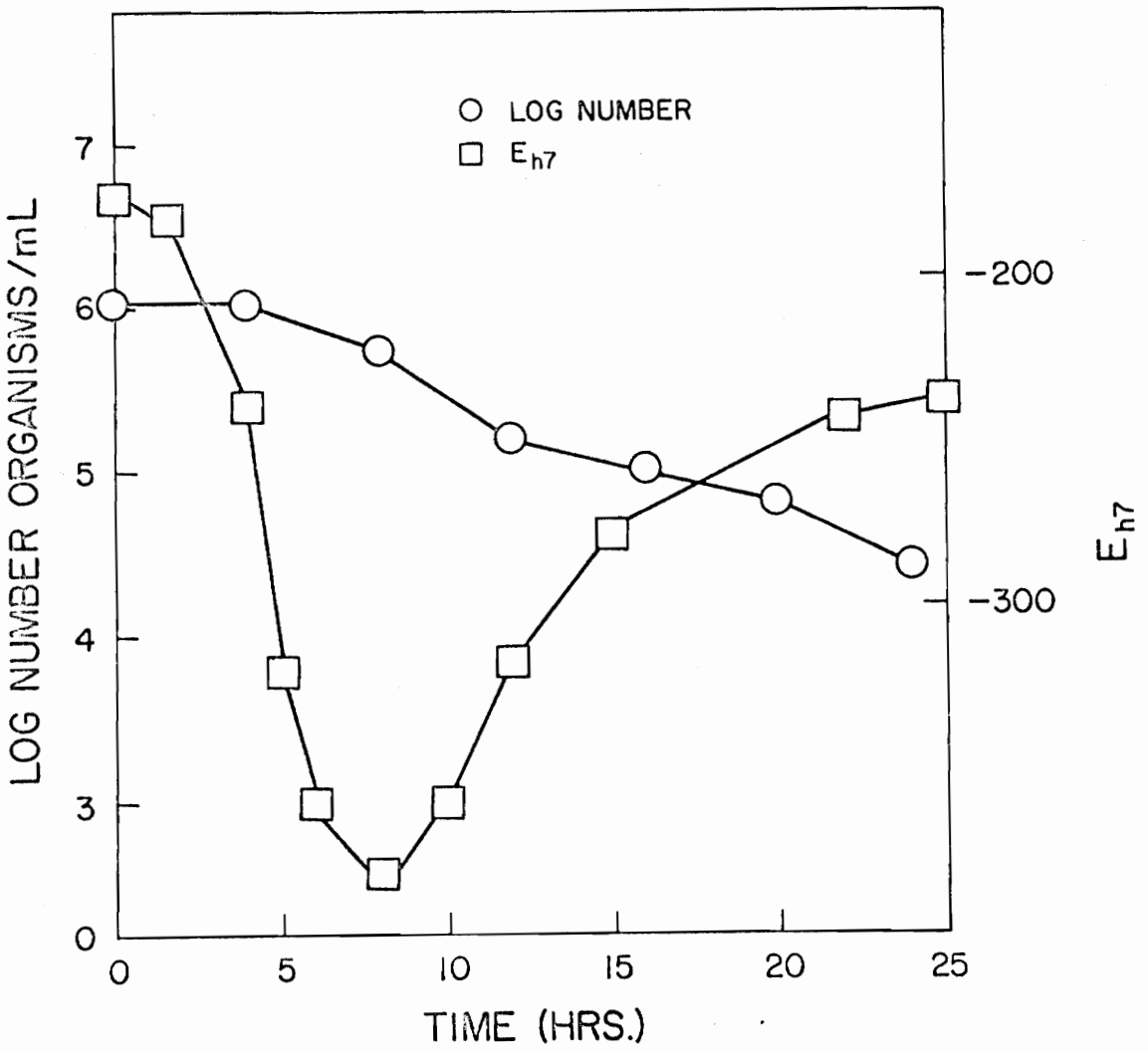

Fig. 32. Growth of C. botulinum type E and Eh7 changes in prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 20 µg/ml penicillin G (1585 units/g).

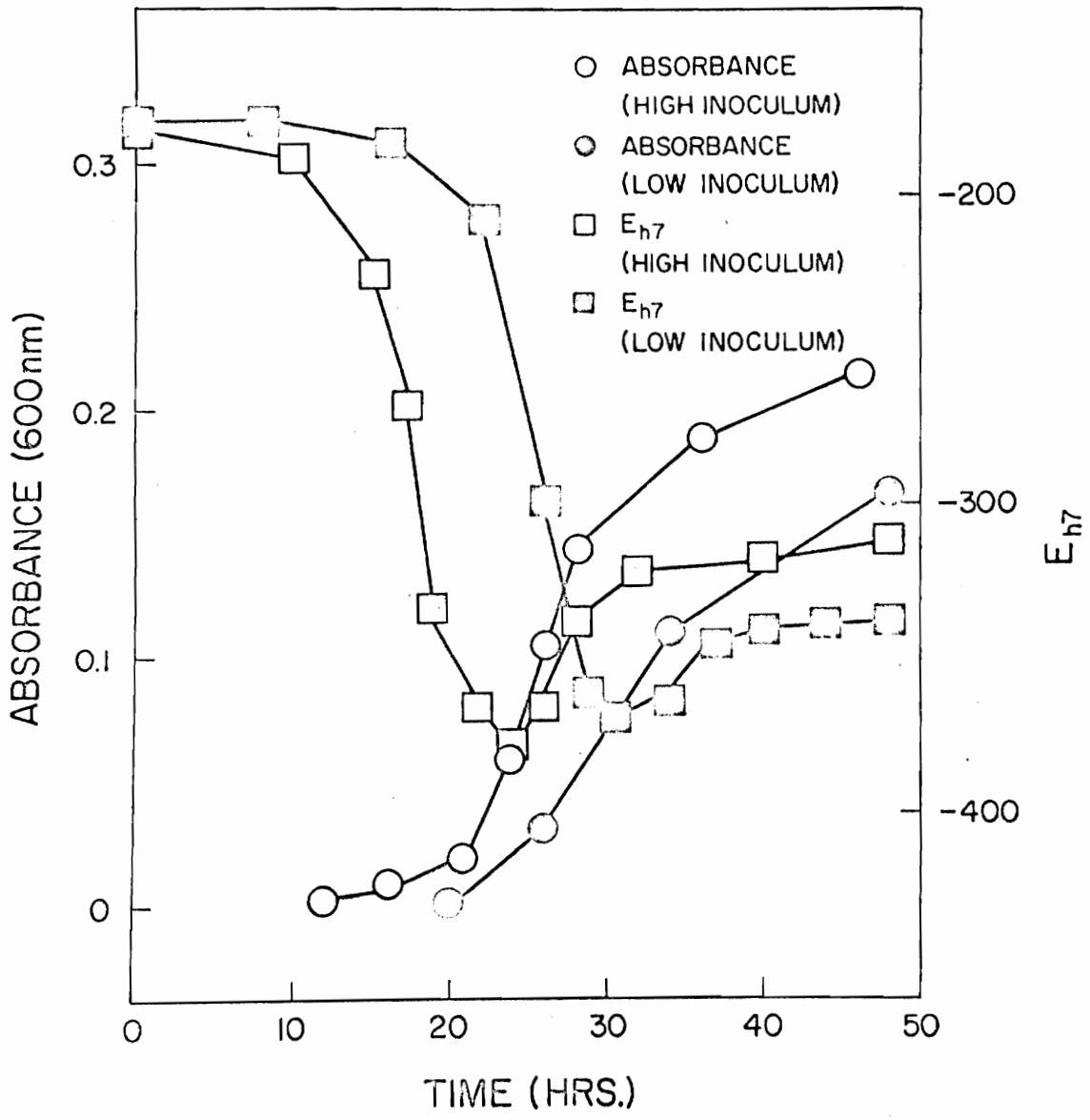

Fig. 33. Growth of C. botulinum type E and Eh7 changes in prereduced anaerobic trypticase soy broth plus 0.05% cysteine and 30% sucrose inoculated with a large population (approx. 10^4 spores/ml) and a small population (approx. 10^2 spores/ml).

and grow in the medium of high sucrose. Spencer (1966) also found that the growth limiting concentration of sodium chloride for C. botulinum was a factor of inoculum size--the larger inocula tolerating more salt. Such a condition has been shown to exist in vegetive cells, especially if the culture is not synchronized in growth (Smith, 1972). The limiting Eh for growth of cells depends on the metabolic state and the inoculum size. In a population of unsynchronized cells, each organism would possess a different metabolic state depending on its age. Smith (1972) has stated that the larger the inoculum the higher is the limiting Eh for growth of C. perfringens. "This results from the endogenous metabolism of the cells in the inoculum which increases the electron density in the neighborhood of the inoculum and thus provides a nidus of low potential and low pH from which active growth can progress."

Spore populations are known to possess varied heat resistances among individuals of the same population. It is proposed that they possess varied abilities to grow under a certain set of conditions (e.g. Eh, pH, salt concentration). Hambleton and Rigby (1970) have suggested such a distribution of sensitivity to molecular oxygen for both the germinative processes and biochemical processes leading to emergence of the spores.

The general effect of Eh7 on outgrowth of the test organism in inhibitory media can be interpreted as follows: There exists in each spore population a certain low percentage of spores capable of outgrowth under adverse inhibitory conditions. This percentage is lowered each time the medium is made less optimal for growth, e.g. increased

concentration of inhibitory chemical or increased Eh7 of the medium. Eventually a combination of less optimal conditions results in few if any organisms in the population capable of growing.

SUMMARY AND CONCLUSIONS

The growth and chemical inhibition of C. botulinum type E as a function of oxidation-reduction potential (Eh) and oxygen concentration were investigated. To study only the effect of Eh and not adventitiously introduced oxygen, strict anaerobic procedures and prereduced media were used. An air tight flask containing electrodes for measuring Eh and pH and attached connections for exchange and mixing of various gases was utilized.

Oxidation-reduction potential measurements were made of TSB containing several concentrations of the following reducing agents: sodium formaldehyde sulfoxylate (SFS), 2-mercaptoethanol (MER), cysteine hydrochloride (CYS), sodium thioglycolate (THIO), and ascorbic acid (ASC). These potentials were adjusted to pH 7.0 (designated Eh7) by the formula of Leistner and Mirna (1959). TSB containing no added reducing agents exhibited a potential measuring -141 mv. ASC at all levels and THIO at low concentrations produced an Eh7 higher than the TSB containing no added reducing agents. The addition of CYS, MER, and SFS to TSB resulted in a reduction of Eh7 compared to the system without added reducing agents. The reductions were more effective at the lower levels of CYS, MER, and SFS (up to 0.03%). At higher concentrations the drop in the Eh7 was less pronounced. The order of relative reducing intensity for the reducing agents used was as follows: SFS, MER, CYS, THIO, and ASC.

Growth of the test organism was determined in TSB containing various concentrations of the reducing agents at pH 7.0 ± 0.2 by measuring absorbance changes at 600 nm in anaerobic cultures tubes.

These growth responses were compared to the reducing agent concentrations and Eh7 measurements for each type of medium. A direct correlation was found to exist between growth of the test organism and -Eh7 X -log concentration of the reducing agent. It can be concluded that maximum growth occurs at a low oxidation-reduction potential and a low concentration of reducing agent. Since all of these experiments were performed in the absence of oxygen, the Eh of the system alone or the chemical components of the system which the Eh level reflects can be concluded to have a definite effect on the growth of the test organism.

Growth and Eh7 changes during growth were measured in the air tight electrode vessel. Under uninhibited growth conditions there were no significant growth differences between the oxidized (autoclaved under air) and reduced (autoclaved under nitrogen) media. However, inhibition by sodium chloride, hydrogen ions, and sucrose revealed an increased lag phase and a decreased maximum absorbance for growth in the oxidized medium compared to growth in the reduced medium. Media poised at a high Eh7 (-80 mv) by dissolved oxygen also exhibited no retarded growth when compared to the reduced media except during sucrose inhibition. Since the oxygen poised system and the oxidized system had similar Eh7 levels (-80 mv) and the lag times and maximum growths were similar, it can be concluded that molecular oxygen does not have an increased inhibitive effect compared to the effect of simply a raised Eh7 at least in the range studied. The addition of exogenous hydrogen gas to the media reduced or eliminated the growth differences described above for sucrose inhibition in the reduced, oxidized and oxygen poised media. The added

hydrogen gas may have produced a lower potential in the medium resulting in an enhanced growth response compared to the control system without added hydrogen. In the oxygen poised medium, added hydrogen undoubtedly reduced the partial pressure of oxygen in the system and allowed for much better growth.

It was shown that the Eh7 drop observed during growth of the test organism probably occurred during emergence or outgrowth of the spores and not during germination. In addition, this drop was coincident with the production of metabolic hydrogen gas by the organism. The evolution of metabolic hydrogen by most anaerobes may explain the observed low redox potentials established in growing cultures of such organisms. Hewitt (1950) has suggested that typical anaerobes can reduce the Eh to a level of -0.4 volts or even lower. Evidence presented in the present study indicates that the presence of hydrogen gas in the medium would reflect an extremely low potential which was obtained artificially as a result of the local ionization of the hydrogen at the platinum tip of the Eh measuring electrode. This effect may obscure any actual reduction in Eh. This contention is supported by the observation that some strains of the strict anaerobe Bacteriodes fragilis do not produce hydrogen and have been shown to have a higher culture Eh when compared to other anaerobic bacteria (Vennesland and Hanke, 1940).

An increased lag phase was observed when a small inoculum (10^2 spores/ml) was used compared to a large inoculum (10^4 spores/ml). This difference in lag was similar to that observed between the reduced and the oxidized or oxygen poised media. It is hypothesized that the variations in growth between the oxidized, oxygen poised and reduced media are due

to a distribution of sensitivity to adverse conditions (low pH, high sucrose, high salt, high Eh). Such a distribution exists within each spore inoculum population.

Data were presented to indicate the wide range of Eh7 values existent among various canned food products. Definite conclusions about the relative abilities of these foods to support the growth of C. botulinum cannot be made since the growth has been shown to be a function of not only the Eh7 but also the reducing agent concentration of the medium.

In order to make further conclusions about the relationship of Eh alone to the growth of C. botulinum in food systems, it would be necessary to measure growth responses of the organism in various foods. The final answer may not lie with the Eh intensity but with the ability of the system to poise the oxidation-reduction potential at a low or optimal level.

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VITA

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Smith, M. V., and Muldoon, P. J. 1974. Campylobacter fetus subspecies jejuni (Vibrio fetus) from commercially processed poultry. Appl. Microbiol. 27: 995-996.

THE EFFECT OF OXIDATION-REDUCTION POTENTIAL ON THE OUTGROWTH AND CHEMICAL INHIBITION OF *CLOSTRIDIUM BOTULINUM* TYPE E SPORES

by

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(ABSTRACT)

The effect of oxidation-reduction potential (Eh) and oxygen concentration on the outgrowth of *Clostridium botulinum* type E spores was studied. An airtight flask containing electrodes for continuous measurement of Eh and pH and attached tubing for the exchange and mixing of various gases (i.e. prepurified nitrogen, anaerobic carbon dioxide, hydrogen, oxygen) were utilized. Prereduced media and anaerobic procedures were employed to insure controlled conditions and reproducible results.

Eh measurements were made of Trypticase Soy Broth containing several concentrations of the following reducing agents which are listed in what was found to be the order of their reducing intensities: sodium formaldehyde sulfoxylate, 2-mercaptoethanol, cysteine hydrochloride, sodium thioglycolate, and ascorbic acid. Outgrowth of *C. botulinum* type E spores was shown to be directly related to a low Eh and a low concentration of added reducing agent.

Outgrowth and Eh changes were measured in the electrode vessel described above for several growth conditions. Eh levels in the media were poised by sparging prereduced media with nitrogen gas or 0.1%

oxygen in nitrogen gas or by sparging with nitrogen gas in oxidized media (media which was autoclaved under air). Under uninhibited growth conditions there were no significant growth differences between the oxidized (Eh = -80 mv), oxygen poised (Eh = -80 mv), and reduced (Eh = -190 mv) media. However, inhibition by sodium chloride (3.5% and 4.0%), hydrogen ions (pH 5.11 and 5.05), and sucrose (30% and 35%) revealed an increased lag phase and a decreased maximum absorbance for growth in the oxidized medium compared to growth in the reduced medium. Media poised by dissolved oxygen also exhibited retarded growth when compared to the reduced media. The addition of exogenous hydrogen gas to the media reduced or eliminated the growth and lag time differences described above for sucrose inhibition.

It was shown that the Eh drop observed during growth of the test organism probably occurred during emergence and outgrowth of the spores and not during germination. In addition, this drop was coincident with the production of metabolic hydrogen gas by the organism. Data indicated that the presence of hydrogen gas in the medium would reflect an artificially low potential as a result of the local ionization of the hydrogen at the platinum tip of the Eh measuring electrode. This effect may obscure any actual reducing in Eh.

An increased lag phase was observed when a small inoculum (10^2 spores/ml) was used compared to a large inoculum (10^4 spores/ml). This difference in lag was similar to that observed between the reduced and the oxidized or oxygen poised media. It is hypothesized that the variations in growth are due to a distribution of sensitivity within each

spore inoculum population to adverse conditions (e.g. low pH, high sucrose, high salt, high Eh).