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# Oxidation-reduction potential and growth of *Salmonella* spp. and *Pseudomonas fluorescens*

by

James Leslie Oblinger

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Food Technology

Approved:

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Iowa State University
Ames, Iowa

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# INTRODUCTION

Among environmental factors influencing bacterial growth, oxidation-reduction potential (Eh) has received relatively little attention over the last several years. This lack of attention may be due, in part, to difficulties in measurement. It is also probable that the confusion relating to meaningful interpretation of experimental data pertaining to redox phenomena in bacterial cultures is an important obstacle to increased study in this area. The redox potential of a given bacterial culture is, to say the least, a complex parameter not at present understood (Wimpenny and Necklen, 1971). This has been the case for at least the last sixty years.

With the observation by Potter in 1911, that inoculated culture medium had a lower electrode potential than sterile culture medium interest in oxidation-reduction potentials in bacterial cultures began. The association of an increasing negativity of measurable oxidation-reduction potentials with the amount of bacterial growth was established by Burrows (1941). Usually the lowest electrode potential is reached when the metabolic activities of the bacteria are most intense in the logarithmic phase of growth (Hewitt, 1950). By using oxidation-reduction potentials, we have a quantitative measure of the reducing intensities of bacterial systems.

Very limited investigations of the redox potentials developed by *Salmonella* spp. were reported by Burrows and Jordan (1935). These workers were among the first to report that potentials were "characteristic" of species of various genera of bacteria. Since that time, various other workers have suggested many possible applications of redox potentials of bacterial cultures. More recently, Zador (1961) proposed that such measurements be considered as an aid to present methodology used in the classification of bacteria.

The significance of Eh has acquired increased importance to the food microbiologist with the advent of the packaging of various food products, particularly meat products, in an ever-increasing array of materials. Depending on the particular gas permeability characteristics of a given packaging material, the Eh of the system may play a vital role in influencing the microbial flora that exists, or, in time, develops. There seem to be definite values of Eh that determine if a given organism can initiate growth and(or) maintain its survival. Values of this type have been reported for anaerobes, particularly the clostridia. There is no reason why similar values might not apply to the aerobes, and, in particular, organisms that have public health or spoilage significance.

This study was undertaken to investigate the relationships of oxidation-reduction potential, pH, and growth in pure

and mixed cultures of *Salmonella* spp. and *Pseudomonas fluorescens*. It was hoped that this work might contribute to understanding of growth relationships of these organisms, particularly with regard to vacuum or gas-packaging of various food products.

# LITERATURE REVIEW

## Definition and Background Material

Oxidation and reduction processes are defined in terms of electron migrations. Besides the addition of oxygen, or the withdrawal of hydrogen, oxidation is also the withdrawal of electrons with or without the withdrawal of protons. Reduction is the addition of electrons, with or without the addition of protons, or the withdrawal of oxygen, or the addition of hydrogen (Clark, 1960).

The readiness with which substances part with, or take up, electrons determines the intensity level of their oxidizing or reducing functions. In order to measure these functions quantitatively, it is necessary to measure the so-called "electronic escaping tendency" (Hewitt, 1950). Since oxidation-reduction reactions are electronic migrations involving exchanges of electric charges, it is clear that the quantitative study of oxidation-reduction processes can be accomplished by measuring the electric potential differences.

Hewitt (1950) stated that, theoretically, for every oxidation there is a reduction, although this is difficult to demonstrate in complex biological systems, e.g., bacterial cultures.

It is not possible to measure a single potential difference (E) at an electrode as this constitutes only a half-

cell; but if the circuit is completed by including a standard half-cell, the electromotive force of the entire cell is readily measurable. The second half-cell to which electrode potentials are referred is the normal hydrogen electrode, which is taken as the standard of reference. Electrode potentials referred to the normal hydrogen electrode are measured in volts or millivolts and designated Eh.

Eh is a measure of oxidation-reduction (O-R) intensity and not the oxidation-reduction capacity of a given redox system. The capacity term for Eh is poising action and is similar to the buffer effect in acid-base equilibria. A solution, or in this case, a bacteriological medium, is well poised when it resists changes in Eh under either oxidizing or reducing conditions.

This brings us to the general electrode equation:

$$Eh = E_0 + \frac{RT}{nF} \ln \frac{[\text{Oxidants}]}{[\text{Reductants}]}$$

where:

$E_0$ = a constant for the system, measured in volts

R = the gas constant of 8.315 joules/mole/°C

T = the temperature in degrees absolute

n = number of electrons involved in the process

F = Faraday's constant = 96,500 coulombs

The above equation is used primarily in chemical systems wherein salts or metals in ionized forms are in either oxidized or reduced states (Clark, 1960).

The pH affects oxidation-reduction systems by altering

ionic equilibria. This factor was taken into account by Leistner and Mirna (1959) when they studied the redox phenomenon involved in curing solutions. These workers developed an equation for the calculation of Eh of complex biological systems by including a pH correction term that allowed comparison of various data from numerous experiments, all standardized to a pH of 7.0. The Eh at a pH of 7.0 is symbolically referred to as $Eh_7$. The equation is as follows:

$$Eh_7 = E_m + E_{ref} + \frac{RT}{F} \ln(pH_x - 7.0)$$

where:

$E_m$ = the measured potential of the system

$E_{ref}$ = the potential of the saturated calomel reference electrode

R = the gas constant as defined previously

T = the temperature in degrees absolute

F = Faraday's constant of 96,500 coulombs

$pH_x$ = the measured pH of the system

As mentioned previously, hydrogen-ion concentration has definite effects on electrode potentials, so that, strictly speaking, potentials can be compared directly only with others at the same pH. The effect of pH on the electrode potential is not constant but varies both with different systems and at different ranges of pH in the same system (Hewitt, 1950). Clark and Cohen (1923) reported that for each increase in the hydrogen-ion activity by a power of 10

(1 pH unit), the Eh of the system becomes more negative by 60 millivolts (mv).

In an effort to avoid confusion regarding effects of pH on Eh, Clark and Cohen (1923) introduced the term rH, which includes Eh and pH. The rH of a given system is equal to the logarithm of the reciprocal of the partial pressure of hydrogen gas in equilibrium with the system. Hewitt (1950) showed the simple relationship between Eh, pH and rH in a reversible system at 30C as follows:

$$Eh = 0.03\ (rH - 2\ pH)$$

It was hoped that this equation would enable investigators to derive rH values when Eh and pH were known, or to obtain Eh when rH and pH were given. Unfortunately, as one surveys the literature wherein rH values are reported, the authors seldom mention either the Eh or the pH, or both. Consequently, Clark (1960) suggested that the term rH no longer be used.

The use of dyes or indicators for the measurement of redox changes has been well documented and is discussed at length in Clark's book dealing with oxidation-reduction potentials of organic systems (1960). Perhaps the most well-known use of indicator dyes in biological systems is the methylene blue (or resazurin) test for contaminated milk (American Public Health Association, 1967). According to

Hewitt, an ideal dye for the determination of redox potential should possess the following characteristics:

(1) become reduced at a suitable range of Eh.

(2) of distinctive color, preferably blue, to avoid confusion with the natural color of the system studied.

(3) not change color with pH changes

(4) of intense color so that very low concentrations may be used, thus not over-poising the system being studied.

(5) with no direct participation in the system studied, i.e., it should not catalyze biological oxidations or have toxic effects on cells or tissues, and should not combine with components of the system.

Due to the limitations imposed, and despite the continued work of Clark, no simple reliable indicator method has been worked out for use with biological systems. It is for these reasons that electrometric methods have gained such prominent usage in this area.

## Methods of Eh Measurement and (or) Control

The oxidation-reduction potential of a given system can be observed quantitatively by measurement of the potential at a so-called "unattackable" electrode where electrons tend neither to enter nor leave the system. Or, in other words, the unattackable electrode serves as an inert conductor of electrons. Electrodes used by workers in the past have included platinum (Hewitt, 1950; Tabatabai and Walker, 1970),

gold (Dixon and Quastel, 1923; Lepper and Martin, 1931; Longsworth and MacInnes, 1935), iridium (Lepper and Martin, 1930a), tungsten (Flexner and Barron, 1930), graphite (Flexner and Barron, 1930; Tuttle and Huddleson, 1934), mercury (Clark et al., 1925; Michaelis and Flexner, 1928), and palladium (Hakomori, 1931). Platinum and gold electrodes are most commonly used with present-day instrumentation. The Eh when referred to a standard of references such as the normal hydrogen electrode, or a saturated calomel electrode, may then be measured potentiometrically in volts.

Methods for studying oxidation-reduction potentials have been described thoroughly in the texts of both Hewitt (1950) and Clark (1960). Numerous investigators have reported methods that offer special advantages to the particular systems being studied. In the area of bacterial cultures, Knight (1930a, 1930b) described an apparatus for maintaining a low oxygen tension in the gas phase above the medium. This method was intended for Eh control of a medium before the germination of spores of *Clostridium tetani*. In 1943, Hanke and Katz used an electrolytic method while working with *C. sporogenes* for controlling the Eh to within 10-20 mv and the addition of alkali for pH control to within 0.2 pH unit. The electrolytic method was not used in later work by these investigators partly because of the large pH changes induced by electrolysis, and partly because it was

suspected that the products of electrolysis were toxic. Hanke and Bailey (1945), in a study of several species of *Clostridium*, passed a controlled stream of very dilute oxygen in nitrogen into the medium. These workers adjusted the concentration and flow of the gas mixture until there was an equilibrium established between the rate of reduction of the broth system and its rate of oxidation by the oxygen with the eventual attainment of a constant potential.

The apparatus described by Dobson and Bullen (1963) controlled the pH value of their medium containing bicarbonate by the continuous control of the carbon dioxide tension in the gas above the liquid. The Eh was controlled by the intermittent addition of oxygen to offset a drift towards reducing conditions which was inherent in the medium used. Other apparatus for control of redox potential has been described by Daniels et al. (1966) and Williams (1966).

Tabatabai and Walker (1970) have described an apparatus that yields highly reproducible observations on Eh, pH, and cell numbers in pure and mixed bacterial cultures. This apparatus was used in the investigations reported herein and will be described in detail under Materials and Methods. This particular system allows for control of initial Eh values through the use of various gas mixtures. Tabatabai and Walker (1970) made no attempt to maintain the Eh or the pH at a predetermined value throughout the experiment.

Rather, the effect of growth of pure and mixed cultures on these parameters was studied.

Other investigators have used various techniques for stabilization or reduction of the potential of different bacteriological media as shown in Table 1, taken from Borromeo (unpublished thesis, 1969).

Table 1. Methods of reducing and(or) stabilizing the Eh of medium

| Method | Reference |
|---|---|
| Addition of various materials: | |
| calcium sulfate | Arnaudi and Rapette (1938) |
| glucose | Raynaud and Viscontini (1945)<br>Reed and Orr (1943) |
| Cooked meat particles | Arnaudi and Rapette (1938)<br>Hewitt (1950)<br>Lepper and Martin (1929) |
| cysteine | Dubos (1929)<br>Reed and Orr (1943) |
| thioglycolate | Reed and Orr (1943) |
| ascorbic acid | Reed and Orr (1943) |
| sodium formaldehyde | Reed and Orr (1943) |
| sulfoxylate | Reed and Orr (1943) |
| small amounts of agar | Reed and Orr (1943) |
| Boiling of broth with immediate cooling | Dubos (1929) |
| Bubbling of $N_2$ with evacuation of medium | Raynaud and Viscontini (1945) |

However, this investigator feels that attempts at controlling such factors as Eh and pH throughout a given experiment are misguided. That is to say, such alterations of the "natural" conditions that exist in culture may further complicate the interpretation of data obtained from such experiments. The criteria for the use of indicators in redox systems as outlined by Hewitt (1950) and discussed previously may well have application here. There may be poising and(or) toxic effects associated with such control or monitoring of Eh. Dubos (1929) reported that oxidized indophenols and methylene blue were bacteriostatic for pneumococcus and haemolytic streptococci of human and bovine origin, while at the same time, the indigoes, malachite green, and litmus were not toxic. Dubos also stated that methylene blue and indophenols were not toxic when they occurred in the reduced form. Alier (1965) observed that sodium sulfite retarded the growth of _C. perfringens_ and prolonged the period of time necessary for detection of these organisms. The control of pH by various means may also result in data that are difficult to interpret. The metabolism of organisms involved in such experiments may be altered when such artificial agents are employed; the medium itself may be affected (Hewitt, 1932). The primary purposes of the work must be kept in mind, the present investigation has its objectives given in the Introduction.

## Bacteriological Implications and Applications of Eh

Potter (1911) first noted that the electrode potential of an inoculated medium was lower, that is, more reducing, than the sterile medium. It is common for bacterial cultures to develop reducing conditions. The redox potentials of bacterial cultures begin to fall soon after a fresh subculture is made if environmental conditions such as temperature, pH, and available nutrients are favorable. Burrows (1941) observed that in most instances the rapid negative drift from the potential of the uninoculated medium coincided approximately with the logarithmic phase of culture development, slowing down and coming to an end after 12 to 36 hr incubation, the time depending upon the size of the inoculum, etc.

Coulter and Isaacs (1929) studied the oxidation-reduction potentials of aerobic cultures of *Bacillus typhosus* (*Salmonella typhosa*). These workers stated that the reducing properties manifested by bacterial cultures must be regarded as only one phase of a series of processes that come within the scope of the concept of oxidation-reduction phenomena. The reduction potentials of *B. typhosus* in culture in bouillon which was given access to atmospheric oxygen showed a negative drift that attained the values found in sterile bouillon when deaerated with nitrogen: Eh -85 to -95 mv at

pH 7.6. Coulter and Isaacs (1929) concluded that the bacteria influenced the potentials in the first period of their growth by exhaustion of oxygen from the culture, thus permitting the "characteristic" potential of the medium to develop. The bacteria were not believed to contribute the substances responsible for the observed potentials at that point. When the decline in potential to values more negative than those of the culture medium occurred, it was theorized that at that time the rate of death of the bacteria approached and exceeded the rate of multiplication. It was further suggested that dissolution of bacteria liberated reductive substances which then influenced the observed negative potentials. Coulter and Isaacs (1929) also reported that the potential of cultures through which oxygen is passed continuously did not show a negative drift at any time. Reductive substances of bacterial origin apparently did not influence the electrode potentials in the presence of oxygen. These workers confirmed the importance of bacterial respiration as the means for the removal of oxygen and the consequent establishment of characteristic reduction potentials in cultures of _B. typhosus_.

On the other hand, Hewitt (1950) stated that if one assumes that the cell can affect the medium only by 1) removing free oxygen dissolved in the medium, and 2) liberating reducing substances from autolyzed cells, this would ig-

nore a number of facts gleaned from studies of bacterial metabolism. Hewitt maintained that bacteria effect reduction as a natural result of metabolic activities. To obtain the energy necessary for proliferation, food materials in a medium must be oxidized, and for this purpose free oxygen, combined oxygen, hydrogen acceptors (or electron acceptors) or any suitable type of oxidizing agent is utilized by the cell. The actual agents used by a particular cell for these oxidations will necessarily depend on the enzymes and catalysts available, and the oxidizing systems will be reduced sequentially as the cell brings its various enzymatic activities into use.

Hewitt (1930) was sharply critical of those investigators who, in his opinion, "confuse symptoms with functions" when considering the reducing phenomena of bacterial cultures. Hewitt viewed the utilization of oxygen in a culture as a _symptom_ of the dehydrogenation of nutrient materials by the organisms rather that the use of oxygen as a _function_ of the particular organisms. Definite redox potentials are established in cultures, but investigators are not fully aware of what substances are responsible for these potentials in many cases, nor what reversible oxidation-reduction processes are actually being measured.

Yudkin (1935) found that protecting the Eh electrode from coming into contact with bacterial cells made no significant

difference in the observed potentials. This work lends itself to the hypothesis of Hewitt just described that it is the multiple actions of the bacteria that affect the potential.

Lepper and Martin (1930b) have suggested that a rapid drop in Eh coincides with the formation of hydrogen gas, particularly in the case of *Clostridium perfringens*. They proposed that the low potentials recorded were the result of activation of the platinum electrode by the presence of molecular hydrogen and that a hydrogen electrode was formed. However, Cannan et al. (1926) have reported that hydrogen is produced only after low Eh values are attained.

The conditions governing the potentials established in bacterial cultures are complicated and among the factors that affect these potentials are the following: 1) the kind of organism -- class, strain, etc.; 2) the condition of the organism -- previous history; 3) growth phase of the organism; 4) nature of the cultivation medium -- types and quantities of constituents; and 5) conditions of cultivation.

As Hewitt (1950) so aptly stated: "Perhaps the greatest difficulty in dealing with biological systems generally, and bacterial cultures in particular, is that we have not rigidly defined static systems of fixed characteristics but constantly changing systems forever in a state of flux." In a bacterial culture, not only is the oxidation-reduction potential

changing, but also the pH, the chemical composition, the number and condition of the organisms, the amount of dissolved oxygen or other gases present, etc. By its very nature, a bacterial culture is a constantly changing system, and if any factor is held constant, e.g., the Eh or the pH as discussed previously, the culture is even less of a "natural" system with which the microbiologist has to deal.

Depending on their oxygen requirements, bacteria can be classed as aerobes or anaerobes, with various degrees between these classes. Hewitt (1950) has observed that the reducing activity of aerobes is greatest at Eh levels of +400 to +200 mv whereas a range of +50 to -400 mv is given for anaerobes. It is reasonable to say that the reducing activities or capacities of bacteria depend on the enzymes they are capable of producing. The aerobes possess enzymes capable of utilizing oxygen whereas the anaerobes do not, e.g., the anaerobes, such as the clostridia, lack catalase and cytochrome systems. According to Hewitt (1950) the lack of these systems prevents the anaerobes from obtaining energy by the oxidation of nutrients at the high oxidation-reduction potentials at which the systems function. Also, oxygen is not passed onto other enzyme systems containing iron porphyrins, so that there may be a scarcity of acceptable hydrogen acceptors for metabolic reactions. It is also plausible that anaerobes require the absence of air to avoid

the oxidation of sulfhydryl groups on certain enzymes.

## Electrode Potentials Observed in Bacterial Cultures

Rather limited investigations have been conducted in the area of oxidation-reduction potentials in bacterial cultures. Much of the work that has been done has concerned itself with the anaerobes, particularly the clostridia. For the purposes of this discussion, a survey of results that have been obtained with various species of bacteria will be reported. Finally, work pertaining to redox potentials in cultures of *Salmonella* spp. and *Pseudomonas* will be presented.

Gillespie (1920) was the first to suggest that certain groups of bacteria might be characterized by definite "reduction potentials". These observations were made while he was studying the reduction potentials of bacterial cultures and of water-logged soils. He observed the markedly different final potentials which developed in cultures of strict aerobes as contrasted with those of facultative anaerobes or of mixed cultures containing strict anaerobes. From his studies with *Bacillus subtilis*, *B. coli* (*Escherichia coli*), and *B. mycoides*, Gillespie hazarded the opinion that these differences (of up to 500 mv) might apply generally to the two distinct groups of bacteria, i.e., aerobes and anaerobes. Gillespie also emphasized the point that oxidation and

reduction potentials were the intensity factors of O-R measurement as opposed to capacity factors. For the facultative anaerobe, B. coli, and for mixed cultures of unidentified soil organisms grown in deep layer, the values for the reduction potentials were very near that of the normal hydrogen electrode, i.e., approximately -600 mv. With B. subtilis and B. mycoides, which the author considered representative of aerobes, reduction potentials showed an increasing negative drift in potential but at no time did the reduction potentials approach the normal hydrogen electrode potential.

The positive limit of oxidation-reduction potential required for the germination of spores of Bacillus tetanus (Clostridium tetani) in vitro was the subject of an extensive study by Fildes in 1929. This worker felt that the term "anaerobe" implied that a particular organism required a certain reducing intensity in its surroundings before growth could commence. The absence of molecular oxygen was not the essential factor except in so far as oxygen under ordinary circumstances of growth tended to prevent the necessary degree of reducing intensity from developing. Fildes further stated that should the reducing intensity of the surroundings be great, the desired conditions might be attained even in the presence of air, and growth of an anaerobe might then take place. He felt that the chemical significance of Eh was

that it is a direct measure of the energy of hydrogenation of the oxidant of the system involved. Fildes used thionin, indophenols, indigos, methylene blue, and safranin to detect reduction of the growth medium. He found that, upon exclusion of air, the rapidity and extent of development of reducing intensity depended upon the constitution of the medium and the pH. He concluded that pH did not affect the lag of *B. tetanus* at 38C, except through its effect on the reducing intensity of the medium. At pH 7.0, no germination of spores was observed at Eh levels more positive than +10 mv. It was also determined that the greater the reducing intensity of the medium, the shorter the lag prior to germination, until a zone was reached where there was no germination.

Dubos (1929) found that oxidation processes bore a definite relation to the size of inoculum required to initiate growth of *Pneumococcus*, *Streptococcus*, and *Staphylococcus* strains in plain broth. He attributed this relationship to the presence of reducing autoxidizable substances in the broth. In contact with air, it was observed that these substances gave rise either to the oxidized form of a reversible oxidation-reduction system, or, by irreversible oxidation, to highly oxidized substances (peroxides). The system in question was not identified by this investigator. The organisms studied by Dubos required a medium with a definite range of O-R potential (unspecified) before cell multi-

plication occurred.

Also in 1929, Lepper and Martin reported that the disappearance of oxygen from culture media containing cooked meat was principally due to its energetic absorption by the autoxidation of the unsaturated fatty acids (especially linolenic) of the lipid catalyzed by the haematin of the muscle. This phenomenon was attributed to the presence of the reversible system: para-haematin-haemochromogen. These workers also reported that the fragments of meat pack together in a tube in such a way as to minimize convection. Accordingly, the entrance of oxygen was limited to the relatively slow process of diffusion, and about 1-2 centimeters below the surface the rate of its fixation exceeded the rate at which it arrived. This would account for the usual low Eh of cooked meat media, generally near an initial Eh of -200 mv. The work just discussed was done using dyes to determine Eh levels. Lepper and Martin (1930a) electrometrically confirmed the results of this previous work using both gold and iridium electrodes for the measurement of Eh.

Later in the same year (1930b), these workers reported on the oxidation-reduction potential of cooked meat following the inoculation of bacteria. Both aerobes and anaerobes were used in these studies. The aerobes included: *Staphylococcus aureus*, *Streptococcus faecalis*, *B. pyocyaneus* (*Pseudomonas aeruginosa*), *B. coli*, *B. paratyphosa A* (*Salmonella*

paratyphi), B. paratyphosa B (S. schottmuelleri), and B. proteus vulgaris (Proteus vulgaris). The anaerobes included: B. welchii (Clostridium perfringens), B. enteritidis sporogenes (C. sporogenes), B. chauvoei (C. chauvoei), B. oedematieus (C. oedematiens), and B. tetani (C. tetani).

With the exception of the last two aerobes listed, the Eh of pure cultures fluctuated ± 10 mv for the first 10 hr after inoculation and during the next 48 hr, slowly became 50 mv more negative. Cultures of the last two aerobes showed more negative drift, with an observed Eh of -400 mv after the first 24 hr after inoculation. During the next 24 hr, the Eh rose to a level of -350 mv. Both B. paratyphosa and B. proteus vulgaris produced sulfides in the medium and the significance of this will be touched upon shortly.

As for the anaerobes examined, the Eh of these cultures fell very rapidly and after 3 hr, the Eh had reached or exceeded that of the hydrogen electrode. This effect was attributed to the production of hydrogen gas by these organisms. These workers concluded that bacteria produced only slight effects on the Eh of meat tubes unless the microbes produced either nascent hydrogen or sulfides from the meat. Both gold and iridium Eh electrodes were used in this study and sets of these electrodes agreed within a "few" millivolts where there was no hydrogen gas production. In cases where hydrogen was produced, iridium was the most

sensitive; Eh readings with the gold electrodes varied little due to the insensitivity of gold to the hydrogen.

The oxidation-reduction potential of sterile meat broth was also studied by Knight (1930a). In these experiments, the broth had been deaerated and buffered over a pH range of 4.0 to 10.5. Knight measured the OR potential of this system not only with a redox dye (indigo) but also with electrometric methods and found that the two methods did not indicate the same potential level; the dye indicated a more negative potential than was shown by an electrode in the broth. Knight (1930b) investigated the artificial poising of bacteriological media and found that the negative potential drift of the medium was balanced by passing a stream of dilute oxygen in nitrogen through the system. The concentration and rate of flow of the gas mixture was adjusted until equilibrium between the rate of reduction of the broth system and its rate of oxidation by the oxygen was reached. After this equilibrium had been reached, the potential remained constant. Unfortunately, the author made no mention of the flow rates of the particular gases that were employed.

Knight and Fildes (1930) examined the positive limit of O-R potential that was required for the germination of spores of _B. tetani in vitro_. These workers found that germination was completely inhibited at potentials more positive than +110 mv at a pH of 7.0 to 7.65. This value is

100 mv greater than that reported by Fildes (1929). This may have been due to the use of different media in the two studies. Fildes (1929) used a meat infusion broth plus 1.0% glucose. Knight and Fildes (1930) used a peptone-beef infusion medium highly buffered with $KH_2PO_4$. The acid limit for germination was reported to be a pH value of 6.0, while the alkaline limit of germination was a pH value of 8.5. The medium used in these determinations was a peptone-beef infusion medium that had been buffered with $KH_2PO_4$.

The influence of bacteria on the oxidation-reduction potential of milk was reported by Frazier and Whittier (1931a,b). The first paper was concerned with the influences of pure cultures of milk organisms on this parameter. Organisms used in these studies included: *Streptococcus lactis*, *S. citrovorus*, *S. paracitrovorus*, *S. mastitidis*, *S. thermophilus*, *S. bovis*, *S. liquefaciens*, *S. faecalis*, *E. coli*, *Aerobacter aerogenes*, *C. welchii*, *Lactobacillus bulgaricus*, *L. helveticum*, *Bacillus albolactis*, and *B. subtilis*. Under similar conditions each of the organisms studied produced changes in O-R potential characteristic of that organism. With many of the organisms studied, a rapid drop in Eh was coincidental with the beginning of the rapid rise in numbers of bacteria. With some of the bacteria, e.g., *S. faecalis*, *S. mastitidis*, and *S. thermophilus*, it was

necessary to have large numbers of actively growing cells in order to bring the observed potentials to their most negative value. Experiments with *S. lactis* at two temperatures (25 and 37C) showed that, with vigorously growing cultures, the same number of organisms were present at the end of the rapid drop in Eh regardless of temperature. A slower growing strain, which also produced less total acid, had more organisms present at a corresponding place on the Eh curve than observed with the rapidly growing cultures.

*E. coli* brought about an O-R system which was easily thrown out of balance by the introduction of air. *A. aerogenes* cultures showed no sensitivity to air nor did *S. lactis*. Other streptococci showed sensitivity to air similar to that displayed by *E. coli*. Cultures of *C. welchii* developed reducing conditions after a lag phase of 9 hr at 37C with an inoculum of $10^5$ cells/ml.

The Eh of the sterile milk medium was +180 mv as measured and calculated to be +150 mv at a pH of 6.6. Frazier and Whittier (1931a) also reported that in milk between a pH of 4 and 7, Eh versus pH was a straight line function and that the Eh increased by 0.06 volt for each decrease of one pH unit at a temperature range of 25 to 40C. These results were in agreement with the previously cited work of Clark and Cohen (1923).

The second paper (1931b) by these workers was concerned

with the influence of associated cultures of milk organisms on the O-R potential of milk. It was found that E. coli, E. communior, and A. aerogenes, when grown with S. lactis, all exerted a restraining influence on the rapid Eh drop usually observed in pure cultures of S. lactis. The larger the proportion of the coliform types, the greater was the restraining action.

When C. welchii was grown with S. lactis in milk, the lactic organism controlled Eh changes during the first part of growth, but C. welchii brought about a final Eh of -320 mv (initial Eh was +180 mv).

B. subtilis and B. albolactis did not materially affect the Eh changes when grown with S. lactis in milk. The observations with B. subtilis are in good agreement with the observations of Gillespie (1920), as reported earlier.

Allyn and Baldwin (1932) examined oxidation-reduction potentials developed by cultures of rhizobia in a yeast-water mannitol medium. They studied four species of Rhizobium: R. japonicum, R. meliloti, R. trifoli, and R. leguminosarum. Studies were carried out at 28C and an initial pH of 7.0. Initial Eh of the medium was +400 mv. R. japonicum followed a potential-time course in a region very positive to that of the other three cultures of rhizobia. Final Eh for R. japonicum was +100 mv after 18 days of incubation. The other three species of rhizobia manifested an Eh of between -150 to -200 mv after the same period of

time. From their work, Allyn and Baldwin concluded that the oxidation-reduction potentials which limit bacterial growth seemed to be somewhat dependent on the other factors characteristic of the medium. A more suitable ion-balance or a more available nitrogen source, for example, appeared to facilitate wider adjustments of unfavorable potentials by the bacteria themselves.

Oxidation-reduction potentials and ferricyanide reducing activities in peptone (1.0%) cultures and suspensions of *E. coli* were the subject of a lengthy discussion by Clifton *et al.* (1934). These workers reported that the O-R potential developed in a 1.0% peptone solution under aerobic conditions was poorly poised in the neighborhood of +250 mv (pH 7.0) and on deaeration with a stream of nitrogen gas slowly fell to an equilibrium value near +125 mv. During the period of active growth of *E. coli* under aerobic conditions a characteristic drop in potential of 400 to 500 mv was observed. The maximum reducing potential developed in or near the maximum stationary growth phase of the culture was considerably more negative than that developed in either a deaerated peptone solution or in the filtrate of a portion of the culture. Vigorous aeration of the culture resulted in the temporary establishment of a more positive potential. Stirring with nitrogen had little effect on the observed Eh. A positive drift in potential was observed, concurrent with

a decrease in the viable count as the age of the culture increased.

Clifton *et al.* (1934) proposed a mechanism for the development of the potentials observed in peptone cultures of *E. coli* as follows: As growth began an aerobic type of metabolism occurred for a varying time during which the bacteria utilized oxygen and other oxidizing agents in the medium. As the concentration of these reagents was decreased and the total metabolic activities of the cells increased, the potential fell and an anaerobic type of metabolism predominated in the culture. The potential reached a maximum reducing value when the total metabolic requirements of the cells were at a peak value and all available oxidation-reduction systems were employed to meet these demands. In continuous flow cultures this potential was maintained as long as oxidizing agents were supplied and in stationary cultures a positive shift occurred as the metabolic requirements decreased. This theory bears remarkable resemblance to that outlined by Boyd and Reed (1931) and Hewitt (1932).

The first report of the use of redox potential for the purpose of differentiating closely related species was published in 1934 by Sayen *et al.* These investigators correlated O-R potentials of two species of plant pathogens (*Phytomonas tumefaciens* and *P. rhizogenes*) and found that these two *Phytomonas* species showed a tendency to establish

potentials at different levels of intensity.

Longsworth and MacInnes (1936a) observed a correlation between the apparent oxidation-reduction potential and the rate of acid production by Lactobacillus acidophilus at a constant pH. A maximum in the rate of acid production corresponded to a minimum of the observed potential. The Eh of the medium was reported to have been very much affected by slight amounts of oxygen present in the saturating gas. In a second paper by these workers (1936b) apparent O-R potential, acid production, and population studies with L. acidophilus were examined under anaerobic conditions. When 0.2% oxygen was introduced into the gas mixture used for bubbling, it was found that the actively metabolizing culture was able to remove the oxygen as fast as it entered the solution. Consequently, the minimum Eh of -180 mv was not very different from that attained in anaerobic growth. Longsworth and MacInnes concluded that trends of Eh curves were definitely related to the rates of acid production in the cultures of lactobacilli used.

Gillespie and Rettger reported on the differentiation of species of spore-forming anaerobes (1938a) and lactobacilli of diverse origin (1938b) using a potentiometric technique.

Evidence presented showed that cultures of Clostridium botulinum and C. tetani exhibited marked and constant dif-

ferences in O-R potential, with respect to both maximum reduction intensity and trend of potential drift. The authors also noted that not only species differences were apparent, but that strains within a species exhibited a certain degree of individuality. _C. tetani_ produced and maintained a more intense final reduction potential than did _C. botulinum_, although cultures of the latter grew more profusely in the peptone water used as the medium of cultivation. Experiments were of four days duration at 37C. At the end of the experimental period, _C. tetani_ had developed a final Eh of -350 mv whereas _C. botulinum_ had developed a final Eh of -300 mv. These results are in direct opposition to those reported earlier by Plotz and Gelso (1930) who indicated that all of the clostridia species studied by them reached and maintained very nearly the same degree of reduction intensity.

Gillespie and Rettger also correlated changes in pH and titratable acidity with reducing intensities developed by oral and intestinal lactobacilli. In a tomato juice medium with an initial Eh of -35 mv and an initial pH of 7.03, the typical oral _Lactobacillus_ (_L. bulgaricus_) developed a low Eh of -221 mv after 6 hr at 37C and maintained this level for the duration of the five day experiment. _L. acidophilus_ was characterized as the typical intestinal species of _Lactobacillus_ and slowly developed a minimal Eh of

-110 mv. All strains of the oral type were much more active with respect to both rapidity and intensity of growth than the intestinal types.

Gillespie and Porter (1938) presented data that suggested that cultures of Aerobacillus (Bacillus) species could be differentiated using potentiometric methods. At the time, A. polymyxa and A. macerans were differentiated on 1) temperature limits, 2) ability to ferment rhaminose and sorbitol, 3) production of acetyl methyl carbinol, and 4) agglutinative antigen components. The striking physiological differences suggested to Gillespie and Porter that O-R potential differences might also be quite different. They found that the potentiometric grouping coincided with that previously established on cultural, fermentative, and serological grounds. The experimental evidence suggested that differences in carbohydrate metabolism were responsible for differences in reduction potentials established by various strains of these organisms. From an initial Eh of 0 mv, eight strains of A. polymyxa produced a final Eh of -200 mv and six strains of A. macerans produced a final Eh of -300 mv.

Cultivation of anaerobes and the importance of oxidation-reduction potentials was discussed by Reed and Orr (1943). In these investigations, the influence of various ingredients of the medium on the O-R potential that was measured with a

polished platinum electrode was correlated with a minimum inoculum in an effort to induce the growth of clostridia. These workers were instrumental in the development of the use of various reducing agents to poise the medium at favorable O-R potential levels. Agents used included ascorbic acid, sodium thioglycollate, sodium formaldehyde, sulfoxalate or use of these and agar. Special reducing agents were not necessary for anaerobic growth in media where reducing sugars were present. These workers reported that a large inoculum might cause a localized negative drift in potential. The results in this paper agree with the previous work that has been cited in this discussion that a favorable O-R potential is essential for the proliferation of an organism. It was shown that the optimum Eh for fifteen species of the genus Clostridium was in the vicinity of -200 mv. Clostridium species tested included: C. tetani, C. welchii, C. sporogenes, C. septicum, C. histolyticum, C. novyi, C. fallax, C. aerofoetidum, C. capitovalis, C. carnis, C. difficile, C. fallax, C. paraputrificum, C. sordellii, C. tetanomorphum, C. tetrium.

Hanke and Bailey (1945) used two different media for the cultivation of C. welchii in an effort to determine the growth-limiting oxidation-reduction and pH requirements of this and other clostridia. Cultures were grown in both peptone medium and in gelatin-yeast medium. Results obtained

with both media were the same. Under strict anaerobic conditions, growth occurred after 5 hr. From this work it was concluded that the growth-limiting Eh and pH requirements of the three different species (*C. welchii*, *C. sporogenes*, and *C. histolyticum*) were markedly different. At a pH of 6.4, *C. histolyticum* needed more reduced conditions than the other two species. Limiting potentials above which growth was not possible were as follows: *C. histolyticum*, +85 to +90 mv; *C. sporogenes*, +145 to +150 mv; and *C. tetani*, +160 mv. These workers also reported on the effect of pH and its relation to the limiting Eh observed. For the species tested the limiting pH range was 6.4-6.6.

The effects of oxidation-reduction potential and pH on the immunizing activity of vaccines of a weakly proteinase-producing strain of *Streptococcus pyogenes* were studied by Stamp (1953). This investigator found that immunizing activity was decreased when cultural conditions in the digest broth showed a low O-R potential and an acid pH. These conditions were found to favor the production and activation of the proteinase. No such relationship was demonstrated with batches of media not favoring the production of active proteinase. Stamp found that the most potent vaccines were obtained from cultures grown under raised oxygen tensions, i.e., aerated cultures.

Direct antagonism in mixed bacterial cultures and possible

relation to Eh was studied by Charlton (1955). In these experiments, the antagonism of *B. subtilis* and *Aerobacter aerogenes* was evaluated. *A. aerogenes* rapidly lowered both the Eh and the pH of the glucose medium (initial Eh not specified, pH = 7.0), and at 24 hr a pH of 5.5 and an Eh of -200 mv were attained. After 24 hr incubation the pH began to rise slowly, but the Eh remained low. Growth of *B. subtilis* took the Eh to a level of approximately -300 mv after 7 hr. In simultaneously inoculated mixed cultures, the early growth of *A. aerogenes* and *B. subtilis* was very similar to that in the pure cultures of each: neither organism appeared capable of preventing the growth of the other. Charlton reported that in the later stages of growth the results were not reproducible, e.g., on two occasions, when *B. subtilis* lowered the Eh in the early stages of growth, *A. aerogenes* declined to extinction in 4-5 days; on the third occasion, it survived. With *B. subtilis*, the numbers of viable cells declined to extinction in 48 hr on one occasion; on another, they declined in a fashion similar to the pure culture; and on a third occasion, the number of viable organisms increased gradually.

Charlton repeated his experiments in nutrient broth and found that both organisms behaved as in pure culture, neither affecting the growth or survival of the other.

Barnes and Ingram (1956) studied the effect of redox

potential on the growth of Clostridium welchii strains isolated from horse muscle. Limiting potentials for these organisms were investigated. At the onset of rigor, the potential of the horse muscle was -50 mv. These workers reported rapid multiplication of these organisms with the onset of rigor. Actual Eh limits of growth were studied by adjusting oxygen levels. No harmful effect was noted until the Eh reached +231 mv at pH 6.0-6.1. At this point, the organisms being studied died. Yet, at an Eh of +216 mv, considerable growth took place. Oxygen content at this level of potential was determined to be 1.05%. The possible relationship between initial Eh and duration of lag phase was also examined. It was found that at an initial Eh of -36 mv, the lag phase was 30-45 min; at +39 mv, 2 hr; at +115 mv, 2 hr; and at +216 mv, a 6 hr lag was observed. Reed and Orr (1943) were able to get growth of C. welchii at +220 to +250 mv at pH 7.6 when a very heavy inoculum was used but not when small inocula were used.

More recently, Smirnov (1960) studied the redox potential in cultures of enteropathogenic and non-pathogenic strains of Escherichia coli using the redox indicator dye ammonium molybdate. A higher O-R potential could be observed in cultures of enteropathogenic strains during the first two days of development as compared to the non-pathogenic types. These changes in the redox potential of the

cultures were illustrated by characteristic changes in the color of the medium. The author felt that through the use of electrometric methods of measuring redox potential, differential diagnostic media with redox indicators for different species of pathogenic bacteria could be developed. This author would have to disagree with this idea since it seems that such work would involve a duplicity of efforts. Why use the potentiometric technique in conjunction with dyes when the electrometric method by itself is sufficient?

Zador (1961) felt that redox potential measurements involving bacterial cultures should be used as an aid in classification. He arrived at this conclusion while studying the effect of temperature on the redox potential developed in bacterial cultures. His results showed that microorganisms do not conform to the laws of thermodynamics as physical systems do. In Zador's work, it was evident that the organisms under study (*E. coli*) had an optimal temperature range in which the fall in oxidation-reduction potential was maximal within a time unit. This observation would apply to other bacteria as well.

Another paper that dealt with the differentiation of bacteria on the basis of oxidation-reduction potentials was published by Winberg and Novak in 1961. These workers studied members of the Family *Enterobacteriaceae*, namely, *E. coli*, *P. vulgaris*, *Salmonella typhosa*, and *Shigella dysenteriae*.

It was concluded from their research that O-R potentials of bacteria in liquid media were related to the rate of substrate utilization and were not solely accounted for by pH changes. Their results indicated that organisms may be differentiated on an electrometric basis using substrates that emphasize differences in metabolic capabilities. Winberg and Novak suggested that such a scheme might find application in the clinical laboratory where rapid identification of organisms is desired. The medium that was used in these studies consisted of the following: nutrient broth base, urea, lactose, maltose, methylene blue, and phosphate buffer. Using this medium, these workers were able to separate the test bacteria within 5 hr using potentiometric techniques. As a last note, it was mentioned that it was necessary to equilibrate the medium 18 hr prior to inoculation to get reproducible results. This last feature may severely inhibit this technique in gaining wide usage.

In 1964, Horn and Jacob described continuous recording of physiochemical test values in microorganism cultures. Among the parameters that were discussed were turbidity, dissolved oxygen content, oxidation-reduction potential, pH, bacterial growth, bacterial metabolism, and antibiotic production. Oxidation-reduction potentials, pH, and $pCO_2$ in bacterial cultures could be correlated with definite stages of growth of bacteria and thus allowed a certain degree

of characterization of their metabolism. These workers were able to regulate the O-R potential in bacterial cultures by adding oxygen. Organisms used in these studies were *P. vulgaris* and *S. aureus*.

The growth and development of bacteria in algal cultures as affected by oxidation-reduction potentials was reported by Maksimova and Fedenko (1965). It was reported that a decrease in numbers of bacteria in cultures of *Chlorella vulgaris* was due to the high oxidation-reduction potentials which were established in rapidly growing algal cultures. Artificial reduction of the O-R potential of the medium completely eliminated the toxic effect of the algae upon the bacteria.

As reported by Kovacs and Kokai (1965), instantaneous inhibition of the drop in the rH in a growing bacterial culture is regarded as a suitable indicator of sensitivity to an antibiotic. For example, if streptomycin was added to an agitated culture of streptomycin-resistant organisms, the rH continued to fall, while in a sensitive culture the rH did not change.

Kellen *et al.* (1967) discussed the tetracycline inhibition of changes in the redox potential during the logarithmic phase of growth of sensitive microorganisms. Once again, the drop in rH values observed during the growth of microorganisms was correlated with the accumulation of reduced

substances or substrates in the medium. Cultures of resistant and non-resistant *S. aureus* and *E. coli* were used. As the authors pointed out, in a growing bacterial culture, the rH is negatively correlated with the growth curve, the maximum drop being reached during the log phase of growth. Antibiotic inhibition of this decrease in the rH in a growing culture is then direct evidence of immediate bacteriostasis.

Kellen *et al*. eliminated the influence of partial oxygen pressure in the medium by saturation through continuous aeration. In this way it was possible to measure only rH changes caused by metabolic redox processes in the culture. As a result of their experimentation, these workers observed that the addition of oxytetracycline (OTC) to aerated cultures of *E. coli* and *S. aureus* during the logarithmic phase of growth immediately arrested the steady decrease in redox potential in OTC-sensitive variants while no changes in rH values occurred in the resistant variants.

Borromeo (unpublished thesis, 1969) reported on the oxidation-reduction potentials in cultures of *S. faecalis*, *C. perfringens*, and *Lactobacillus plantarum* when grown in APT broth. Low negative Eh (-68 mv) apparently affected the lag phase of *L. plantarum*. The more negative the initial Eh at inoculation was, the longer the lag phase. This was not true for *S. faecalis* or *C. perfringens*. In mixed cultures of *S.*

*faecalis* and *C. perfringens*, both organisms exerted slight inhibitory action on each other as exhibited by decreased viable cell counts. The Eh and pH trends of the anaerobe were evident. In mixed cultures of *S. faecalis* and *L. plantarum*, the streptococcus exerted its influence on the Eh and pH of the system. In this case, *L. plantarum* was markedly inhibited evidenced by considerable lag in growth and decreased counts.

In work done by Tabatabai and Walker (1970) *C. perfringens* grew more "luxuriantly" at an initial Eh of +200 mv, in the presence of small quantities of oxygen, than at a lower Eh of +40 mv in the absence of oxygen. The medium of cultivation was trypticase soy broth. *P. fluorescens* mixed with *C. perfringens* at an initial ratio of $10^6$ to 10 did not have a marked effect on the growth of *C. perfringens* at initial Eh values of either +200 mv or +40 mv. Eh values for *C. perfringens* in pure culture became more positive at a more rapid rate than those for the mixed culture in the presence of oxygen. In the absence of oxygen, the Eh curves for pure and mixed cultures were nearly identical.

Up to this point in the discussion, work that has been done in the area of oxidation-reduction potentials in bacterial cultures has not to any great extent dealt with measurement of O-R in cultures of *Salmonella* or *Pseudomonas*. The remainder of this discussion will then be primarily

concerned with this particular area. However, several of the findings are similar to those already described for other organisms.

Coulter and Isaacs (1929) studied the relation of the potentials of sterile bouillon to those of living cultures of B. typhosa (S. typhosa) in the same medium. Initial Eh of their medium was +250 mv; initial pH was 7.6. No aeration was provided other than exposure to atmospheric air. In the first portion of the time-potential curve, the small numbers of bacteria introduced as inoculum were without effect on the potentials observed. A second period of growth was characterized by a rapid decline in potential to about -90 mv and at this time, the bacteria had begun to multiply and in their rapid respiration took up the dissolved oxygen in the medium. These workers observed that if the surface of the medium was left undisturbed, the actual concentration of oxygen in the system was reduced to a very low (unspecified) value. These observations led Coulter and Isaacs to report that the removal of oxygen by the respiration of bacteria was thus sufficient to explain the attainment of a level of reduction of about -90 mv in a growing culture. They further stated that removal of oxygen and not the elaboration of reductive products by bacteria was responsible for reduction potentials of this intensity in bouillon.

The investigators also reported that the potential of

cultures through which oxygen was passed continuously did not show a negative drift at any time. The flow rate of oxygen was not specified. Reductive substances of bacterial origin in the case of at least the typhoid bacillus did not influence the electrode potentials in the presence of oxygen and confirmed the importance of bacterial respiration as the means for the removal of oxygen and the consequent establishment of characteristic reduction potentials in cultures.

A paper by Burrows and Jordan (1935) dealt with the determination of oxidation-reduction potentials produced in the cultures of a number of *Salmonella* strains. Nutrient broth was the medium of cultivation with a pH of 7.0 to 7.2. Initial Eh was +350 mv. In a few experiments carried out by these investigators, oxygen-free nitrogen was bubbled through the cultures and the establishment of anaerobic conditions resulted only in a somewhat more rapid negative drift in potential during the first few hr of incubation; thereafter the potential did not differ materially from that produced in cultures in which no precautions were taken to exclude atmospheric oxygen. Preliminary experiments with various species of the *Salmonella* group (*S. cholerae-suis, S. paratyphi, S. typhosa*, *S. schottmuelleri*, and *S. enteritidis*) showed a rapid negative shift to more negative potentials during the first 12 hr of incubation at 37C. There was some fluctuation in Eh up to 30 hr after inoculation but thereafter, until the

termination of the experiments at 72 hr there was little change beyond a slight negative or positive drift. The potentials from 30 to 72 hr were regarded as "characteristic."

These preliminary experiments indicated that several recognized types of *Salmonella* differed widely in their characteristic potential level, varying from +50 mv for *S. cholerae-suis* to -200 mv for *S. enteritidis*. As a result of these observations, these workers tested ten strains of each species. These strains were selected from widely different sources and varied in the length of time they had been cultivated on laboratory media, e.g., from six months to thirty years. The results confirmed those of the preliminary experiments in that strains of the same species showed characteristic Eh levels very nearly the same, but also quite different from a similar group of ten strains of a different species.

Burrows and Jordan also presented curves obtained in some of the experiments during the first twelve hr of incubation. The significance of the initial "bounce" or immediate return to somewhat more positive values after the rapid negative drift of potential had come to an end was declared to be problematical. This phenomenon was apparent in almost all the experiments, particularly in the case of those bacteria with lower potential levels. The authors believed that it might have been found in every case if readings were made at

sufficiently short intervals.

Contrary to the results reported by Coulter and Isaacs in 1929, Burrows and Jordan found that the potential levels developed in bacterial cultures differed considerably from the levels attained by a sterile, deaerated medium. These authors stated that classification by means of serological reactions was dependent, ultimately, upon the metabolic activities of the organism involved. The synthesis of somatic proteins and carbohydrates of a serologically specific nature was, no doubt, dependent upon characteristic, synthetic, anabolic processes.

In this respect, Wurmser (1932) suggested that biological syntheses are reactions involving the transfer of electrons and should yield oxidation-reduction potentials. If one accepts this premise, it follows that different species of bacteria should bring about O-R potentials different from one another because of the difference in bacterial substance synthesized-- but consistent within species.

In a second paper, Burrows and Jordan (1936) reported on the oxidation-reduction potentials characteristic of three additional *Salmonella* spp.: *S. paratyphi*, *S. cholerae-suis* var. *kunzendorf*, and *S. sendai*. The tendency for strains of a single type to attain a characteristic potential was evident again, as in experiments previously reported. The characteristic potentials of *S. cholerae-suis* var. *kunzendorf* and *S.*

*paratyphi* C were very close to one another at a level of -75 mv. This observation is in keeping with the close biochemical similarities of these two types. These workers suggested that no single metabolic or antigenic factor determined the potential level.

Later, Burrows (1941) reported on the relation between characteristic potential and antigenic structure in 31 different *Salmonella* cultures. Data were not definitive, i.e., in a number of instances the sharing of antigens was associated with similar characteristic potentials; but in others, this did not occur.

Subsequently, in 1943, Burrows described the relation of observed potentials to pH in *Salmonella* cultures. With *S. cholerae-suis*, *S. typhosa* and *S. enteritidis* in nutrient broth, the relation of species reduction potentials to pH was of the magnitude of +60 mv/unit pH change. Burrows indicated that though the practice of recording both the observed potential and the pH was a sound practice, it did not allow for the comparison of reducing intensities with one another when the pH varied. The practical point at issue was the relation of such potentials to acidity. Leistner and Mirna (1959) have eliminated this problem by deriving a formula that enables one to compare data that have been obtained from several different experiments by the use of a pH correction term. In this way, experimental data

may be compared at a pH of 7.0; this formula is especially useful in biological data, particularly with oxidation-reduction potentials observed in bacterial cultures. This formula will be discussed under the Materials and Methods section of this discussion.

Fedorov and Sergeeva (1957) examined the effect of oxidation-reduction conditions of various media on the intensity of nitrate reduction by denitrifying bacteria. Nitrate reduction by cultures of *P. fluorescens* and *P. pyocyanea* under various conditions of oxygen supply and different substrates was investigated. Under aerobic conditions, both species utilized oxygen and nitrates simultaneously. The better the aeration, the less was the role of nitrates as hydrogen acceptors and vice versa. The Eh of denitrifying cultures in the presence of nitrates did not fall below +70 to +80 mv.

Gouda (1965) studied the oxido-reducibility of *Pseudomonas fluorescens in vivo* and *in vitro* and found that the physiologic pigment complex occurred in two states, one oxidized with green fluorescence and greenish-yellow color, the other reduced with blue fluorescence and less pronounced color. The relative amounts of these two constituents of this redox system varied according to the speed of the bacterial metabolism which reduced the system, and the oxygen tension which oxidized the system. It was found that during the

logarithmic growth phase, a significant amount of the pigment existed in the reduced state; while the maximum production of pigment (most of it in the oxidized form) occurred towards the end of the log growth phase. The pigment stayed in the reduced form for a considerable time if it was protected from light; otherwise, it was photo-oxidized rapidly.

Perhaps the most recent publication dealing with oxidation-reduction potentials of bacteria, and in particular *Clostridium perfringens* and *P. fluorescens*, was that of Tabatabai and Walker (1970). This work involved the development of a new apparatus for measuring changes in Eh, pH, and cell numbers. Studies were undertaken of both pure and mixed cultures of these organisms in trypticase soy broth (TSB) at 30C. It was observed that *P. fluorescens* could grow at a relatively low Eh of +40 mv (pH 7.0) in pure culture but not in the presence of *C. perfringens* under the same conditions.

The Eh curves for *P. fluorescens* leveled off at approximately +10 mv (pH 7.0) in the presence of oxygen and at -40 mv (pH 7.0) in the absence of oxygen. Initial Eh was +200 mv in the case of the presence of 0.4-0.6% oxygen and +40 mv in the absence of oxygen. The overall trend in the development of less positive values of Eh showed a gradual negative drift throughout the experimental period. There were

no sharp inflections in the Eh curve in contrast to that which was observed with the C. perfringens strains (2) that were examined.

From all that has been reported, it is apparent that the redox potential of a bacterial culture is a complex parameter not clearly understood at the present time, despite the years of work that have been devoted to its study. As has already been discussed, it measures a variety of soluble oxidized and reduced "couples" capable of reacting at the surface of a suitable inert electrode. These couples may be substrates or metabolic products of microbial growth. They may be other medium constituents that do not directly affect microbial growth or they may be minor cellular components that have leaked into the medium. The redox potential is thus a numerical estimate of the oxidizing or reducing nature of the environment.

Wimpenny and Necklen (1971) investigated the redox environment and microbial physiology associated with cultures of E. coli and Klebsiella aerogenes. These workers found the potentials they measured seemed to be cell-oriented parameters and felt that there might be an important connection between the culture Eh and the physiological make-up of the cell. According to these authors, two possibilities exist: that the external Eh is seen by the appropriate cell and appropriate regulatory mechanisms come into play or,

much more likely, that the external Eh is a reflection outside the cell of events occurring in it. These workers reported that it would be satisfying if one of these explanations were completely true, but the whole situation is made more complex by the possibility of redox couples which interact with the electrode but not with the cell. Preliminary studies (not yet published) by these men using other electron acceptors showed that nitrate and possibly nitrite as well as oxygen do not significantly alter the Eh of a culture other than through the metabolism of the cell.

As one familiarizes himself with this area, it becomes increasingly clear that Eh may be one of the most fundamental, though perhaps the most complex, indicators of the physiological state of microbial cultures. But then, that is essentially what Hewitt has maintained since 1930.

# MATERIALS AND METHODS

The apparatus used for the measurement of Eh, pH, and bacterial numbers was essentially that described by Tabatabai and Walker (1970) with slight modifications.

## The Apparatus

The apparatus for measuring Eh, pH, and cell numbers was constructed from a 500 ml, three-neck, round bottom distilling flask, which was further modified by the University glass shop (Figure 1). A screw-capped culture tube with the bottom removed and a Pyrex tube were added onto the flask to provide openings for inoculation (and sampling) and the salt bridge. The salt bridge was constructed from soft glass tubing (1.5 cm inside diameter), heated and drawn out to capillary size at one end. Both the gas inlet and outlet tubes were constructed from Pyrex glass tubing (0.6 cm outside diameter), the latter with two glass bulbs to serve as traps and fitted with a length (approx. 60 cm) of black latex rubber tubing which terminated in a flask of 0.1% Roccal (Hilton-Davis Chem. Co. Div., Cincinnati, Ohio) to receive any overflow of foam from the culture vessel. The gas inlet tube of the apparatus was connected to a gas manifold via: 1) latex rubber tubing (25 cm in length); 2) capillary glass tubing (5 cm in length, 0.1 cm bore); and 3) latex rubber tubing (5 cm in length). The upper end of

Figure 1. Schematic drawing of electrode vessel for measuring Eh and pH

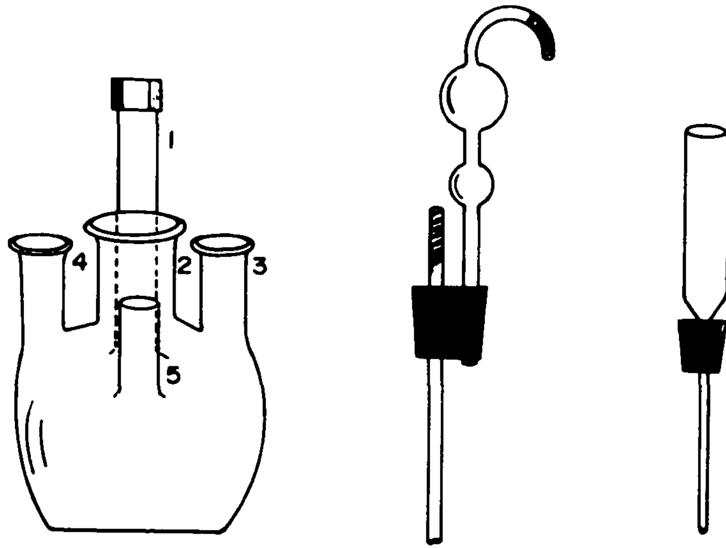
A = electrode vessel

1 = screw-cap tube for introduction and removal of samples
2 = port for gas inlet and outlet assembly (B)
3 = port for glass electrode
4 = port for platinum electrode
5 = port for salt bridge (C)

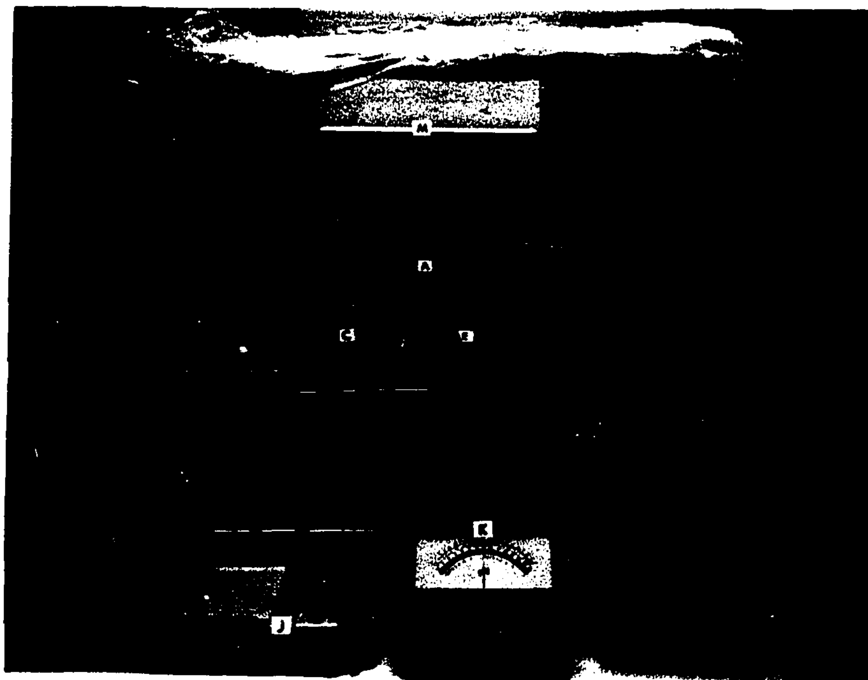
B = gas inlet and outlet assembly
C = salt bridge

Figure 2. Overall view of the laboratory apparatus used for the measurement of Eh and pH

A = gas manifold
B = electrode vessel
C = electrode vessel
D = electrode vessel
E = electrode vessel
F = electrode vessel
G = electrode vessel
H = flow meters
I = controlled temperature bath
J = recorder
K = pH meter
L = pH meter
M = ultraviolet lamp




A. ELECTRODE VESSEL B. GAS INLET AND OUTLET TUBE ASSEMBLY C. SALT BRIDGE



the inlet tube was plugged with cotton to prevent contamination. Fine adjustments of the bubbling rate were made by using a screw clamp on the rubber tubing between the gas manifold and the capillary tubing.

A flow meter (size 11, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.) was used for monitoring the flow of either compressed air or prepurified nitrogen through the electrode vessels. A tube packed with cotton was placed between the gas manifold and the flow meter to serve as a filter.

The Eh was measured with a platinum inlay electrode (Corning 476060 or Beckman 39273) and a saturated calomel reference electrode (Corning 476002 or Sargent-Welch 30080). The pH was measured with a general-purpose glass electrode (Corning 476022 or Sargent-Welch 30080) and a saturated calomel reference electrode. For measurement of either Eh or pH, a Beckman Expandomatic pH meter was connected to a Bausch and Lomb recorder (model VOM-7, B&L, Rochester, N.Y.). The entire laboratory apparatus is shown in Figure 2.

## Standardization of pH and Eh Electrodes

The pH changes in cultures were measured in millivolts (mv) rather than pH units to avoid changing the zero setting on the pH meter. Calibration curves of pH versus millivolt readings were prepared for each set of pH electrodes at each

experimental temperature by using standard buffers of the following pH: 4.01, 6.85, 6.99, 7.40, and 9.14 (Beckman Co., Fullerton, Calif.). To standardize the platinum electrodes, phthalate buffer, pH 4.00, saturated with quinhydrone was used at each of the experimental temperatures; e.g., at 30°C, this system has an Eh of +456 mv.

## Sterilization of the Apparatus

Except where noted, 350 ml of typticase soy broth (Baltimore Biological Laboratory, Baltimore, Md.) was sterilized in the electrode vessel at 121C for 15 min. The gas inlet and outlet assembly and the salt bridge were sterilized at 121C for 25 min.

KCl agar was prepared by dissolving 3g of agar and 35g of KCl in 100 ml of distilled water. This mixture was then autoclaved at 121C for 15 min. The salt bridge was prepared by pouring the hot solution of saturated KCl-agar into the sterile salt bridge, which was held in a near-horizontal position to allow the solution to solidify while slowly flowing out the capillary end.

Platinum and glass electrodes were sterilized by immersion in a solution containing 0.05% sodium hypochlorite for 15 min, and then rinsed four times in sterile distilled water and final drying with a square of sterile cheesecloth prior to insertion in the electrode vessel.

To prevent contamination during assembly of the various components, all sterile materials were placed in a bacteriological hood equipped with a germicidal lamp. The ultraviolet lamp was left on 2 hr prior to assembly of the vessels.

Equilibration of the Medium

After assembly and prior to inoculation, the vessels were placed in the controlled temperature bath (Blue M Electric Co., Blue Island, Ill.) and connected to the gas manifold. Flow rates of the compressed air used for aeration were adjusted to a rate of 25 ml/min/vessel. The compressed air was bubbled through the medium until a stable Eh was observed. This equilibration period was usually 24-48 hr. When prepurified nitrogen was used for aeration, a flow rate of 75 ml/min/vessel was usually used and the vessels required 48-72 hr before a stable Eh was attained.

Calculation of $Eh_7$

The Eh of a system at pH 7.0 is referred to symbolically as $Eh_7$. The formula adapted by Leistner and Mirna (1959) was used to calculate the $Eh_7$: Eh (pH 7.0) = $E + E_{ref} + 2.303 \frac{RT}{F}$ (pH x -7.0), where E = the measured potential; $E_{ref}$ = the potential of the saturated calomel reference electrode at the particular experimental temperature; $2.303 \frac{RT}{F}$ = a constant at a given temperature (e.g., at 30C =

60.1); and (pH x -7.0) = the pH correction term. This formula permits comparison of data of different experiments.

## Organisms

Six species of *Salmonella* and three strains of *Pseudomonas fluorescens* were used. *Salmonella* spp. included: *S. typhimurium*, *S. heidelberg*, *S. infantis*, *S. tennessee*, *S. enteritidis*, and *S. thompson*. *P. fluorescens* strains were designated F21, F17, and 2. All organisms used in these studies were from the departmental stock collection (Department of Food Technology, Iowa State University, Ames) and previously isolated from poultry products.

In all instances, cultures were incubated at the temperature used during the experiments (24 hr at 30 and 37C; 96-120 hr at 15C). Trypticase soy broth was the medium employed for such cultivation unless otherwise noted.

## Inoculation and Enumeration

For pure culture studies at 15, 30, and 37C , three or more vessels were inoculated with the particular *Salmonella* or *Pseudomonas* strain to yield approximately $10^2$-$10^3$ cells/ml. For mixed culture studies at 15 and 30C, four vessels were inoculated with mixtures of selected *Salmonella* and *Pseudomonas* strains to yield different ratios of *Salmonella* to *Pseudomonas* initially.

During the pure culture investigations, trypticase soy

agar (BBL) was used for enumeration of both Salmonella spp. and Pseudomonas. For mixed culture enumeration, both organisms were plated on spread plates on the agar described by King et al. (1954). This medium was evaluated along with trypticase soy agar and brilliant green agar (Difco); little variation was observed in counts with these media. Plates were incubated at either 15, 30, or 37C depending on the temperature used for cultivation.

# RESULTS AND DISCUSSION

## Selection of an Appropriate Medium for Cultivation

As reported by Tabatabai and Walker (1970), uniform Eh values between experimental vessels were obtained after rinsing of the acid-cleaned vessels with 3N HCl and then a deionized water rinse. In the graphs that follow each point represents an average of values obtained from at least three vessels.

Preliminary investigations were conducted using three different cultivation media to evaluate the poising effect of these media. Poising effects have been described earlier in this discussion as the capacity of systems (here, the sterile medium) to obstruct oxidation or reduction processes. This is because the systems themselves have to be oxidized or reduced before the level of electrode potential can be altered appreciably. Nutrient broth (NB, Difco), brain heart infusion broth (BHI, Difco), and trypticase soy broth (TSB, Baltimore Biological Laboratory) were examined. Several of the test organisms were used in conjunction with these media. Results obtained with *Salmonella heidelberg*, were considered representative of the trends that were developed by other *Salmonella* spp. investigated. Figure 3 shows results obtained with NB, and Figure 4, with TSB.

For NB, the initial Eh was +400 mv, and the value quickly

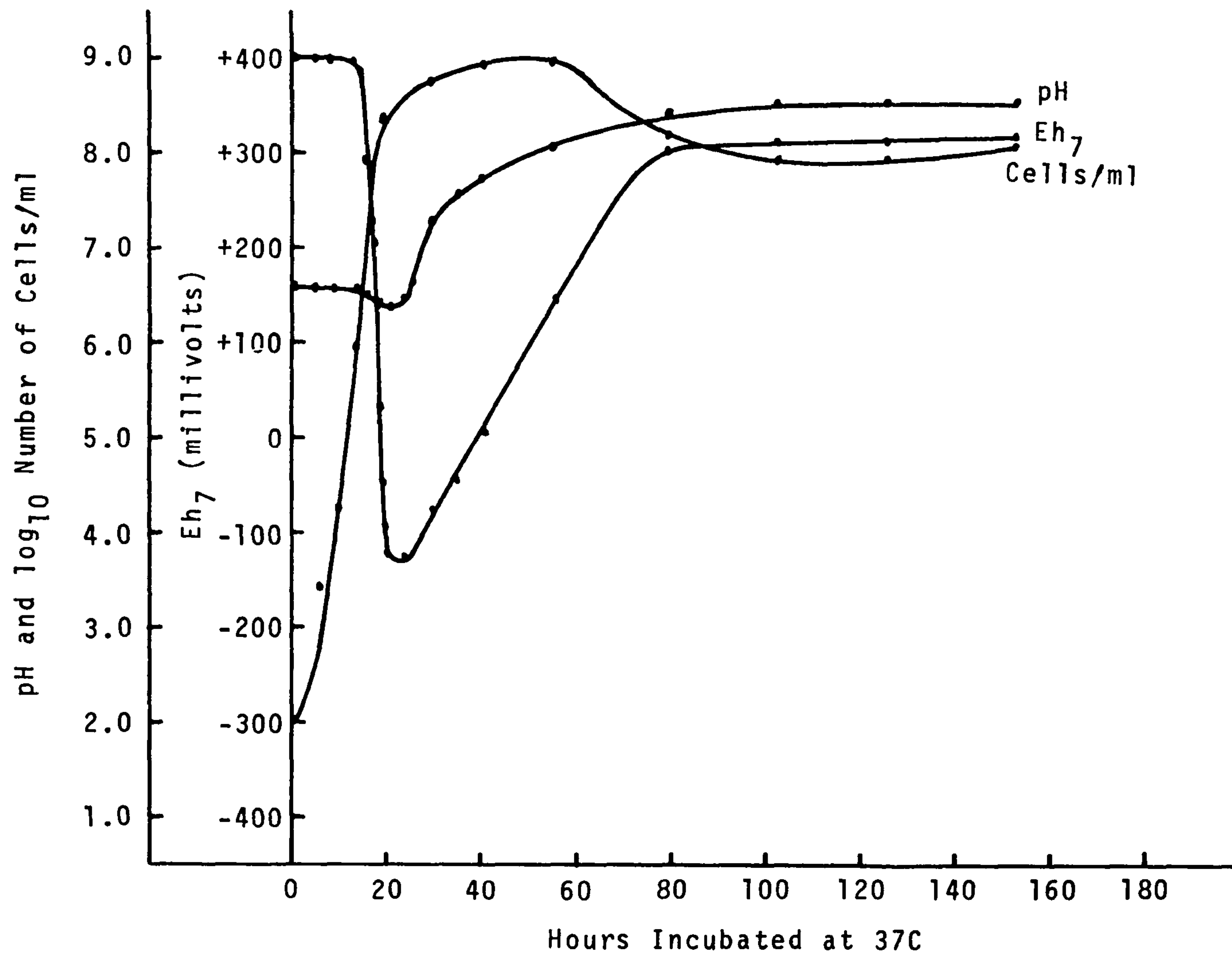



Figure 3. Growth, $Eh_7$, and pH curves of <u>Salmonella heidelberg</u> in nutrient broth (NB) at 37C

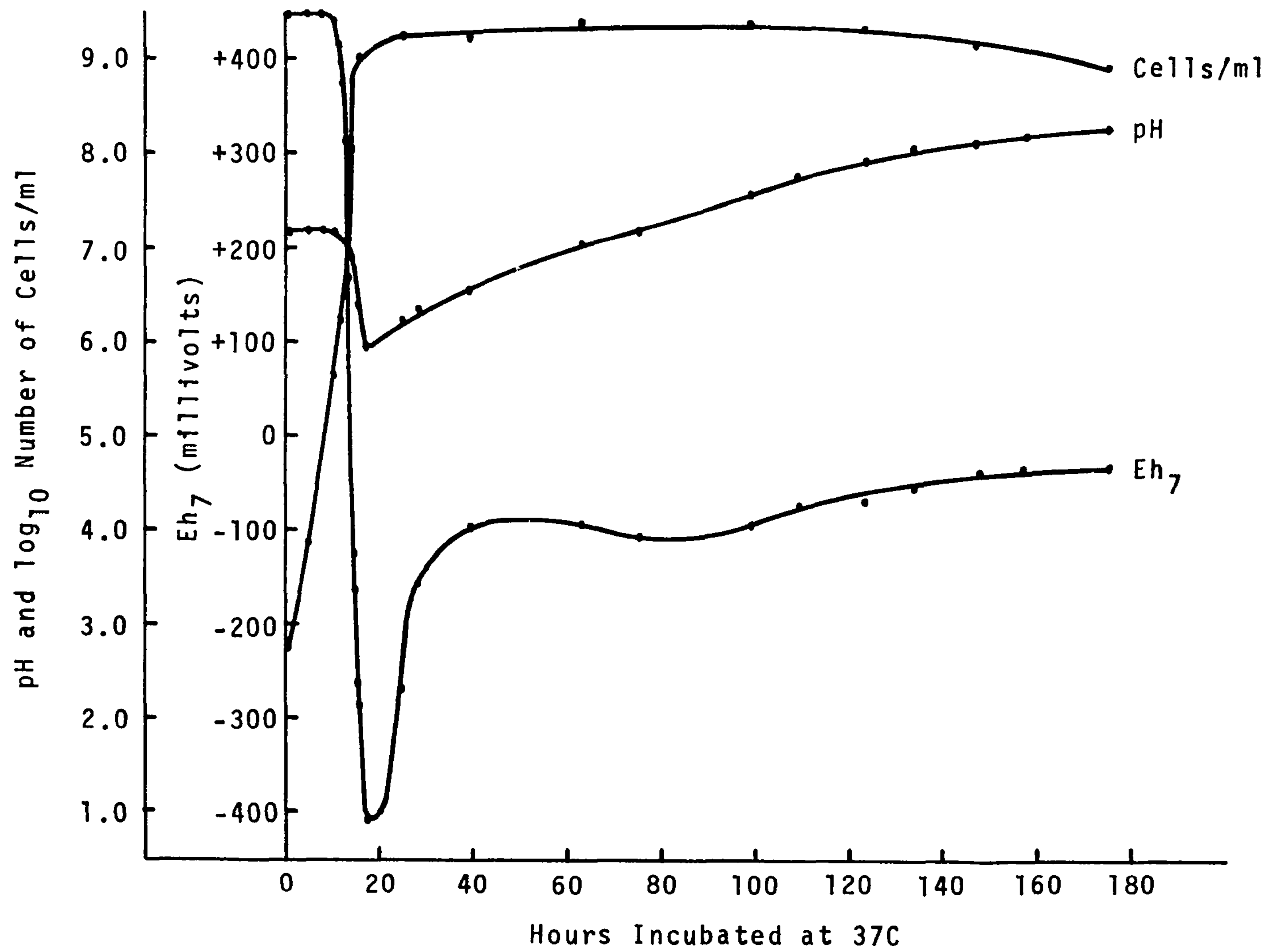



Figure 4. Growth, $Eh_7$, and pH curves of <u>Salmonella heidelberg</u> in trypticase soy broth (TSB) at 37C

fell to -130 mv after the *S. heidelberg* began to grow rapidly after approximately 15 hr of incubation at 37C. Minimal Eh was obtained when cell numbers attained their maximal value of $1.0 \times 10^9$ cells/ml. After 55 hr, however, there was a marked death of the culture, during which viable cell numbers fell to a final concentration of $1.0 \times 10^8$ cells/ml at the end of the experiment. Also, between 24 to 80 hr, the Eh rose rapidly to a value of +315 mv and remained essentially the same until the experiment was concluded. The pH in these cultures fell from an initial value of 6.60 to its lowest value of 6.35 after 22 hr. Thereafter, there was a rise to more alkaline values and a final pH of 8.50.

In contrast, Figure 4 shows results obtained with *S. heidelberg* in TSB, where the initial Eh was +450 mv. A minimal Eh of -410 mv was attained after 18 hr at 37C. A minimal pH of 5.95 also was attained at approximately this time. As with NB, the lowest observed potential was reached when the metabolic activities of this organism were most intense in the logarithmic phase of growth. Maximal cell count was $2.2 \times 10^9$ cells/ml; numbers decreased to a final concentration of $7.9 \times 10^8$ cells/ml after 176 hr. The rise in Eh after the attainment of a minimal value in Eh was much faster, but not as much as that with NB. Final Eh in the TSB culture was -35 mv. Final pH was 8.25.

Experiments in which BHI was used gave results that were essentially identical with those obtained with TSB, i.e., sharp inflections in the Eh and pH curves were noted. After several similar experiments with other *Salmonella* spp., it was decided to use TSB as the medium of cultivation in all future experiments. This decision was based on the evidence that NB seemed to have a greater poising capacity than that of TSB; cultures grown in TSB showed more dramatic changes in oxidation-reduction potentials and pH values.

### Selection of Appropriate Level of Inoculum

Another aspect of these investigations concerned the level of inoculum used in the study of pure cultures of *Salmonella* and *Pseudomonas fluorescens*. Experiments were performed using both high ($10^6$ cells/ml in the electrode vessel) and low ($10^2$ cells/ml in the vessel) levels of inocula to determine if initial cell concentrations caused appreciable differences in the Eh, pH, and later cell numbers observed. With the exception that the cultures entered log phase a bit sooner (usually by 2 to 3 hr) with the greater inoculum and, therefore, changes in Eh and pH occurred more quickly, the over-all patterns of the parameters, i.e., maximal and minimal values, were nearly identical. By virtue of these types of observations, it was decided to use the smaller level

of inoculum to obtain a more characteristic growth curve and accompanying changes in Eh and pH. Frazier and Whittier (1931a) had reported that in some cases, their streptococci required the use of large numbers of cells as inoculum to reach minimal levels of Eh in culture. This was not observed with the cultures that were used in this study. As mentioned previously, Barnes and Ingram (1956) showed that growth of C. welchii (C. perfringens) strains at high Eh levels (+200 to +250 mv) was not possible unless a large inoculum ($10^6$ cells/ml) was used. This may be attributed to the fact that these organisms were anaerobic and would not be expected to be able to grow at such a highly oxidized level.

## Pure Culture Data -- Salmonella spp.

Tables 2 and 3 show data obtained from the six pure cultures of Salmonella at 15 and 37C. Table 2 gives minimum and final Eh values, and Table 3 gives minimum and final pH values attained by these organisms. Individual figures for each of these organisms will be presented and discussed briefly.

Data for S. heidelberg in TSB at 37C has already been presented in Figure 4. Figure 5 shows results obtained with this organism at 15C. As might be expected, there was a considerable lag phase as compared to 37C. After approximately 130 hr, however, changes in Eh and pH began to occur.

Table 2. $Eh_7$ values (mv) of various species of *Salmonella*

| Organism | 15C | | | | 37C | | | |
|---|---|---|---|---|---|---|---|---|
| | Minimum | (hr) | Final | (hr) | Minimum | (hr) | Final | (hr) |
| *S. typhimurium* | -243 | (173) | +63 | (354) | -422 | (15) | -3 | (176) |
| *S. heidelberg* | -220 | (206) | +20 | (406) | -410 | (18) | -33 | (176) |
| *S. infantis* | -285 | (150) | +155 | (406) | -420 | (14) | 0 | (176) |
| *S. tennessee* | +80 | (230) | +175 | (326) | -384 | (19) | -7 | (185) |
| *S. enteritidis* | -85 | (238) | +135 | (402) | -412 | (17) | -15 | (177) |
| *S. thompson* | -315 | (180) | +107 | (404) | -415 | (11) | -25 | (187) |

Table 3. pH values of various species of *Salmonella*

| Organism | 15C | | | | 37C | | | |
|---|---|---|---|---|---|---|---|---|
| | Minimum | (hr) | Final | (hr) | Minimum | (hr) | Final | (hr) |
| *S. typhimurium* | 6.24 | (186) | 7.85 | (354) | 5.73 | (16) | 8.23 | (176) |
| *S. heidelberg* | 6.45 | (207) | 7.90 | (402) | 5.95 | (19) | 8.25 | (176) |
| *S. infantis* | 6.25 | (151) | 8.70 | (406) | 5.85 | (15) | 8.20 | (176) |
| *S. tennessee* | 6.85 | (234) | 8.55 | (326) | 5.90 | (20) | 8.18 | (185) |
| *S. enteritidis* | 5.90 | (240) | 8.25 | (404) | 5.57 | (21) | 8.15 | (177) |
| *S. thompson* | 6.35 | (181) | 8.35 | (402) | 5.95 | (12) | 8.15 | (187) |

After 90 hr, marked changes in viable cell concentration were noted. At 15C, there was a marked negative drift in Eh, although not to the degree observed at 37C, to a minimum Eh of -220 mv at 206 hr. The pH also decreased to a minimum value of 6.45 at 207 hr. The recovery to higher Eh and pH values also

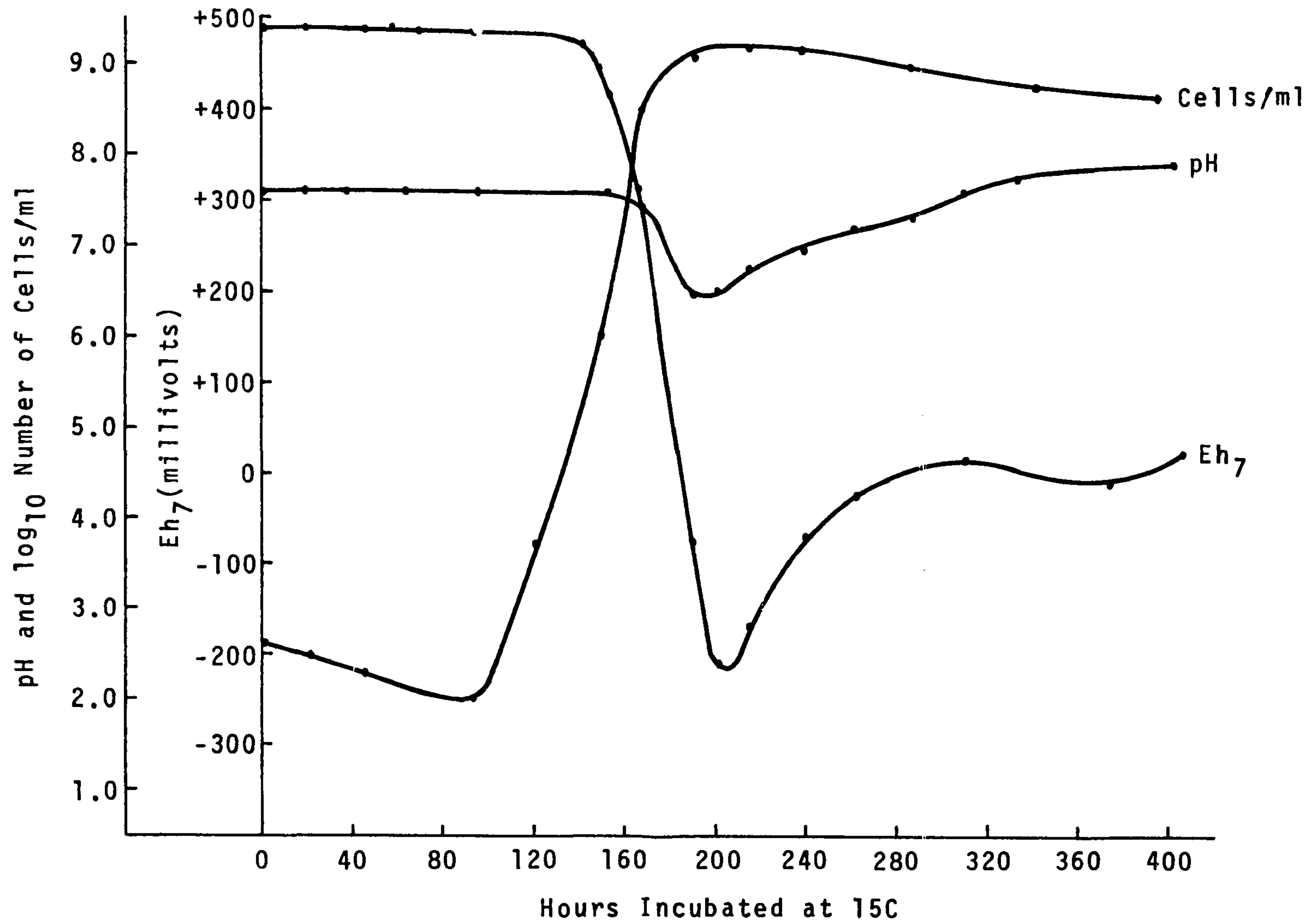



Figure 5. Growth, $Eh_7$, and pH curves of Salmonella heidelberg in TSB at 15C

was obvious. Attainment of stationary growth phase coincided with minimal Eh and pH values. A maximal concentration of 1.2 x $10^9$ cells/ml was noted at 210 hr, afterwhich there was a marked decline in cell numbers during the balance of the 406 hr experiment. Recovery to higher Eh and pH was much less pronounced at 15C than at 37C, or the time to reach more positive values was considerably longer. At 406 hr, final Eh and pH values were +20 mv and 7.90, respectively. As one compares the data obtained at 15 and 37C, the most obvious difference is that at 15C, changes in the oxidation-reduction potentials of the cultures are not as intense or as quantitative as at 37C. Minimal values for Eh, as well as pH, are not as low as observed at the optimal temperature of 37C. This trend will become more evident as data are presented for the other *Salmonella* spp. studied.

Figure 6 presents data that were obtained with *S. typhimurium* at 37C. Intense reducing conditions were established in the culture within 12-15 hr. Very nearly coincidental with the establishment of minimal Eh, the pH reached its minimum value of 5.73. Viable cell numbers also attained a near maximum shortly thereafter. After minimal Eh was observed, there was a sharp rise in Eh for the next 10-15 hr and then a gradual increase to a final value of -3 mv at 176 hr. The sharp drop in pH to a minimum value of 5.73 at 16 hr was followed by a steady increase to a final value

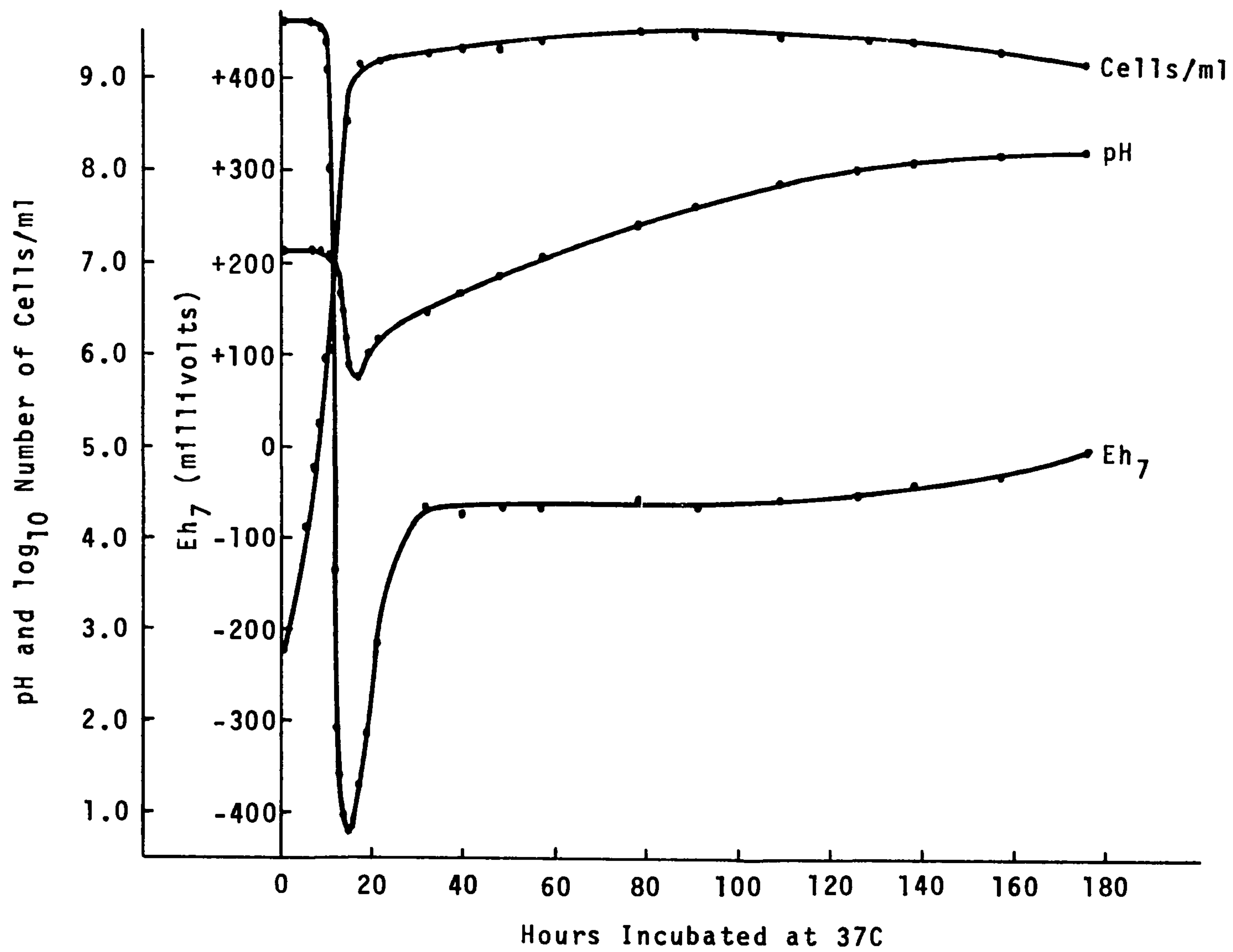



Figure 6. Growth, $Eh_7$, and pH curves of Salmonella typhimurium in TSB at 37C

of 8.23 at 176 hr. From the inoculum of $1.8 \times 10^2$ cells/ml, cell numbers reached a maximum concentration of $3.2 \times 10^9$ cells/ml after approximately 80 hr at 37C and exhibited a slight decrease in viable numbers to a final concentration of $1.7 \times 10^9$ cells/ml after 176 hr.

Growth, Eh, and pH patterns for pure cultures of S. typhimurium at 15C are given in Figure 7. As was observed with S. heidelberg at this temperature, there was an extended lag phase with only slight growth during the first 80 hr of the incubation period. After the bacteria had adjusted to the somewhat adverse environment, significant changes in Eh, pH, and cell numbers began to occur. There was a very characteristic negative drift in Eh, more so than was observed with S. heidelberg. The Eh reached its minimum value of -243 mv after 173 hr. The pH decreased rapidly to a minimal value of 6.24 at 186 hr. The fast recovery to more positive Eh values and more alkaline pH values was observed during the period thereafter, up to the time that the experiment was terminated at 354 hr. Attainment of stationary growth phase also coincided with minimal Eh and pH values. Maximum and final cell concentrations were the same, $2.2 \times 10^9$ cells/ml. with no apparent decrease in population after 360 hr.

Figure 8 shows data obtained with S. infantis at 37C. From an initial Eh of +455 mv, the bacteria were able to

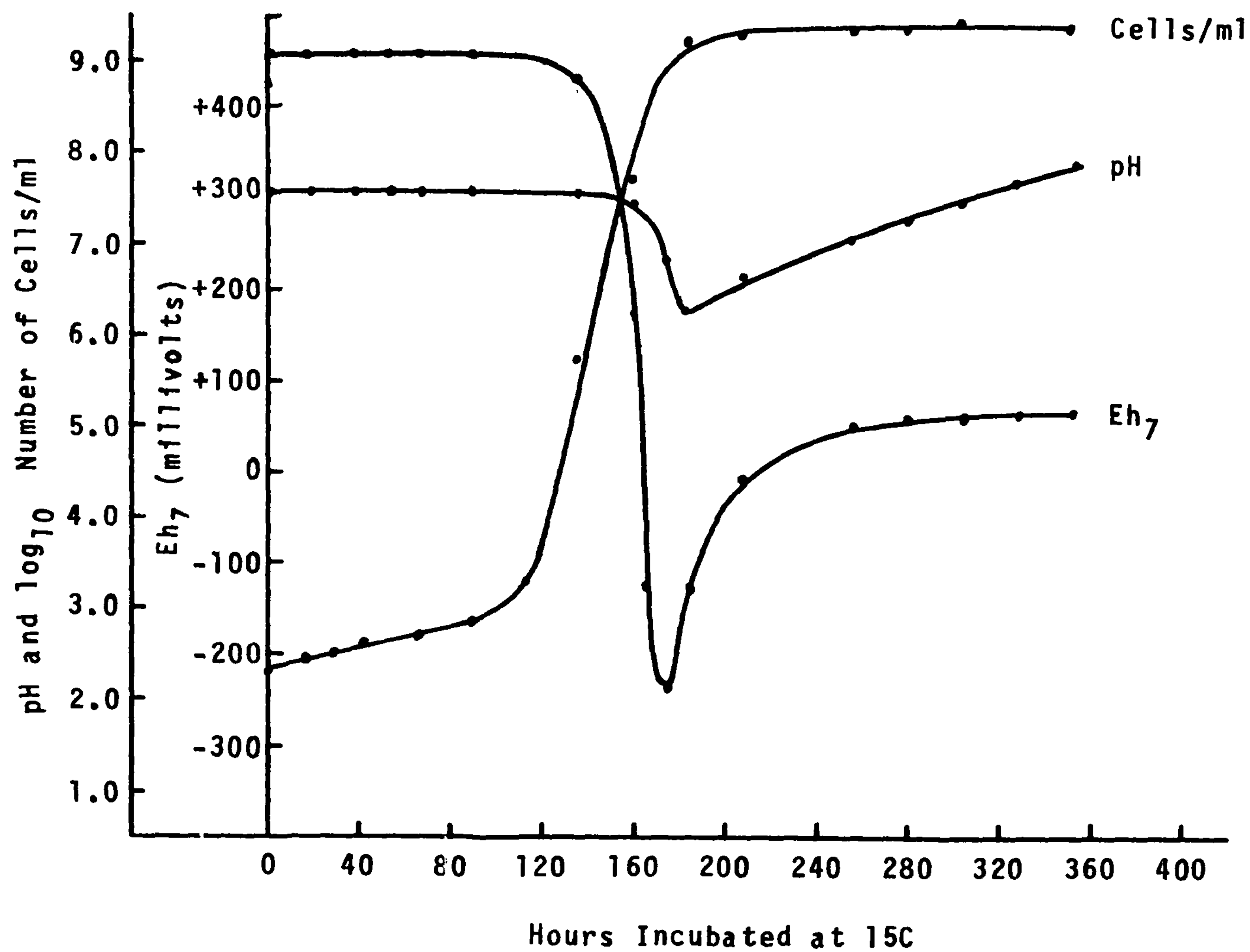



Figure 7. Growth, $Eh_7$, and pH curves of <u>Salmonella typhimurium</u> in TSB at 15C

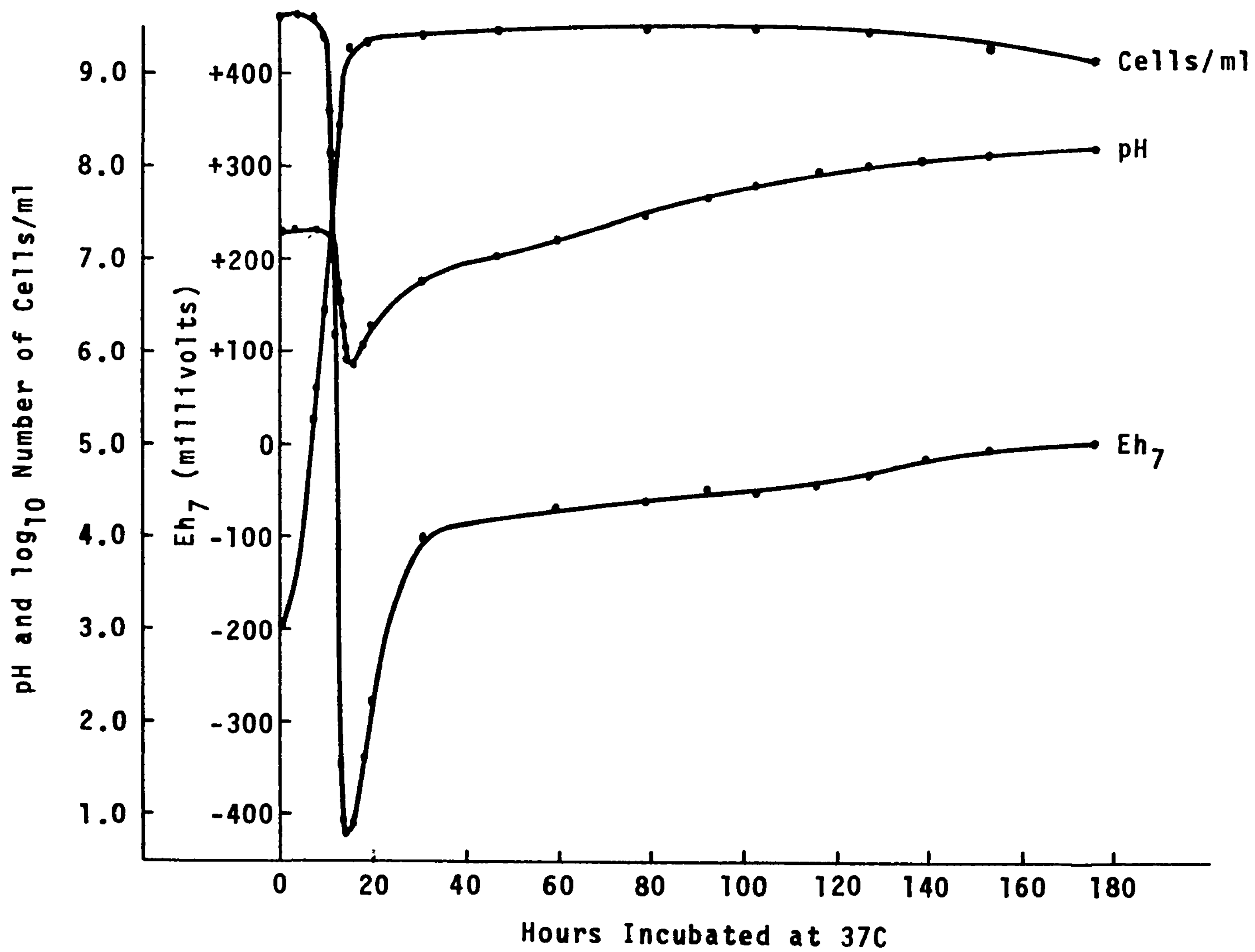



Figure 8. Growth, $Eh_7$, and pH curves of *Salmonella infantis* in TSB at 37C

bring about a marked reduction of the medium to a minimum Eh value of -420 mv after 14 hr of incubation. The minimal pH value was 5.85 and was reached after 15 hr of incubation. The concentration of viable cells rose above $10^9$ cells/ml soon afterward. This level was maintained for the duration of the experiment, showing only a slight dying-off trend after 120 hr until the experiment was terminated at 176 hr. The Eh and pH curves show that once minimal values were reached, there was a rather sharp increase towards more positive Eh values and more alkaline pH values. It will be noted that this was the trend for all of the *Salmonella* spp. examined. This period of sharp increase in Eh lasted about 15 hr after the minimum Eh had been reached, giving an Eh of -100 mv after 30 hr of incubation. Thereafter, there was a gradual upward drift in Eh to a final value of 0 mv at 176 hr. The pH gradually increased from its minimum value to a final value of 8.20.

Data obtained with *S. infantis* at 15C are presented in Figure 9. Of the *Salmonella* spp. tested, this organism adjusted the quickest to the low temperature situation and changes in Eh and pH were noted after 150 hr. A minimum Eh of -285 mv was reached at this time followed by the attainment of a minimal pH of 6.25 an hour later. Cell numbers reached $3.1 \times 10^9$ cells/ml at approximately 240 hr after inoculation. Very little decrease in viable cells was

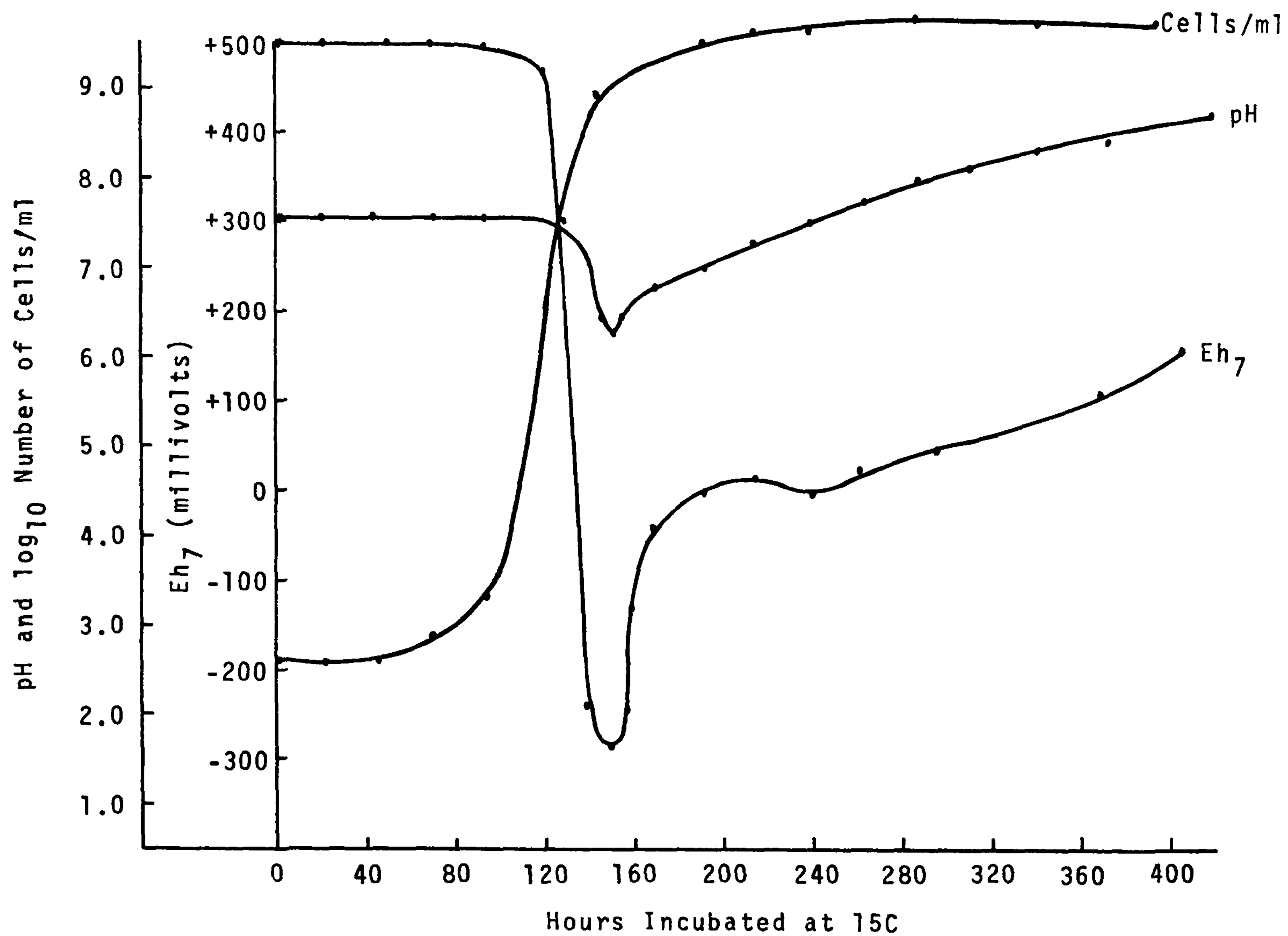



Figure 9. Growth, $Eh_7$, and pH curves of <u>Salmonella infantis</u> in TSB at 15C

detected during the remainder of the experiment. Both Eh and pH values remained more negative for a longer period of time than observed for any of the Salmonella spp. described thus far. Recovery to more positive Eh and higher pH was observed as previously mentioned. Final Eh and pH values were +155 mv and 8.70, respectively, at 406 hr.

Figure 10 presents data obtained with S. tennessee at 37C. Once again, a rapid rate of reduction of TSB occurred. This particular Salmonella was the least reductive strain studied. A minimum Eh value of -384 mv was observed after 19 hr of incubation. The minimum pH value recorded was 5.90 at 20 hr. Recovery of Eh and pH to more positive and alkaline values was also apparent. Cell numbers reached a maximal concentration of $1.9 \times 10^9$ cells/ml at approximately 50 hr and showed a slight dying-off trend during the remainder of the experiment down to a final concentration slightly more than $10^9$ cells/ml.

Figure 11 shows results obtained with S. tennessee at 15C. This organism grew the poorest of all the Salmonella spp. tested at this temperature. There was a pronounced decrease in cell numbers from an inoculum of $7.9 \times 10^2$ cells/ml during the first 180 hr of incubation. The Eh of the cultures began to fall at approximately 140 hr and showed a minimum value of +80 mv at 230 hr followed by a slow increase in observed potential to a final value of +175 mv

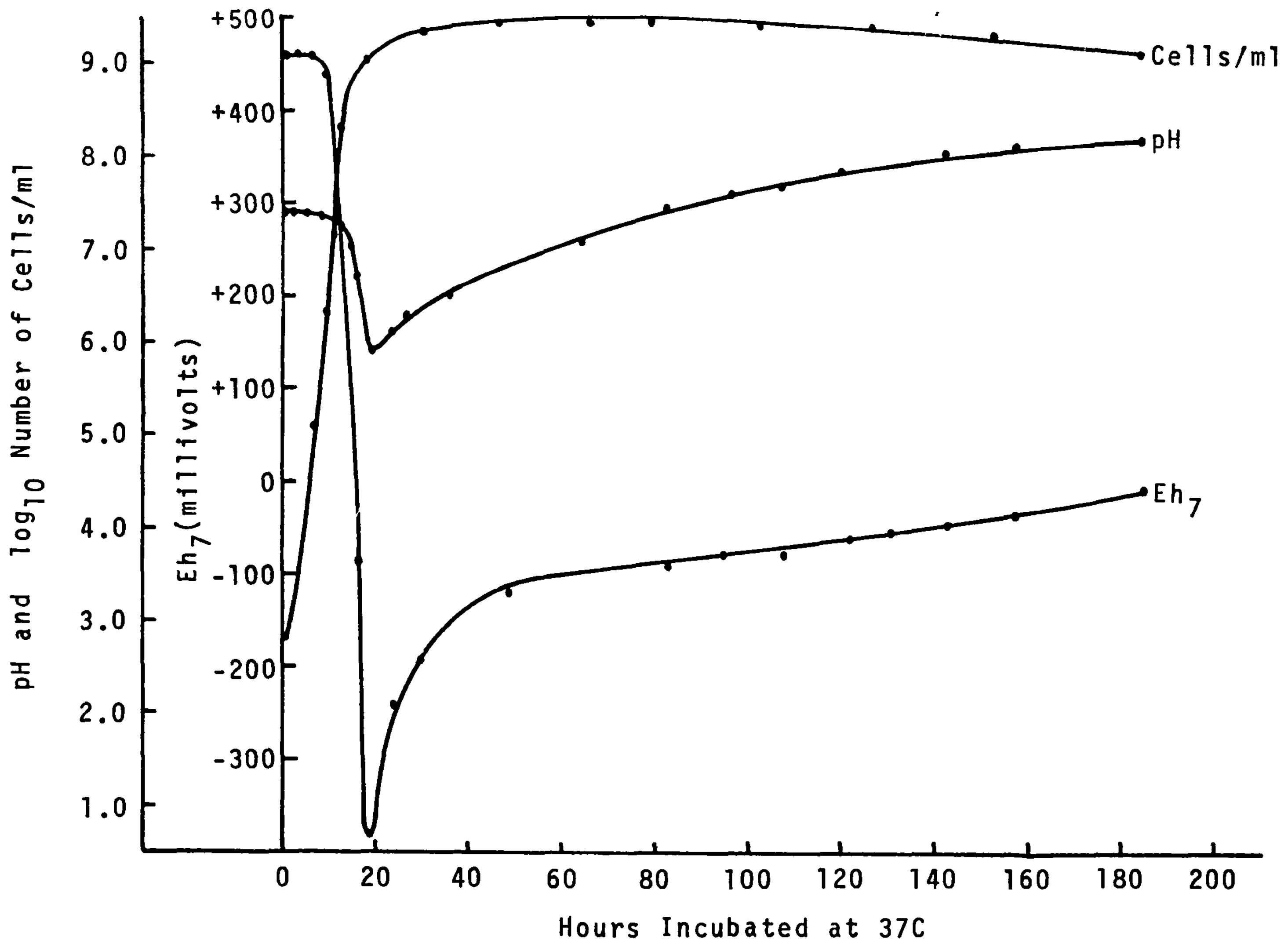



Figure 10. Growth, $Eh_7$, and pH curves of Salmonella tennessee in TSB at 37C

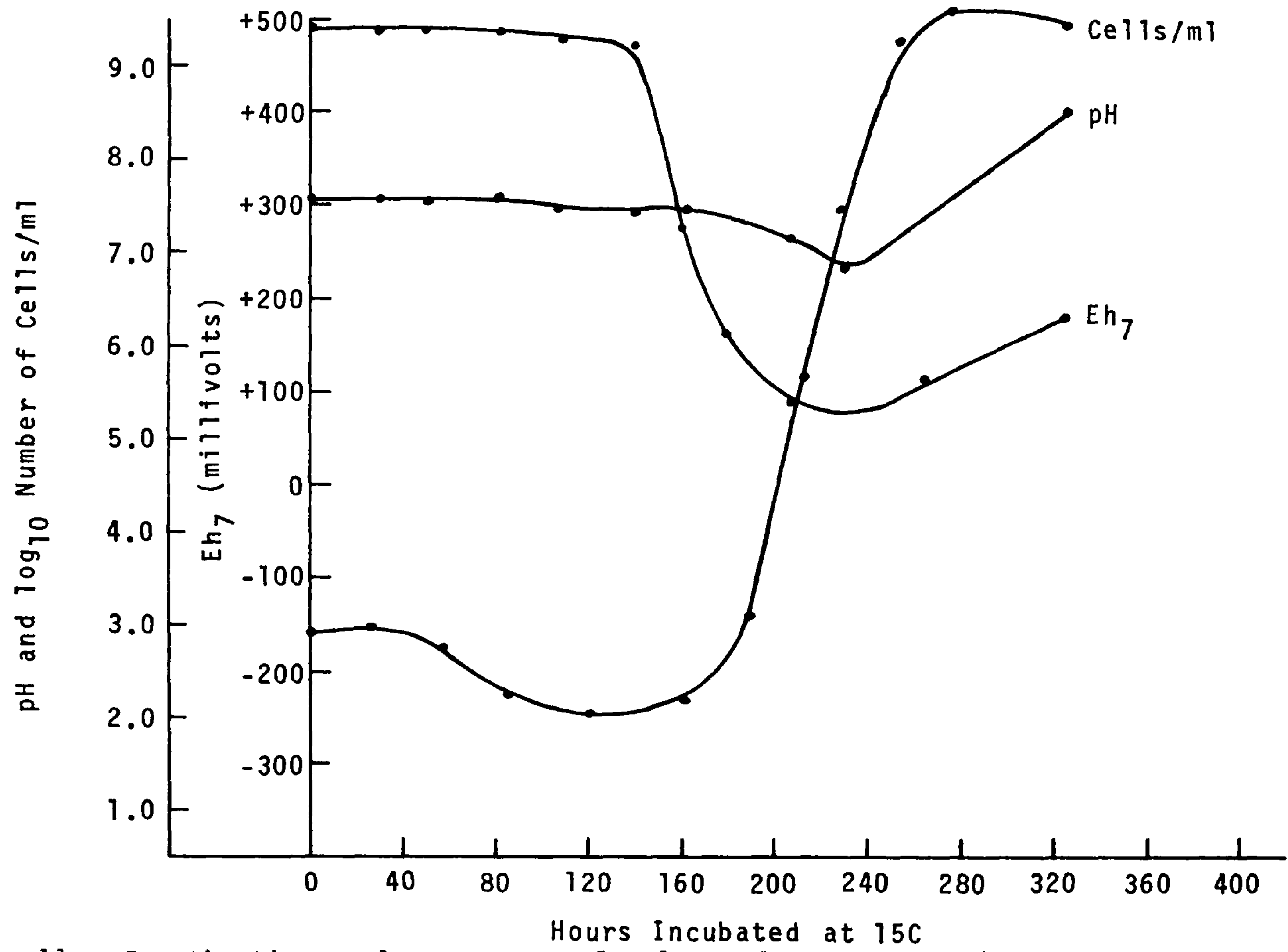



Figure 11. Growth, $Eh_7$, and pH curves of <u>Salmonella tennessee</u> in TSB at 15C

at 326 hr. The pH fell from its initial value of 7.55 to a minimum of 6.85 at 234 hr and then sharply increased to a final value of 8.55 at 326 hr. Cell numbers reached a maximum concentration of 3.5 x $10^9$ cells/ml after 276 hr and then showed a slight decrease during the balance of the experimental period. The most noticeable difference of these data when compared to all of the data obtained with the other Salmonella spp. (and, in fact, the Pseudomonas fluorescens results), is that, in the case of S. tennessee at 15C, the attainment of maximal cell concentrations is not coincidental with the attainment of minimal Eh and pH values. This observation is not readily explainable. It is possible that because of the apparent trouble that this organism had in adjusting to the somewhat adverse environment, there may have been some cell lysis and, in turn, the liberation of reductive substances that would not ordinarily be detected under normal circumstances. Coulter and Isaacs (1929) and Hewitt (1950) have suggested this possibility.

Growth, Eh, and pH curves for S. enteritidis at 37C are shown in Figure 12. As with the other Salmonella spp. studied, intense reduction of the substrate is readily apparent, and within a very short period of time. In this instance, a minimal Eh of -412 mv was recorded after 17 hr, followed by a sharp increase in potential for the next 4-5 hr, and then a very slight increase in observed potential to

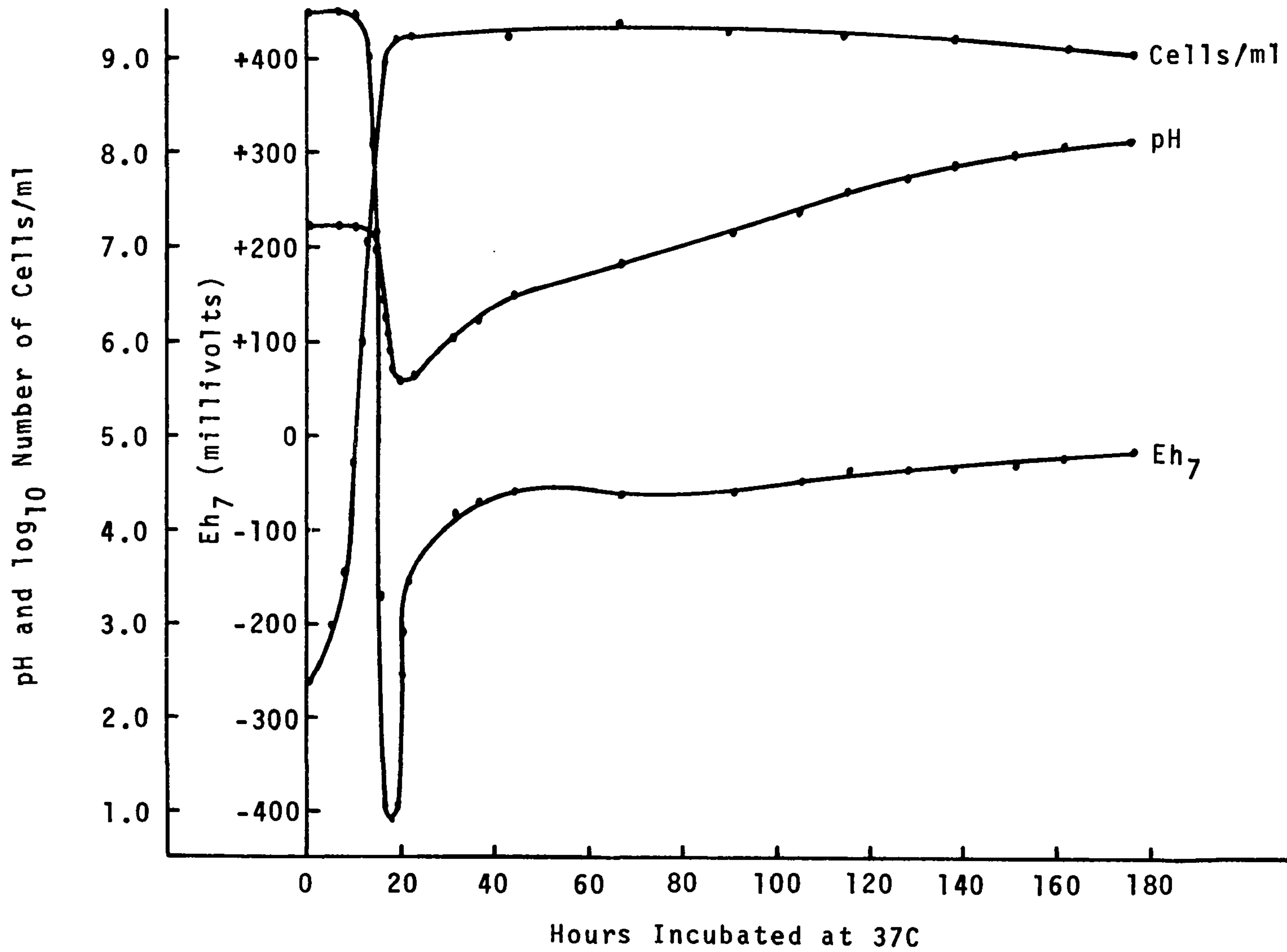



Figure 12. Growth, $Eh_7$, and pH curves of Salmonella enteritidis in TSB at 37C

a final redox potential of -15 mv after 177 hr. Minimal Eh coincided with minimal pH and maximal cell concentrations. The pH fell to its lowest value of 5.57 at 21 hr and gradually increased to a final value of 8.15 at the conclusion of the experiment after 177 hr. A concentration of $1.4 \times 10^9$ cells/ml was recorded at about this time also, followed by only a slight decrease in viable cell numbers. S. enteritidis showed the capacity to produce the lowest pH observed in all the Salmonella cultures examined.

Figure 13 shows data obtained with S. enteritidis at 15C. The general trends of the Eh, pH, and growth curves are essentially similar to those already described for the other Salmonella spp. studied at 15C, with the exception of S. tennessee. Attainment of minimal Eh occurred after 238 hr and its value was -85 mv, the second least reductive value recorded for the six Salmonella studied. There was a slight recovery to more positive potential, followed by a slight decrease in potential and then, finally, a general upward drift in Eh to a final value of +135 mv after 402 hr. A minimal pH of 5.90 was recorded at 240 hr, followed by a gradual drift toward more alkaline values and a final pH of 8.25 at 404 hr. Cell numbers decreased slightly for the first 80 hr after inoculation with $5.6 \times 10^2$ cells/ml. Log growth phase began after about 100 hr of incubation at 15C and maximal numbers were observed to be $2.9 \times 10^9$ cells/

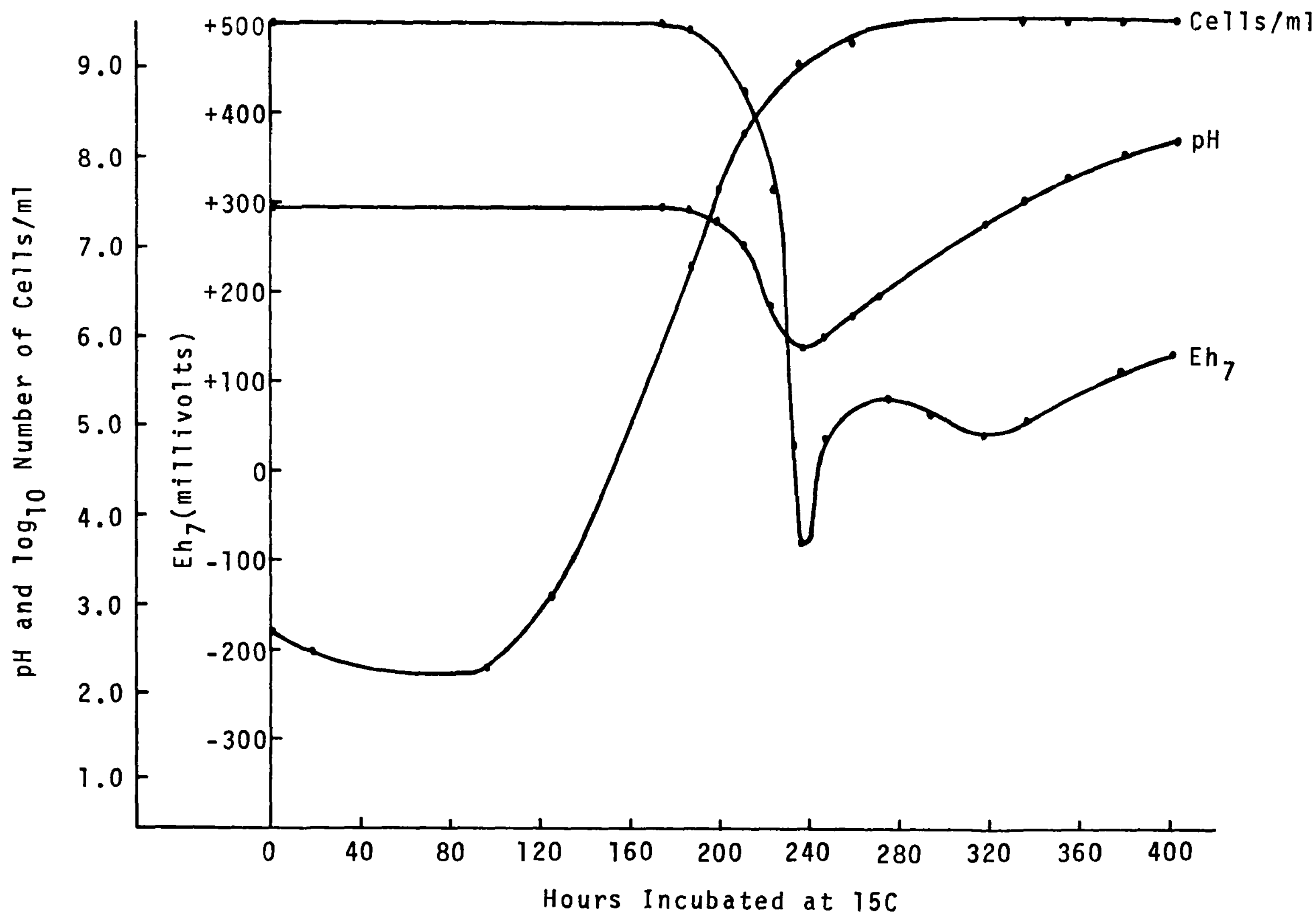



Figure 13. Growth, $Eh_7$, and pH curves of *Salmonella enteritidis* in TSB at 15C

ml after 244 hr. No obvious decrease in viable cell numbers occurred throughout the duration of the experiment.

Curves for _S. thompson_ at 37C are given in Figure 14. This particular organism was the most active _Salmonella_ studied insofar as the attainment of very low redox potentials was concerned. The minimal Eh attained by these cultures was -415 mv after 11 hr of incubation. This minimum value was also maintained for nearly 9 additional hr, followed by the now familiar rise in Eh to more positive values and then a leveling off of potential to a final Eh of -25 mv after 187 hr, the length of the experimental period. A minimum pH of 5.95 was reached after 12 hr followed by a subsequent slow increase to more basic values and a final pH of 8.15 at 187 hr. Cell numbers rose rapidly from an initial concentration of $3.9 \times 10^2$ cells/ml to a maximum concentration of $1.7 \times 10^9$ cells/ml coincidental with the minimal Eh and pH at approximately 18 hr. As with other _Salmonella_ spp. at 37C, _S. thompson_ exhibited no detectable decrease in viable cells throughout the experimental period.

Figure 15 shows results obtained with _S. thompson_ at 15C. Perhaps indicative of its metabolic capabilities, this organism attained the lowest Eh recorded by the six _Salmonella_ investigated at 15C. This Eh was -315 mv and occurred after 180 hr incubation. The sharp return to more positive potentials is also apparent, followed by the

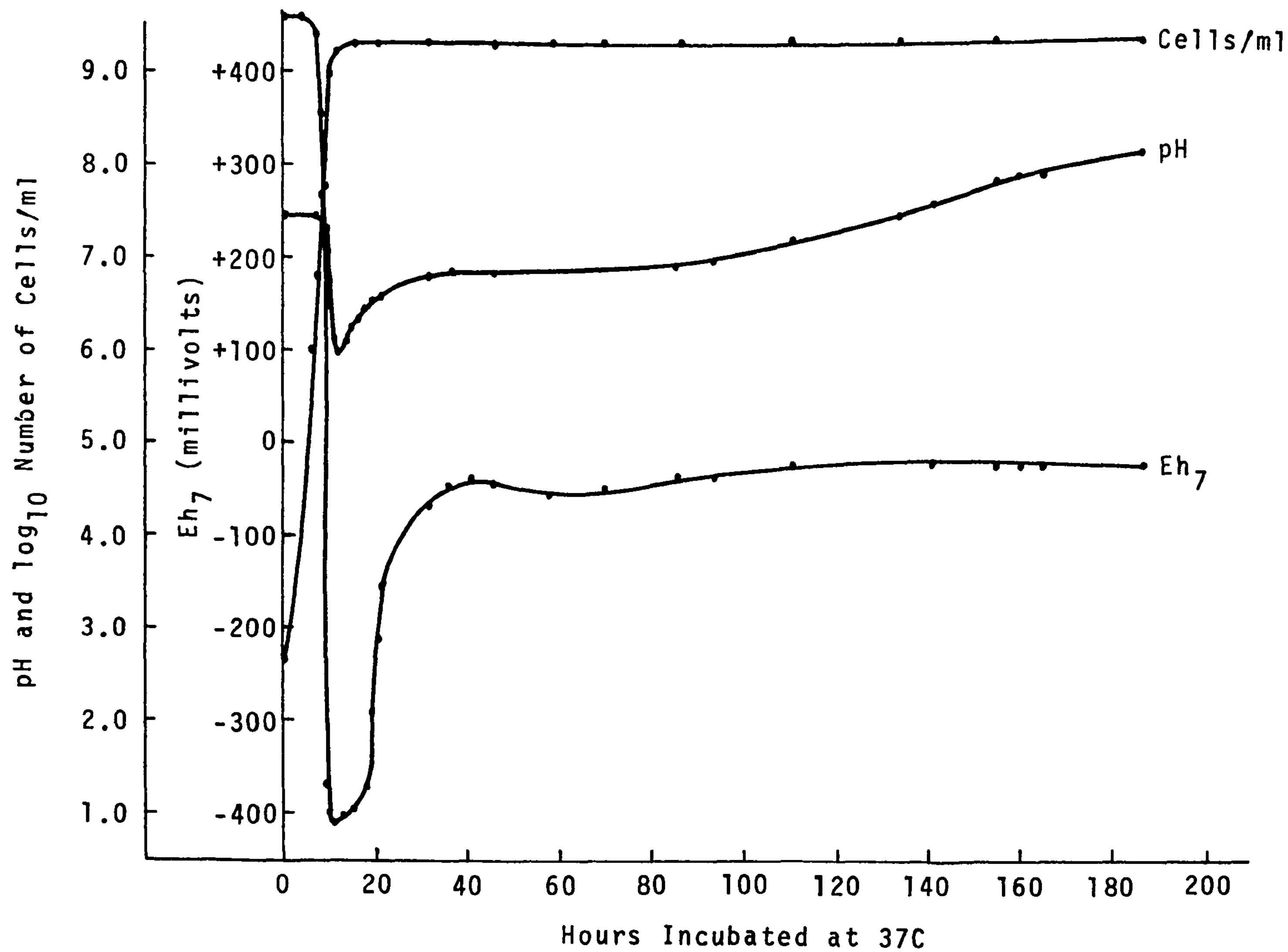



Figure 14. Growth, $Eh_7$, and pH curves of _Salmonella thompson_ in TSB at 37C

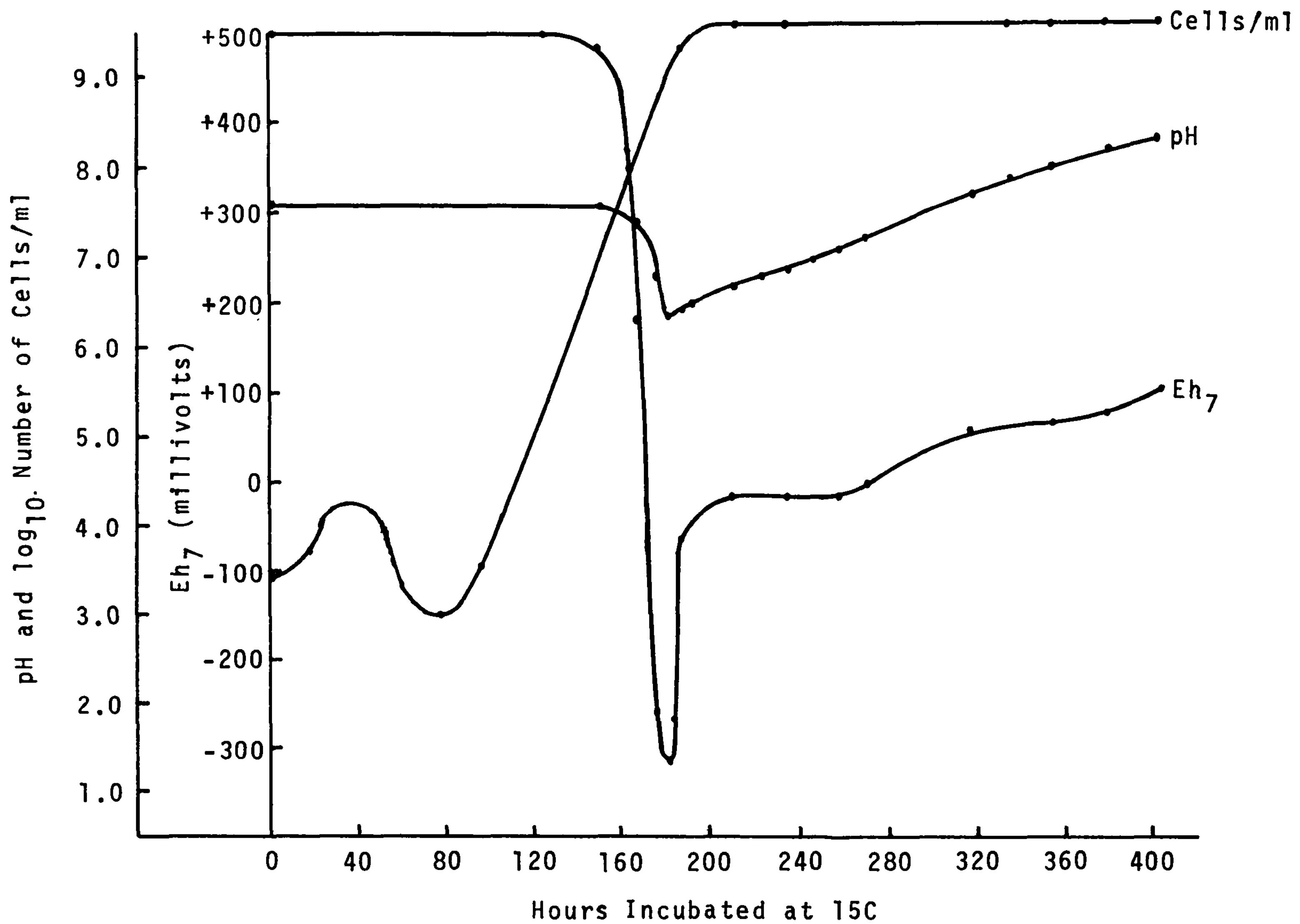



Figure 15. Growth, $Eh_7$, and pH curves of *Salmonella thompson* in TSB at 15C

leveling off towards the end of the experiment to a final value of +107 mv after 404 hr. A sharp drop in pH was observed coincidental with the attainment of minimal Eh. The lowest pH was 6.35 at 181 hr. As has been the rule, there was an increase to more alkaline pH values during prolonged incubation, with a final pH of 8.35 after 402 hr. Cell multiplication was erratic during the first 80 hr of incubation. However, a maximum concentration of well above $10^9$ cells/ml was observed after 180 hr and no obvious decrease in viable cell numbers occurred for the duration of the 404 hr experiment.

## Pure Culture Data -- *Pseudomonas fluorescens*

Tables 4 and 5 show data obtained from three strains of *Pseudomonas fluorescens* at 15 and 30C. Table 4 gives minimum and final Eh values, and Table 5 gives minimum and final pH values attained by these organisms. Individual curves for each of these strains will be presented and discussed briefly.

Growth, Eh, and pH curves for *P. fluorescens* F21 at 30C in TSB are shown in Figure 16. These results differed in several ways from those obtained with the *Salmonella* spp. studied. The reducing capacity of the *Pseudomonas* was much less marked than that of the *Salmonella* spp. For the *Pseudomonas*,

Table 4. $Eh_7$ values of various strains of *P. fluorescens*

| Organism | 15C | | | | 30C | | | |
|---|---|---|---|---|---|---|---|---|
| | Minimum | (hr) | Final | (hr) | Minimum | (hr) | Final | (hr) |
| *P. fluorescens* (F21) | +110 | (354) | +110 | (354) | +72 | (205) | +72 | (205) |
| *P. fluorescens* (F17) | +35 | (354) | +35 | (354) | +40 | (191) | +40 | (191) |
| *P. fluorescens* (2) | +75 | (353) | +75 | (353) | +60 | (194) | +60 | (194) |

Table 5. pH values of various strains of *P. fluorescens*

| | 15C | | | | 30C | | | |
|---|---|---|---|---|---|---|---|---|
| | Minimum | (hr) | Final | (hr) | Minimum | (hr) | Final | (hr) |
| *P. fluorescens* (F21) | 7.05 | (78) | 8.65 | (354) | 7.15 | (64) | 8.37 | (205) |
| *P. fluorescens* (F17) | 7.15 | (80) | 8.50 | (354) | 7.05 | (72) | 8.07 | (191) |
| *P. fluorescens* (2) | 7.10 | (96) | 8.22 | (352) | 7.12 | (24) | 8.20 | (192) |

there was a gradual reduction of the Eh of the medium throughout the period of the various experiments, regardless of strain. Little decrease occurred in the pH during the time that the Eh decreased most rapidly, but a gradual increase to more basic values was observed after the pseudomonad had reached the stationary phase after approximately 64 hr at 30C. Final pH was 8.37 after 205 hr. An inoculum of $2.0 \times 10^2$ cells/ml yielded a maximum concentration of $9.0 \times 10^8$

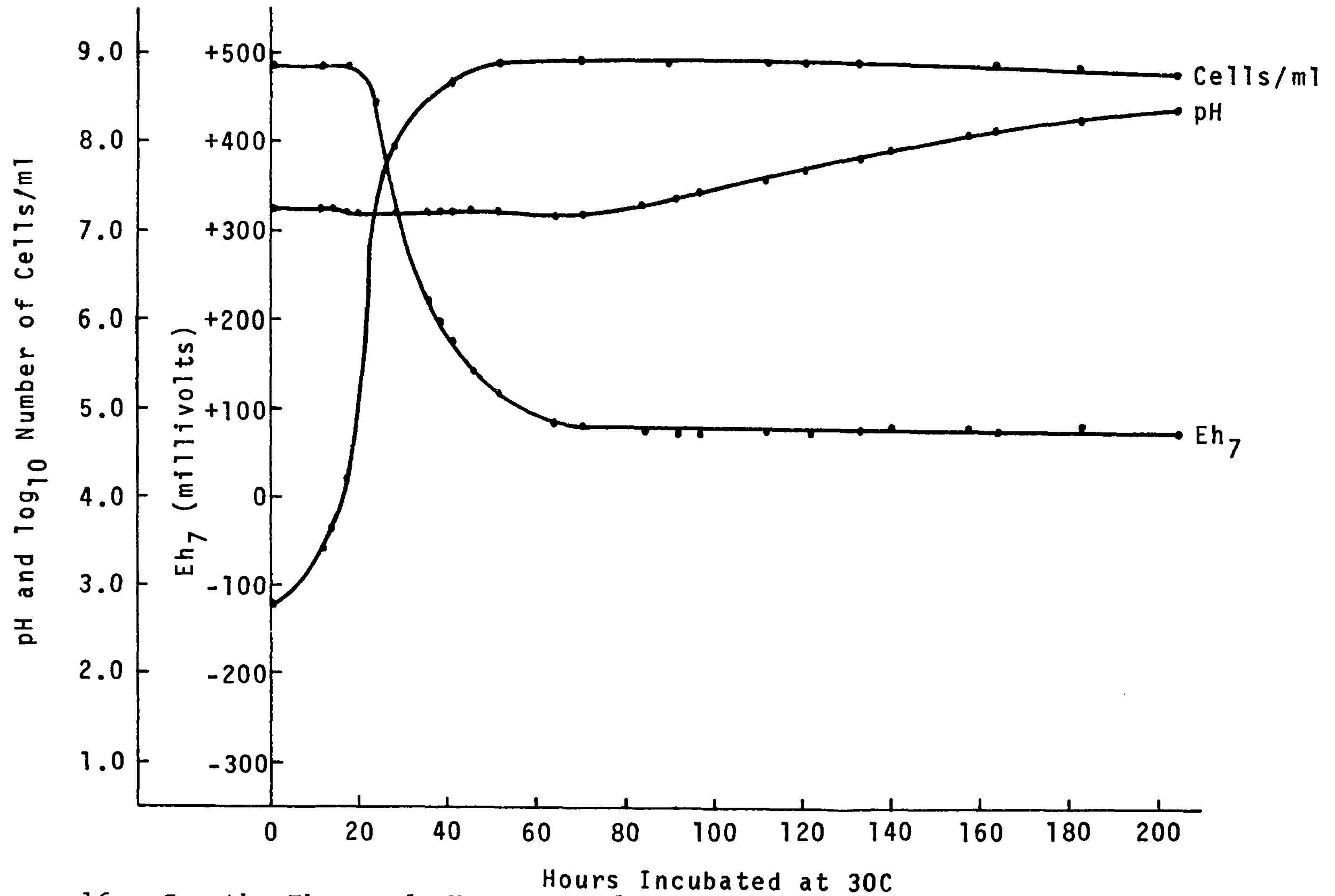



Figure 16. Growth, $Eh_7$, and pH curves of *Pseudomonas fluorescens* F21 in TSB at 30C

cells/ml at 62 hr. There was a slight decrease in viable cells to a final concentration of $6.3 \times 10^8$ cells/ml after 205 hr.

Figure 17 shows data for _P. fluorescens_ F21 at 15C. As with the cultures at 30C, there was a gradual negative drift in Eh down to a final Eh of +110 mv after 354 hr. This value was about 35 mv greater than the final Eh at 30C. There was a more marked variation in pH, in that the value of 7.05 at 78 hr was the minimal value as compared with the pH of 7.15 obtained at 30C. After this point, there was a gradual upward drift in pH values to a final value of 8.65 after 354 hr. Cell numbers reached a maximum of $4.4 \times 10^9$ cells/ml after approximately 240 hr and maintained this level for the duration of the experiment.

Growth, Eh, and pH curves for _P. fluorescens_ F17 at 30C are shown in Figure 18. As was observed with the F21 strain, there was a gradual reduction of the medium from an initial Eh of approximately +460 mv down to a final value of +40 mv at 191 hr. There was only a slight downward drift in pH to a minimum value of 7.05 at 72 hr, followed by a gradual increase in observed pH to a final value of 8.07 at 191 hr. Cell numbers increased from an initial concentration of 4.2 $\times 10^1$ cells/ml to a maximum of $1.7 \times 10^8$ cells/ml at about 80 hr and showed no appreciable decrease in viable numbers over the balance of the experimental period.

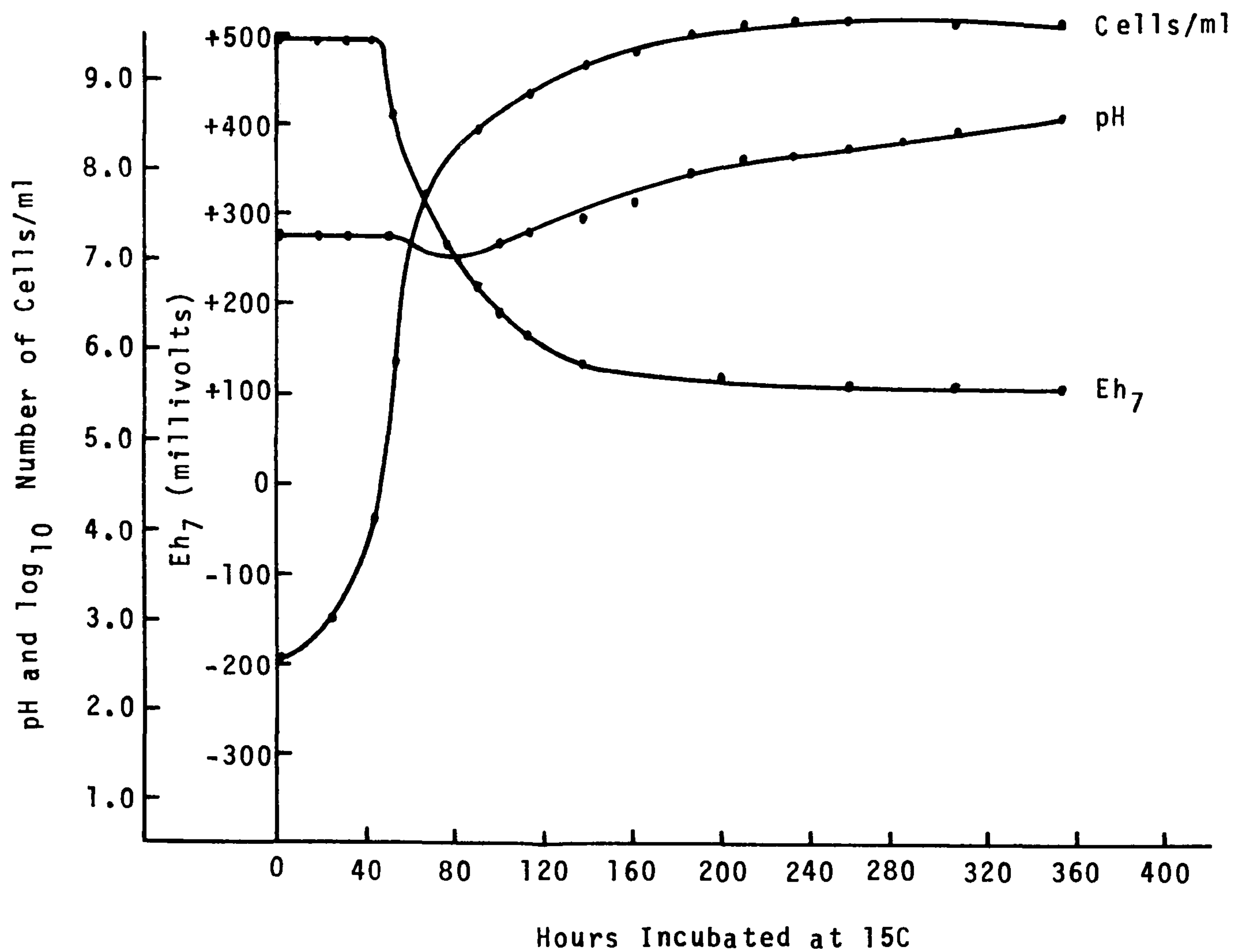



Figure 17. Growth, $Eh_7$, and pH curves of *Pseudomonas fluorescens* F21 in TSB at 15C

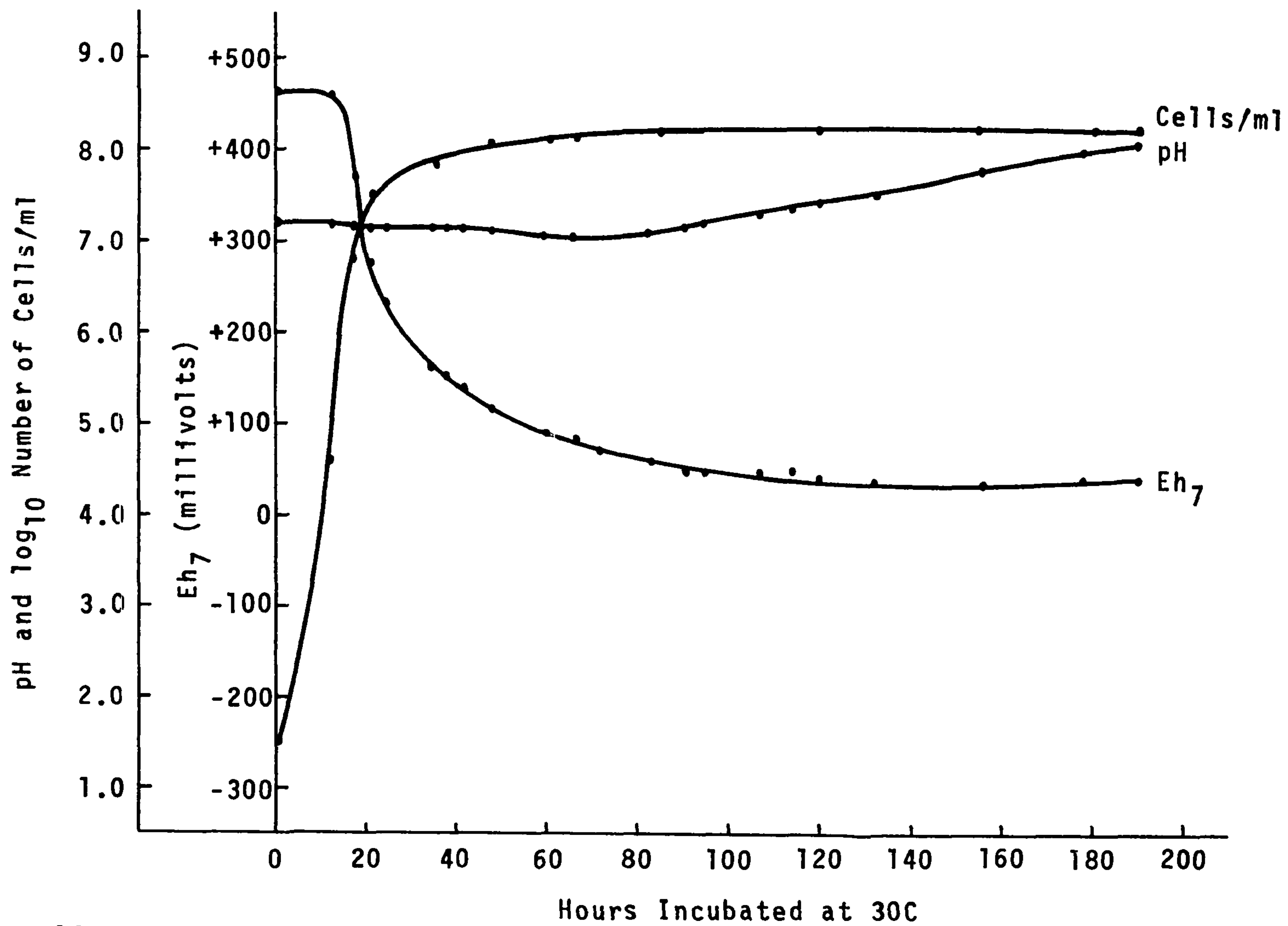



Figure 18. Growth, $Eh_7$, and pH curves of <u>Pseudomonas fluorescens</u> F17 in TSB at 30C

Figure 19 shows results obtained with F17 at 15C. In this case, once again, the gradual lowering of the redox potential is evident, from an initial value of +485 mv down to a minimal and final value of +35 mv. A minimum pH value of 7.15 was noted at 80 hr followed by a return to more alkaline values and a final pH of 8.50 after 354 hr. Viable cells increased from an initial count of 1.0 x $10^2$ cells/ml to a maximum level of 1.2 x $10^9$ cells/ml at the end of the experiment. Entry into stationary phase was gradual and over an extended period of time; cell numbers increased gradually thereafter throughout the experimental period.

*P. fluorescens* 2 strain was the third and final strain that was studied. Growth, Eh, and pH patterns for this organism at 30C are given in Figure 20. As in the case of the other pseudomonads investigated, there was a gradual reduction in the observed Eh of the TSB from an initial value of +485 mv to a final value of +60 mv after 194 hr. The pH reached its minimum value of 7.12 at 24 hr and gradually rose to a final value of 8.20 at the close of the experiment. Concentrations of cells increased from an initial level of slightly over $10^3$ cells/ml to a near maximum concentration of 2.0 x $10^8$ cells/ml after 60 hr and then showed a very slight increase during the remainder of the experiment.

Data obtained on strain 2 at 15C are presented in Figure 21. The Eh fell to its minimum and final value of +75 mv

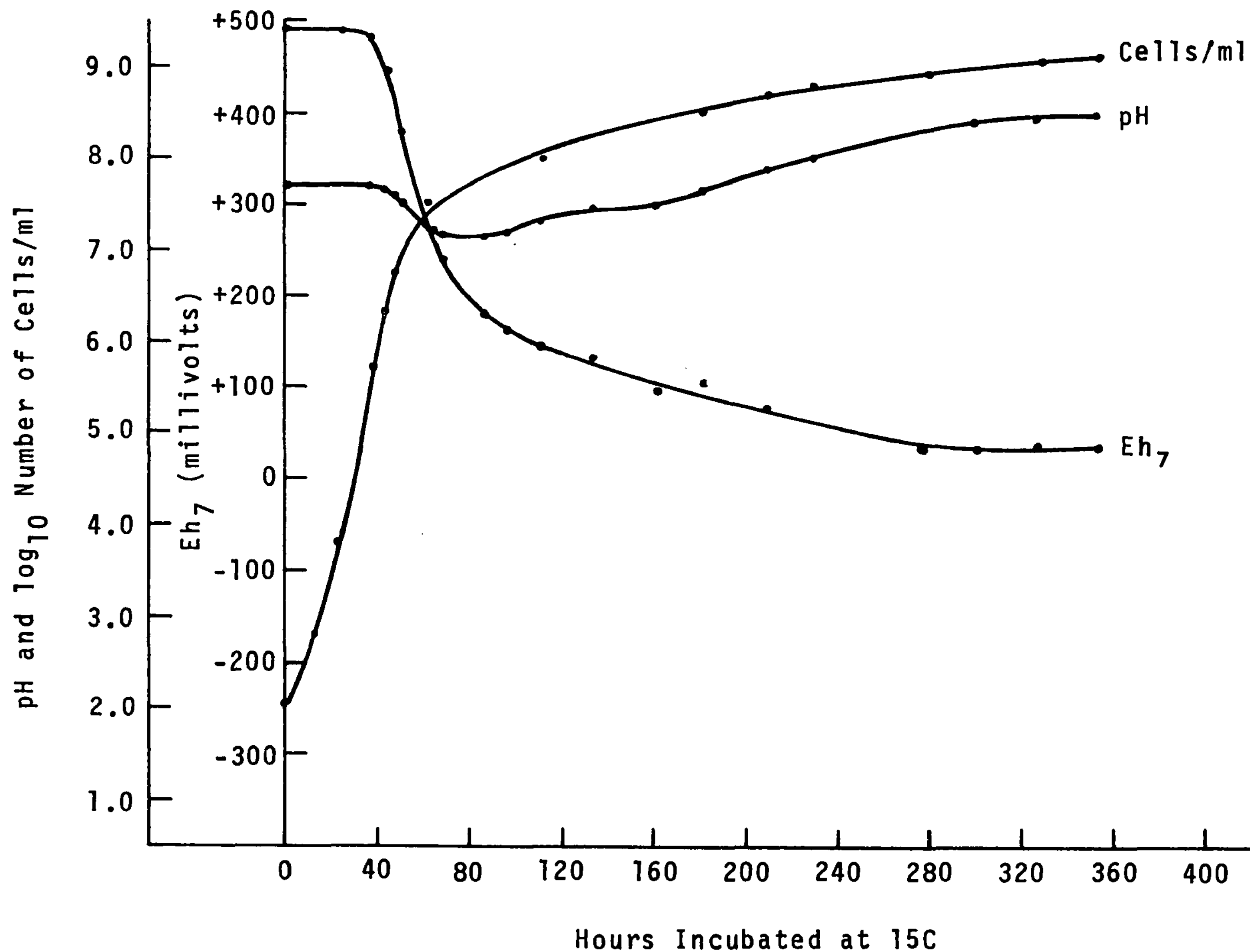



Figure 19. Growth, $Eh_7$, and pH curves of _Pseudomonas fluorescens_ F17 in TSB at 15C

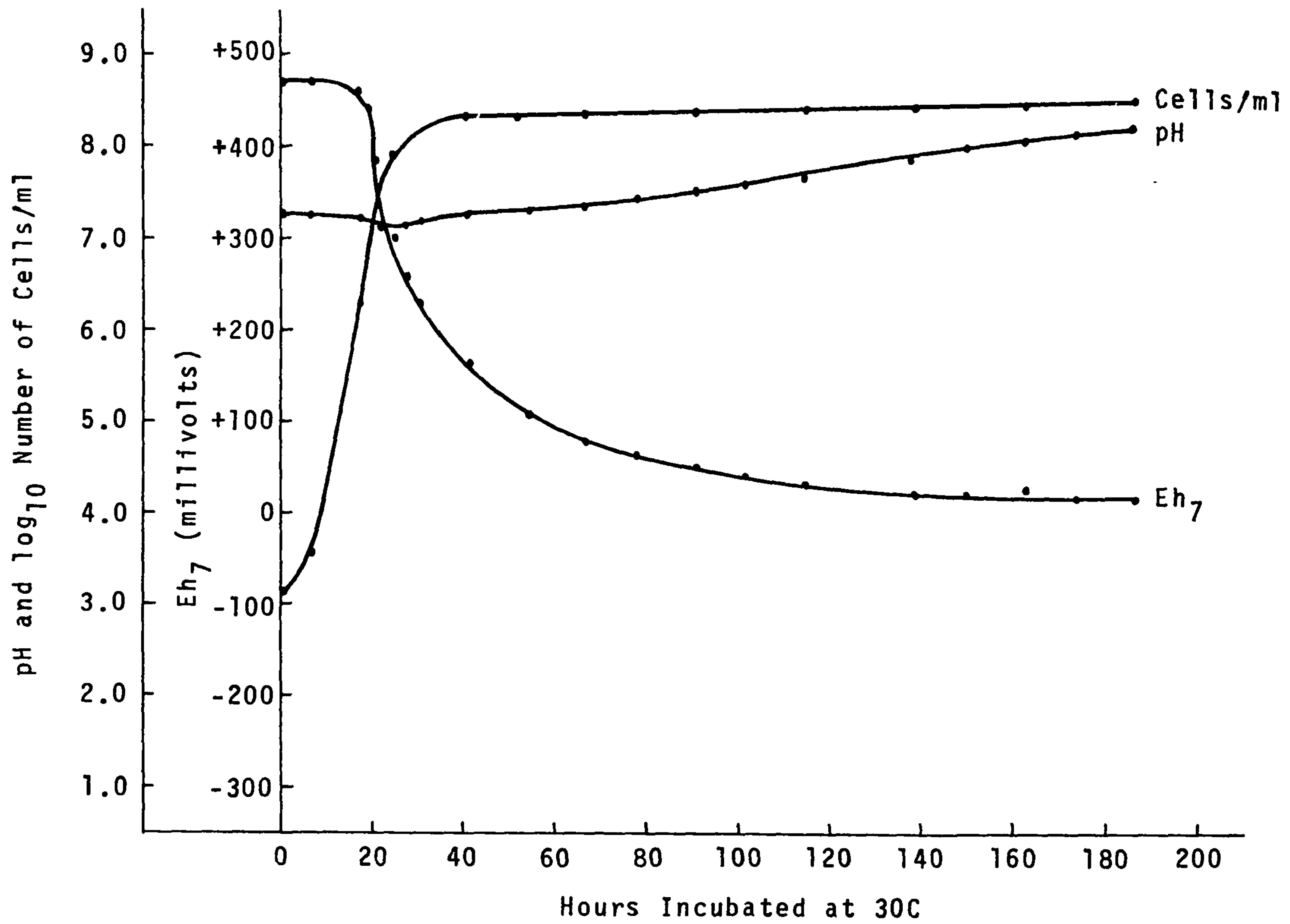



Figure 20. Growth, $Eh_7$, and pH curves of <u>Pseudomonas fluorescens</u> 2 in TSB at 30C

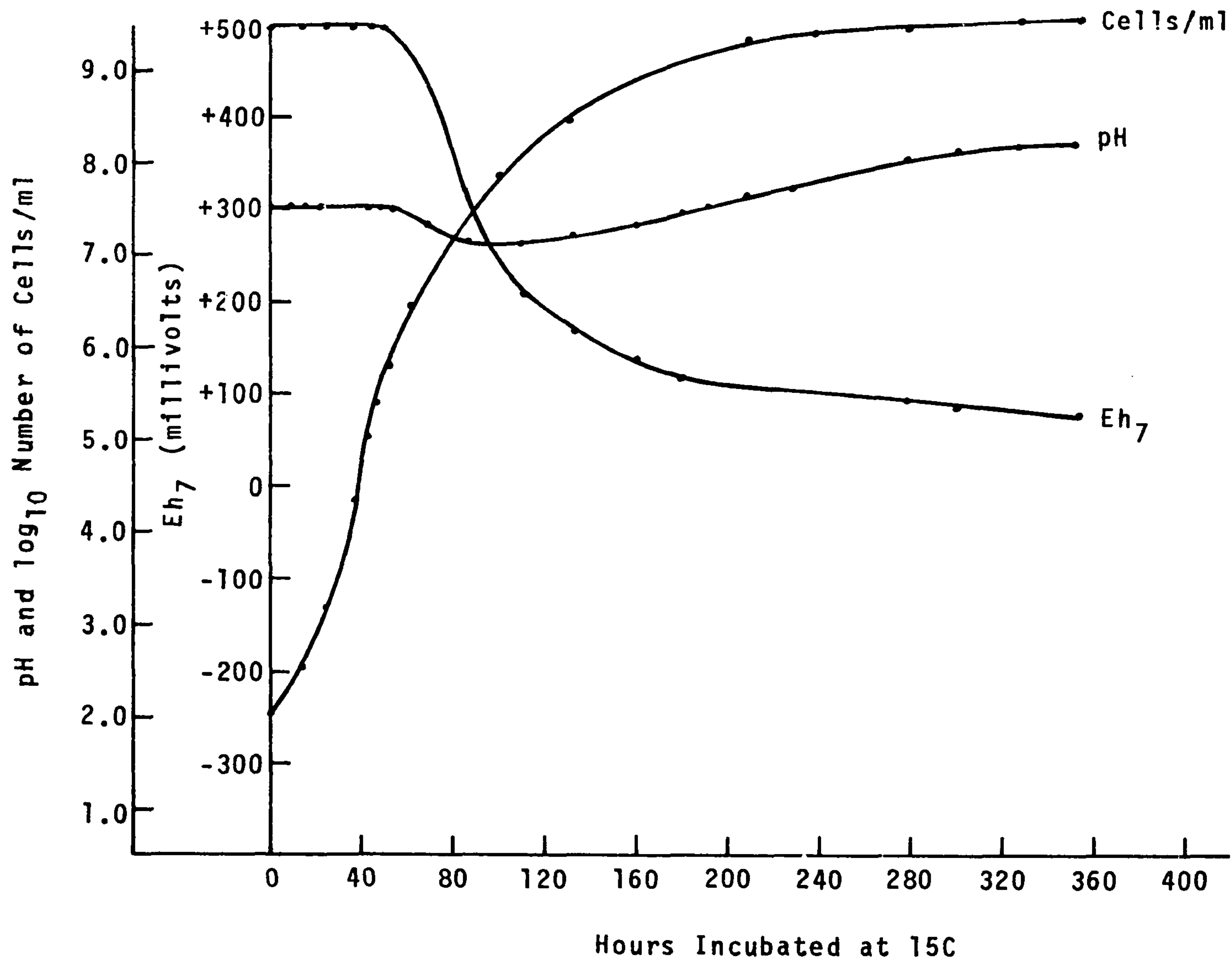



Figure 21. Growth, $Eh_7$, and pH curves of <u>Pseudomonas</u> <u>fluorescens</u> 2 in TSB at 15C

after 353 hr of incubation. The pH fell from its initial value of 7.50 to a minimum value of 7.10 at 96 hr and then grew progressively more alkaline, attaining a final value of 8.22 after 352 hr. As was noted with strain F17, cell numbers steadily increased over the entire experimental period from an initial concentration of $1.0 \times 10^2$ cells/ml to a final level of $3.6 \times 10^9$ cells/ml at 356 hr.

## Effects of Cultural Conditions on Experimental Parameters

### Non-aerated cultures

Experiments also were conducted where there was no aeration of the culture vessel with compressed air. In experiments of this type, the vessels were assembled and treated as if the cultures were to be aerated, but the air supply was not used. It has been reported by Williams and Sullivan (1942) and Clark (1960) that there is a layering of Eh that affects the accuracy of the measurements that are recorded. As a consequence then, it may be expected that the highest Eh observations would be those made at the surface of a given medium.

Absence of added aeration had little effect on early trends of Eh, pH, and growth curves of the six *Salmonella* spp. and three *P. fluorescens* strains studied. Data will be presented that are typical of the results obtained with the various experimental organisms; the first set of data are from

experiments with S. heidelberg; the second set of data presented are from studies with P. fluorescens F21; and the third set of data are from experiments with P. fluorescens F17. Results obtained from experiments of this type indicated that layering of Eh was not a significant problem since measurements were made on the unagitated cultures and then compared with readings taken after slight swirling of the cultures. Measurements, i.e., readings in mv, taken between unagitated and swirled cultures varied in the range of $\pm$ 5 mv.

Figure 22 shows data obtained with S. heidelberg at 15C in TSB. The characteristic recovery of both Eh and pH to higher values was greatly retarded in the absence of aeration. The Eh increased from a low of -385 mv at 80 hr to a final Eh of -35 mv after 418 hr. Maximum cell concentration was $1.0 \times 10^9$ cells/ml at 120 hr, and this concentration slowly decreased to a final figure of $3.2 \times 10^8$ cells/ml at the end of the experiment. The pH increased from its lowest value of 5.80 at 92 hr to a final value of 6.45 at 418 hr. Growth and subsequent changes in Eh and pH at 15C occurred in much less time than did similar changes in pure culture at 15C under aeration (see Figure 4). It may very well be that the Salmonella spp. studied preferred somewhat reduced oxygen tensions for the initiation of growth. More will be said in support of this hypothesis when data from

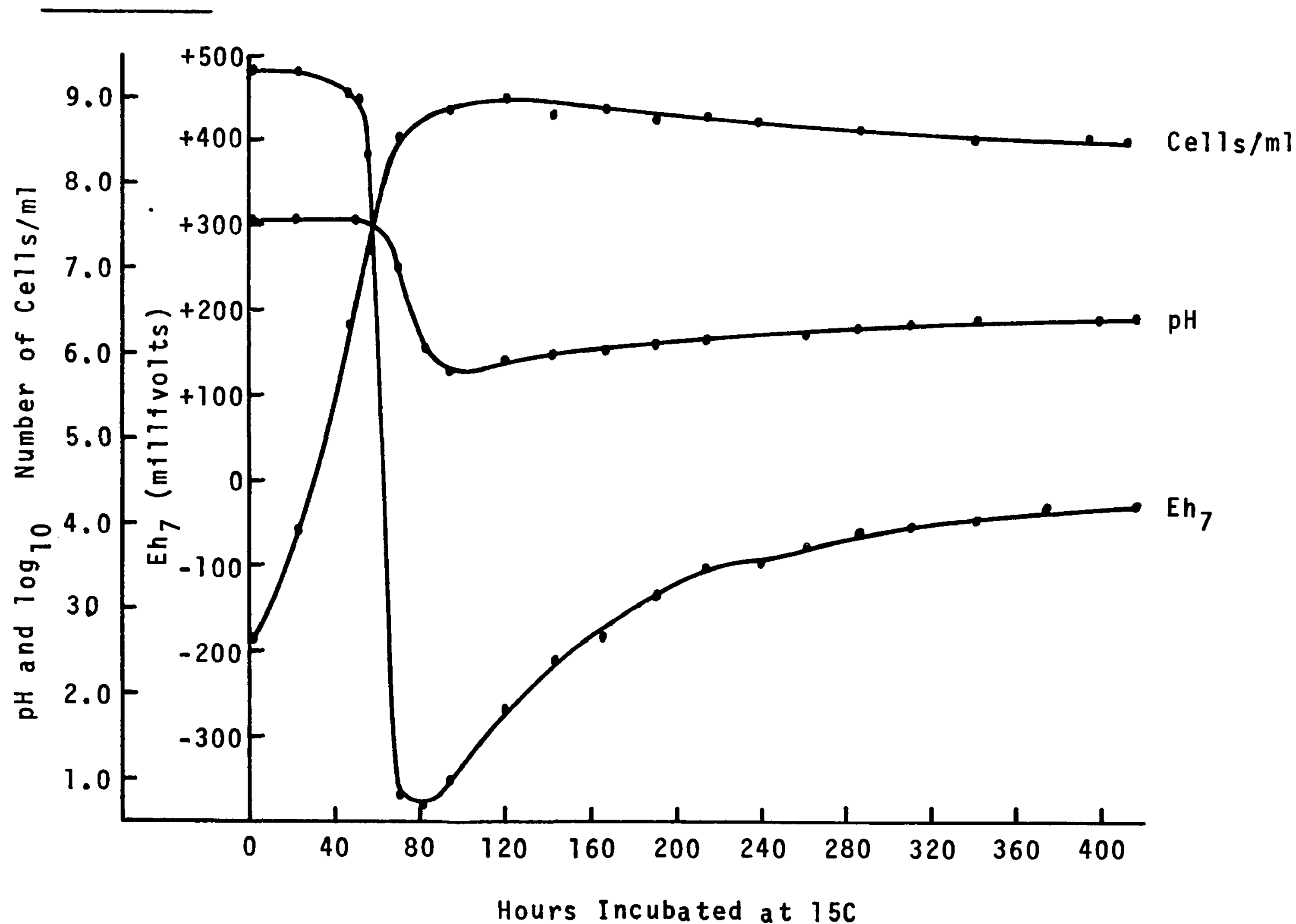



Figure 22. Growth, $Eh_7$, and pH curves of <u>Salmonella heidelberg</u> in TSB at 15C with no aeration

mixed cultures of Salmonella spp. and Pseudomonas fluorescens are presented later in the discussion.

Figure 23 shows results obtained when P. fluorescens F21 was grown at 15C with no aeration of the medium. The patterns of Eh, pH, and growth are markedly different from patterns observed with aeration at this temperature (see Figure 17). General trends were perhaps the least variable for the Eh curves. With no aeration, the Eh gradually became more negative throughout the experiment and, at the conclusion of the study, had a value of -105 mv, considerably less than the final Eh of the aerated culture (+110 mv). In the absence of the oxygen in the compressed air, fewer components are present in the non-aerated medium that have the capacity to oxidize the by-products of cellular metabolism; therefore, more negative Eh values occur. The oxygen that is dissolved in the medium is quickly used up completely, and other, less efficient compounds must be used for such oxidations (Hewitt, 1950). The pH in these experiments became more acidic throughout the experimental run, with a final value of 6.90 at 350 hr. Cell numbers increased to a level of $2.9 \times 10^8$ cells/ml after about 80 hr at 15C, but fell during the rest of the experiment to a final concentration of $2.5 \times 10^7$ cells/ml at the end of the experiment. Also, cell lysis, evident upon examination of the culture microscopically, may account for part of the low Eh and pH values that

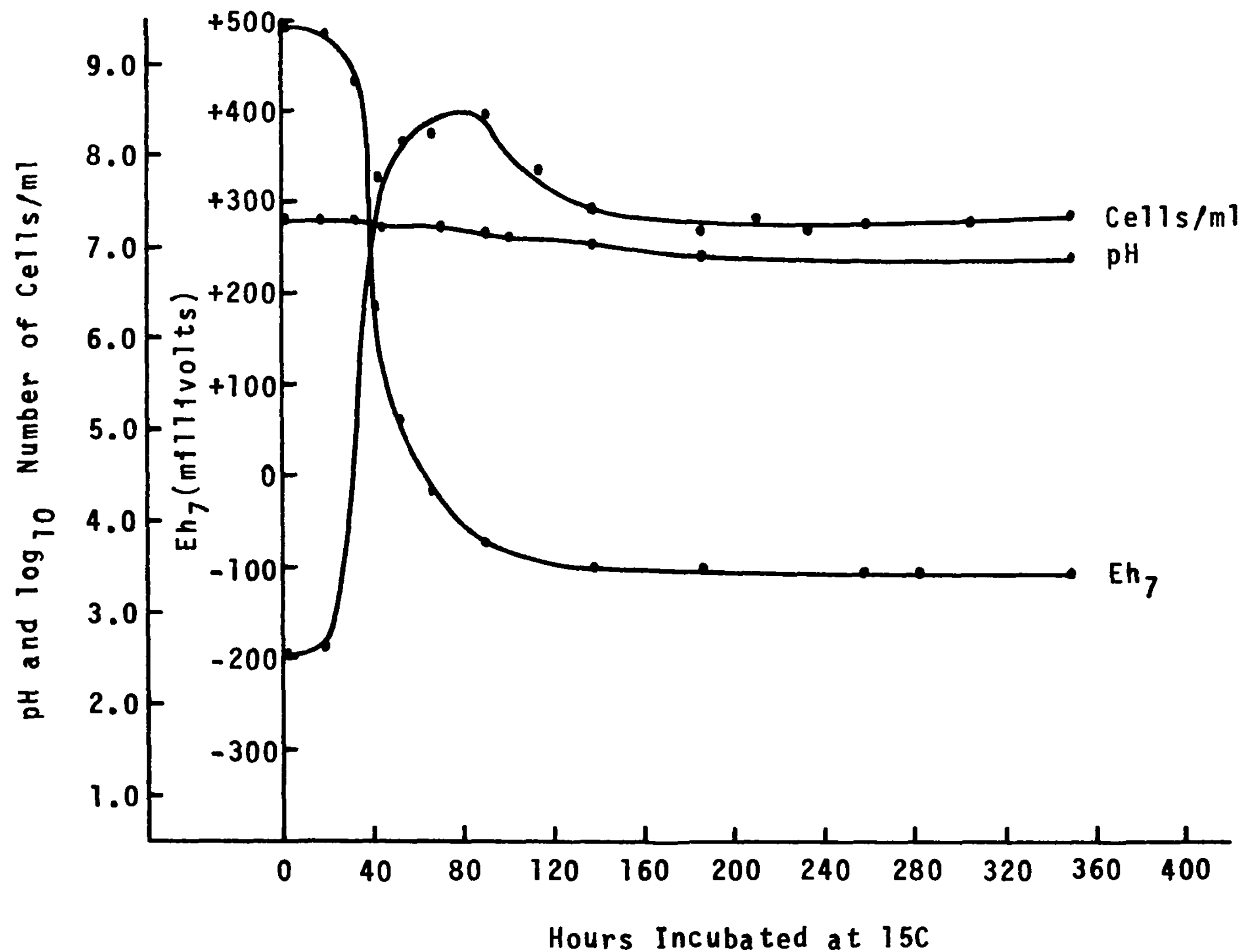



Figure 23. Growth, $Eh_7$, and pH curves of <u>Pseudomonas fluorescens</u> F21 in TSB at 15C with no aeration

were manifested. This lysis could have resulted in the elaboration of reductive substances from the pseudomonads. It is also evident that the highly aerobic metabolism of the *Pseudomonas* strain was not operating with any great degree of efficiency. In any event, it is obvious that the pseudomonad did not survive as well under non-aerated conditions as under aerated conditions.

Curves for *P. fluorescens* F17 at 15C with no added aeration are presented in Figure 24. The patterns here suggest that survival of this organism was better than observed with F21 under similar environmental conditions. Minimal and final Eh in this instance was +10 mv compared with a minimal and final Eh of +35 mv under aerated conditions at 15C. As was the case with F21, the pH values for the cultures of F17 gradually became more acidic throughout the experiment, having a final value of 6.95 after 356 hr. As mentioned, cell numbers increased from an initial inoculum of $6.4 \times 10^3$ cells/ml to a final and maximal concentration of $1.0 \times 10^8$ cells/ml at the end of the experiment. Lack of marked decrease in numbers may have been due, in part, to the use of a somewhat higher inoculum than in other experiments where inocula were in the range of $10^2$ to $10^3$ cells/ml.

## Use of prepurified nitrogen ($PPN_2$)

Experiments were performed using prepurified nitrogen ($PPN_2$) as a means of lowering the initial Eh of sterile media

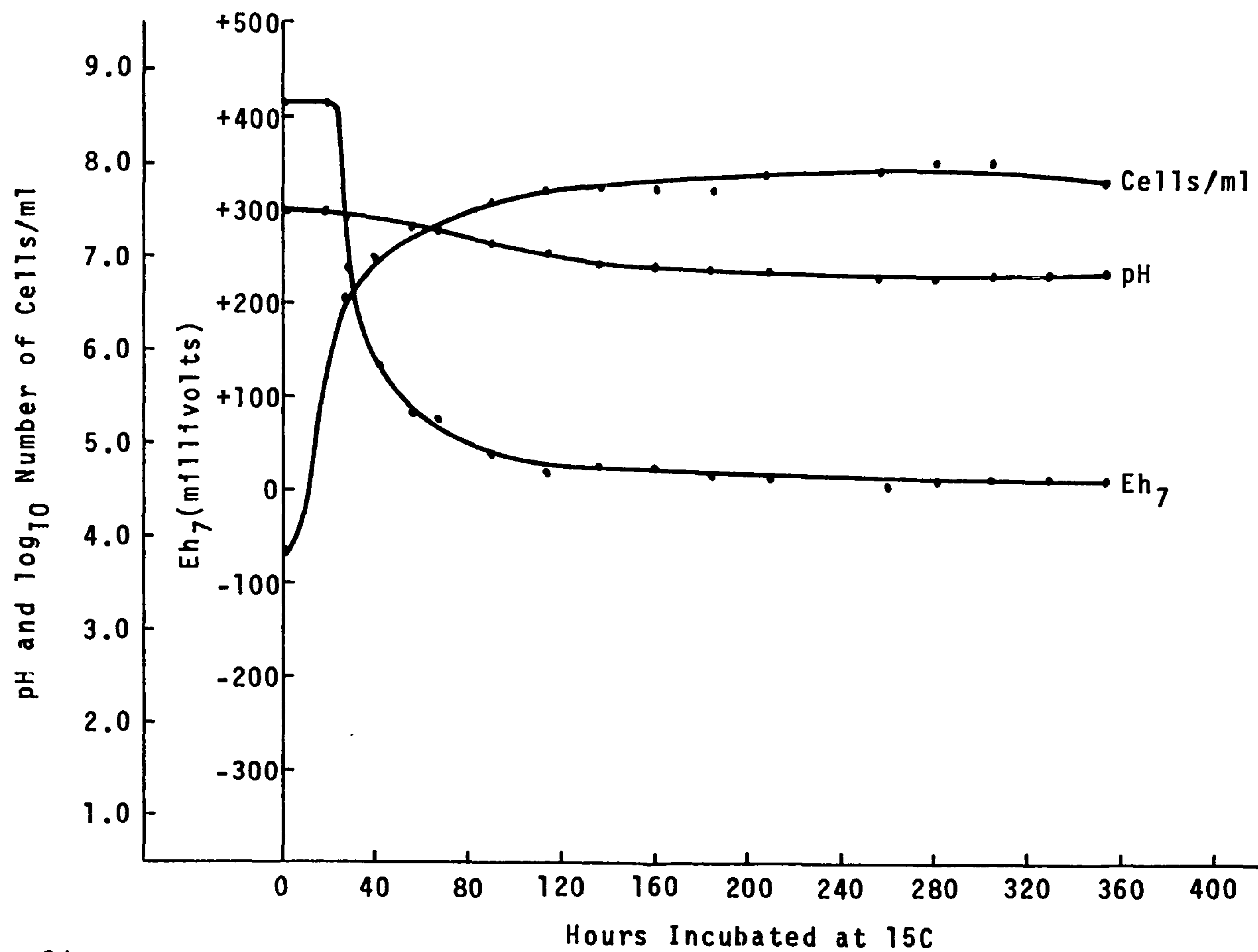



Figure 24. Growth, $Eh_7$, and pH curves of <u>Pseudomonas fluorescens</u> F17 in TSB at 15C with no aeration

and then observing the presence or absence of growth by various organisms after inoculation. Results were compared with those obtained from experiments in which compressed air was used. Tests of this type were designed to determine whether or not there were limiting Eh values below which proliferation of a given organism would not take place, or at least would be greatly hindered. Various levels of Eh could be obtained by varying the flow rate of $PPN_2$ into the cultures. Representative results for a few of these experiments are presented in the following three figures.

As shown in Figure 25, at an initial Eh of +180 mv, _P. fluorescens_ F21 was able to initiate growth and, indeed, reach a population of 1.0 x $10^8$ cells/ml after 354 hr at 15C. This level of initial Eh was obtained by bubbling 65 ml/min/vessel through the cultures continuously. Only slight changes in the Eh of the cultures were observed throughout the experiment. The pH of the cultures began to fall after 236 hr and continued its decline to a final value of 6.77.

Figure 26 illustrates data obtained with the same organism at the same temperature at a slightly lower initial Eh of +158 mv and with only early application of $PPN_2$. A flow rate of 75 ml/min/vessel was needed to attain this level of Eh. Growth proceeded very slowly at this level of Eh, but when the $PPN_2$ supply was shut off after 90 hr of incubation, there was a sharp increase in Eh values up to a level of +320 mv,

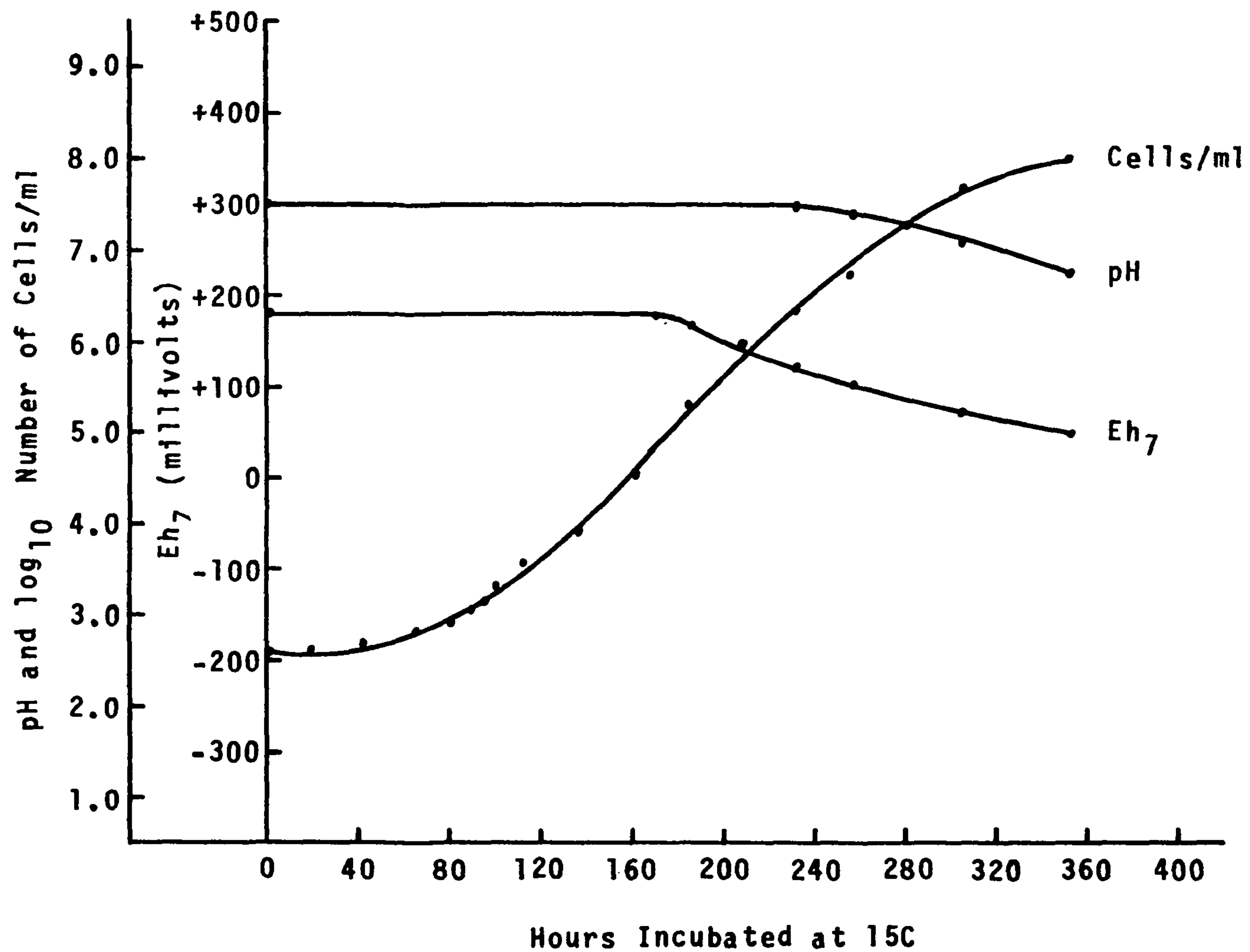



Figure 25. Growth, $Eh_7$, and pH curves of Pseudomonas fluorescens F21 in TSB at 15C in the presence of prepurified nitrogen ($PPN_2$, 65 ml/min/vessel)

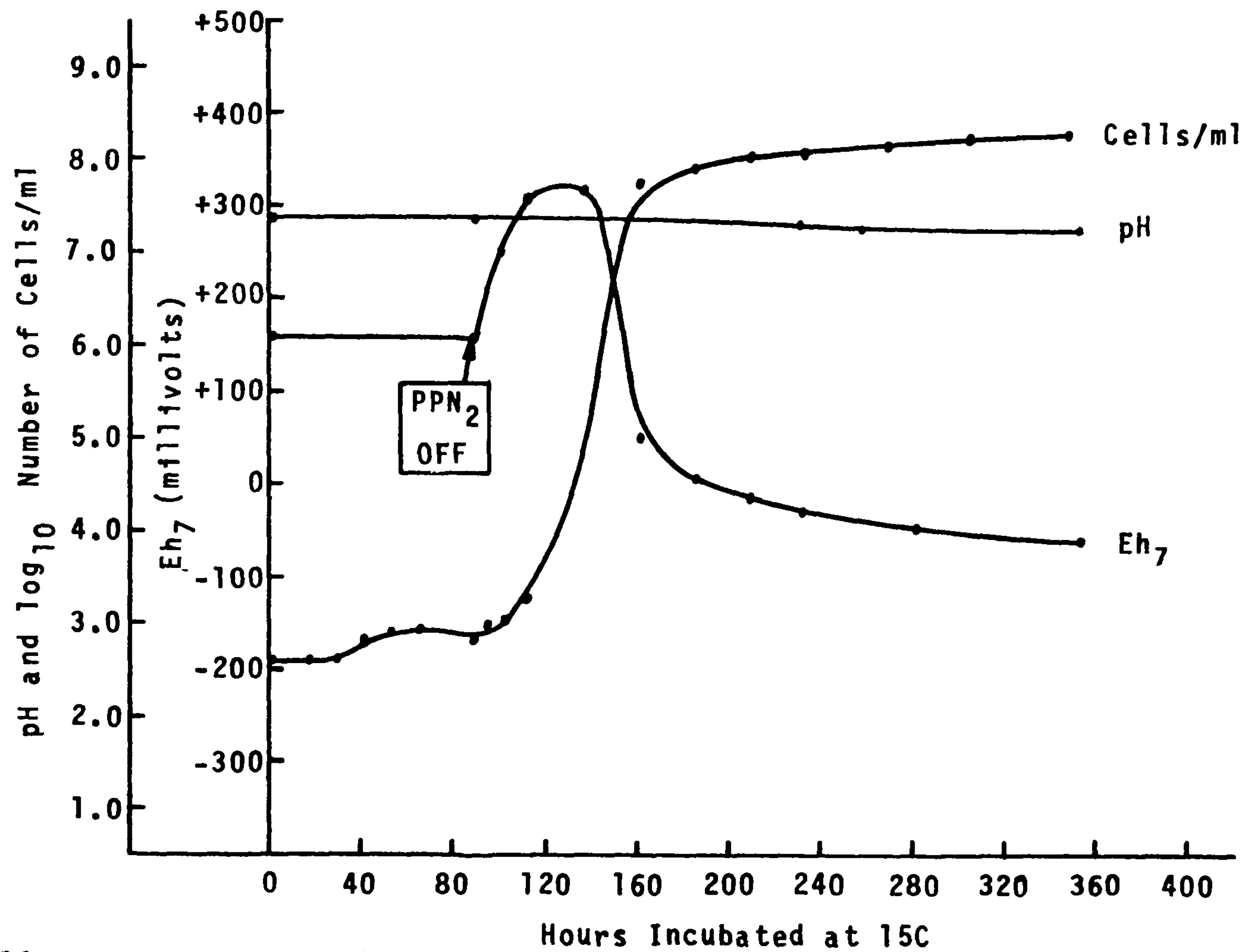



Figure 26. Growth, $Eh_7$, and pH curves of Pseudomonas fluorescens F21 in TSB at 15C in the presence of $PPN_2$ (75 ml/min/vessel) for first 90 hr of incubation

during which time the pseudomonad began to actively multiply, thereby causing the reducing trend that was evident after 140 hr. From this point, Eh, pH, and growth curves were very similar to curves obtained in the non-aerated pure cultures at the same temperature.

Results obtained with P. fluorescens F21 at an initial Eh of +132 mv and 30C are given in Figure 27. A flow rate of 85 ml/min/vessel was used to achieve this level of Eh. As in previous experiments of this type, proliferation of F21 was very slow. Cell numbers increased from an initial concentration of $2.1 \times 10^{2}$ cells/ml to a final concentration of $4.0 \times 10^{5}$ cells/ml after 205 hr of incubation at this temperature. There was only a slight change in pH throughout the study, i.e., initial pH was 7.27; final pH was 7.23. The Eh also changed slightly, from an initial value of +132 mv to a final value of +85 mv.

Similar results were obtained when $PPN_2$ was used to artificially lower the initial Eh of the medium with the other organisms used in the overall study. Under the environmental conditions that were used in these studies, P. fluorescens strains did not grow at Eh levels below +80 mv; Salmonella spp. used in this work were not able to grow at Eh levels lower than +30 mv, although one strain, S. typhimurium, was able to withstand this level of initial Eh and was able to proliferate once the $PPN_2$ supply was exhausted and Eh

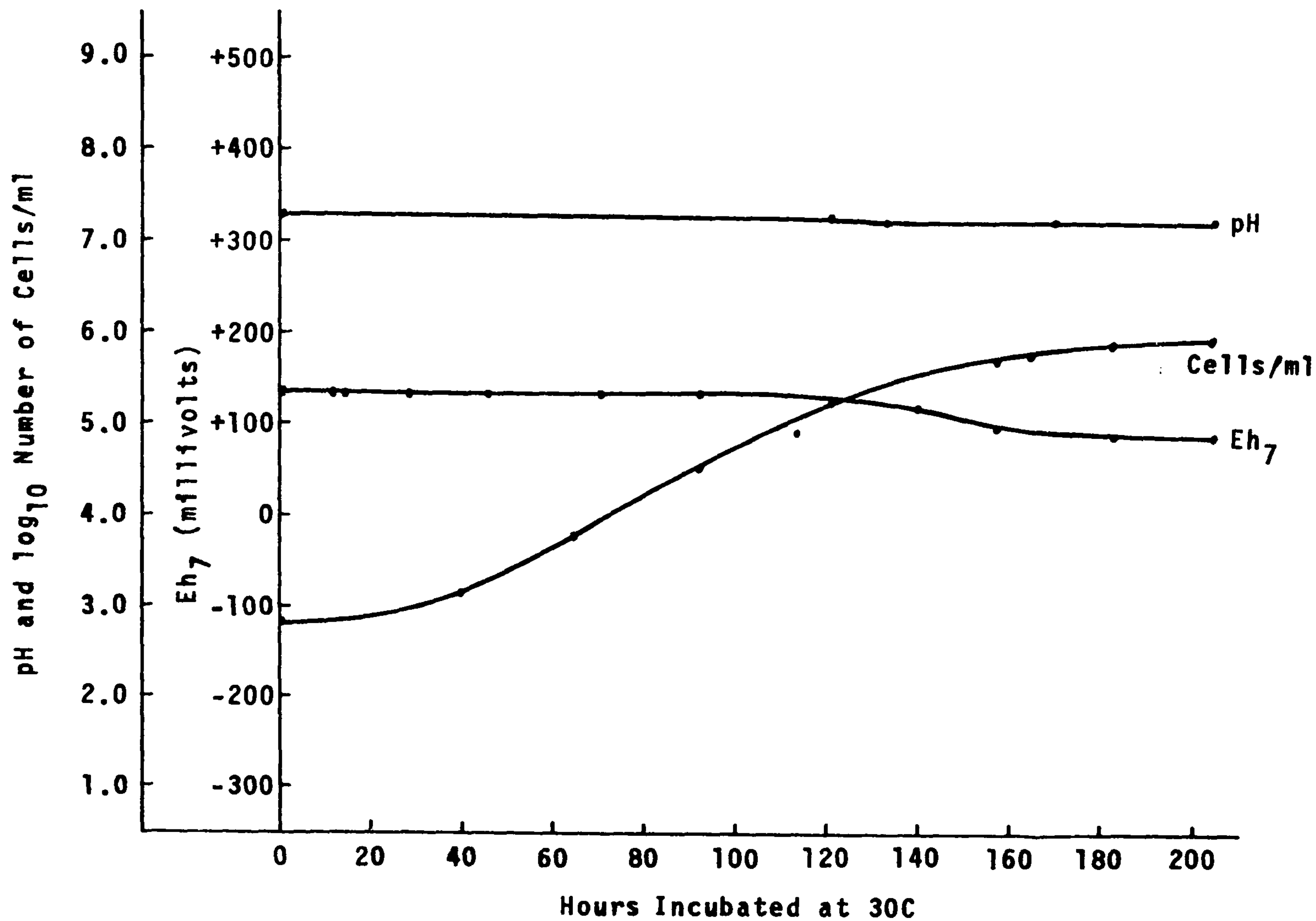



Figure 27. Growth, $Eh_7$, and pH curves of *Pseudomonas fluorescens* F21 in TSB at 30C in the presence of $PPN_2$ (85 ml/min/vessel)

values drifted upward.

By using $PPN_2$, it is possible to lower the initial Eh of the culture medium without having to be concerned about its interference in any of the metabolic processes of the organisms involved as well as interaction with the constituents of the medium. The use of chemicals that act as artificial reducing agents, thereby lowering the Eh, most certainly have drawbacks as to such possible interactions. These have been discussed earlier in the text.

Through the use of $PPN_2$ to remove most of the oxygen present in the cultivation medium, it was demonstrated that *P. fluorescens* F21 has the capacity to grow under conditions where there is very little oxygen present. Similar data to this effect have been reported by Tabatabai and Walker (1970). One might not have expected this capacity from such an aerobe.

### Mixed Culture Data -- Selected *Salmonella* spp. and *Pseudomonas fluorescens*

Limited investigations involving mixed cultures of *Salmonella* spp. and *P. fluorescens* F21 also were conducted. These experiments were undertaken in an effort to detect any inhibition or interaction of these organisms. It has been reported by other workers and mentioned previously in this discussion that the use of redox potential measurements, along

with monitoring of pH and cell growth, may have some application to work in the area of microbial antagonisms. Borromeo (unpublished thesis, 1969) and Tabatabai and Walker (1970) have reported on the use of such techniques in conjunction with assaying for inhibitory activity by other organisms against C. perfringens, among which were Streptococcus faecalis, Lactobacillus plantarum, and P. fluorescens. Oblinger and Kraft (1970) reported on the inhibitory activity of certain Pseudomonas species against Salmonella spp. and other poultry isolates. As a result of this work, Oblinger (unpublished thesis, 1970) undertook investigation of the use of redox methods as a means of detecting interactions among bacteria. The limited studies reported herein represent these endeavors and an attempt to aid in the understanding of such relationships.

Figure 28 shows data obtained when the initial ratio of S. thompson to P. fluorescens F21 was 1:1. Experimental temperature was 30C. Although the temperature was not at the optimal level, it is evident that the Salmonella were affected very little by the presence of the pseudomonad. In fact, the Eh and pH trends that were recorded are essentially that of a pure culture of S. thompson as shown in Figure 14, where the experimental temperature was 37C. One can see the very intense reduction of the medium down to a minimal value of close to -400 mv and a slow, extended return to more

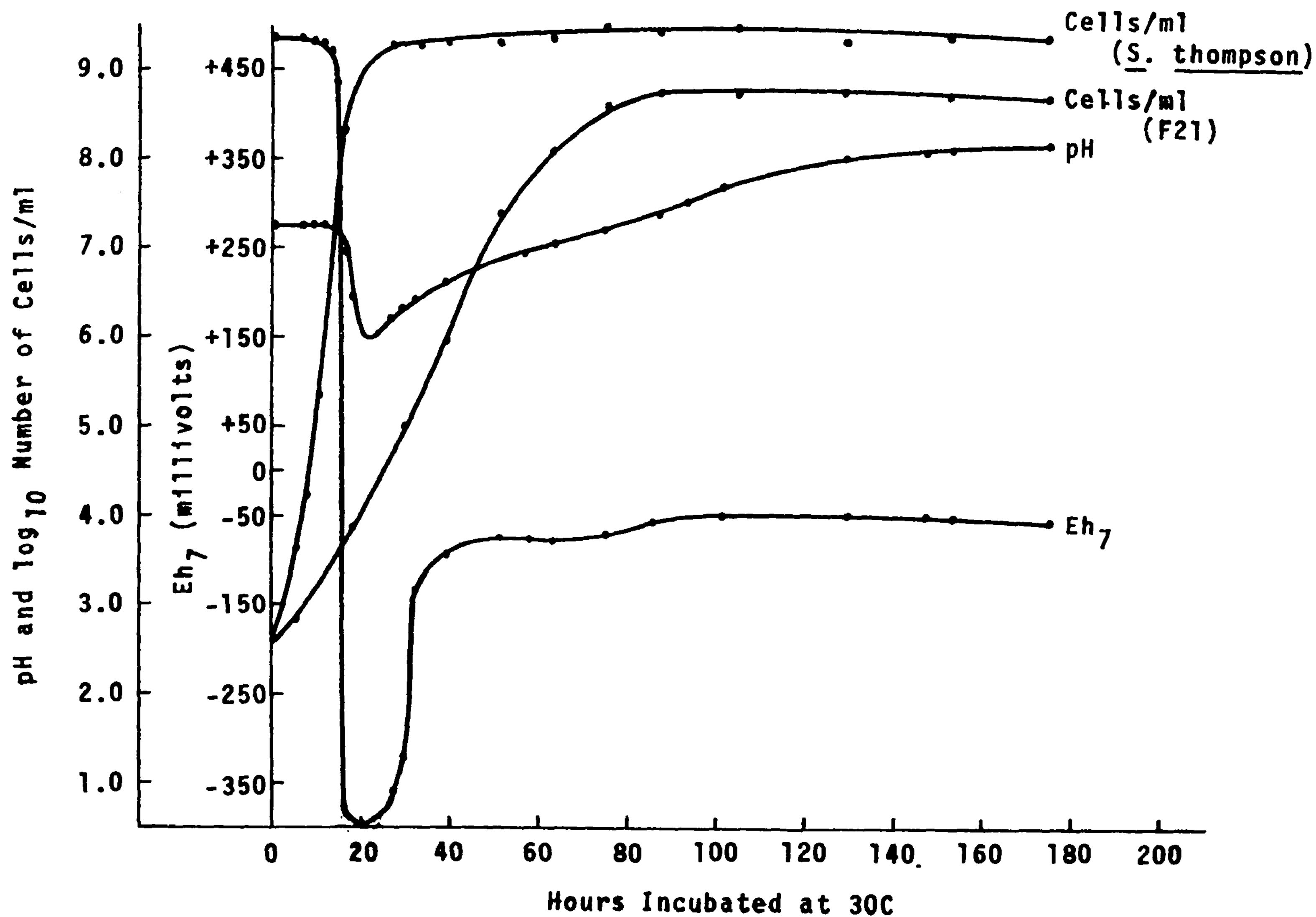



Figure 28. Growth, $Eh_7$, and pH curves of Salmonella thompson and Pseudomonas fluorescens F21 in TSB at 30C at an initial ratio of 1:1

positive values in observed potential. Finally, a leveling off of Eh to a value of -53 mv occurred at 176 hr. The pH values that were recorded were also quite similar to those recorded for the pure culture at 37C, a minimum value of 6.00 at 21 hr followed by a gradual alkaline drift up to a final value of 8.10 after 176 hr. Cell numbers increased as usual and attained a maximal population of approximately $1.8 \times 10^9$ cells/ml after 23 hr with no appreciable decrease in numbers for the duration of the experiment.

On the other hand, there was some form of inhibition of the *P. fluorescens* F21 during the early hours of incubation. If one compares the data obtained in pure cultures of this organism (see Figure 16), it can be seen that, in the case of the mixed culture (Figure 28) , the lag phase was extended a bit and entry into log phase was slower. However, maximal cell populations were nearly identical after approximately 80 hr of incubation at 30C. Neither organism exhibited any decrease in cell numbers over the rest of the experimental period in either pure or mixed cultures under the conditions described.

Figure 29 shows results that were obtained when F21 initially outnumbered *S. thompson* by a ratio of 100:1. Once again, these experiments were conducted at 30C. The Eh and pH curves presented are essentially those of the pure culture of S. *thompson*. The Eh fell to characteristically low levels

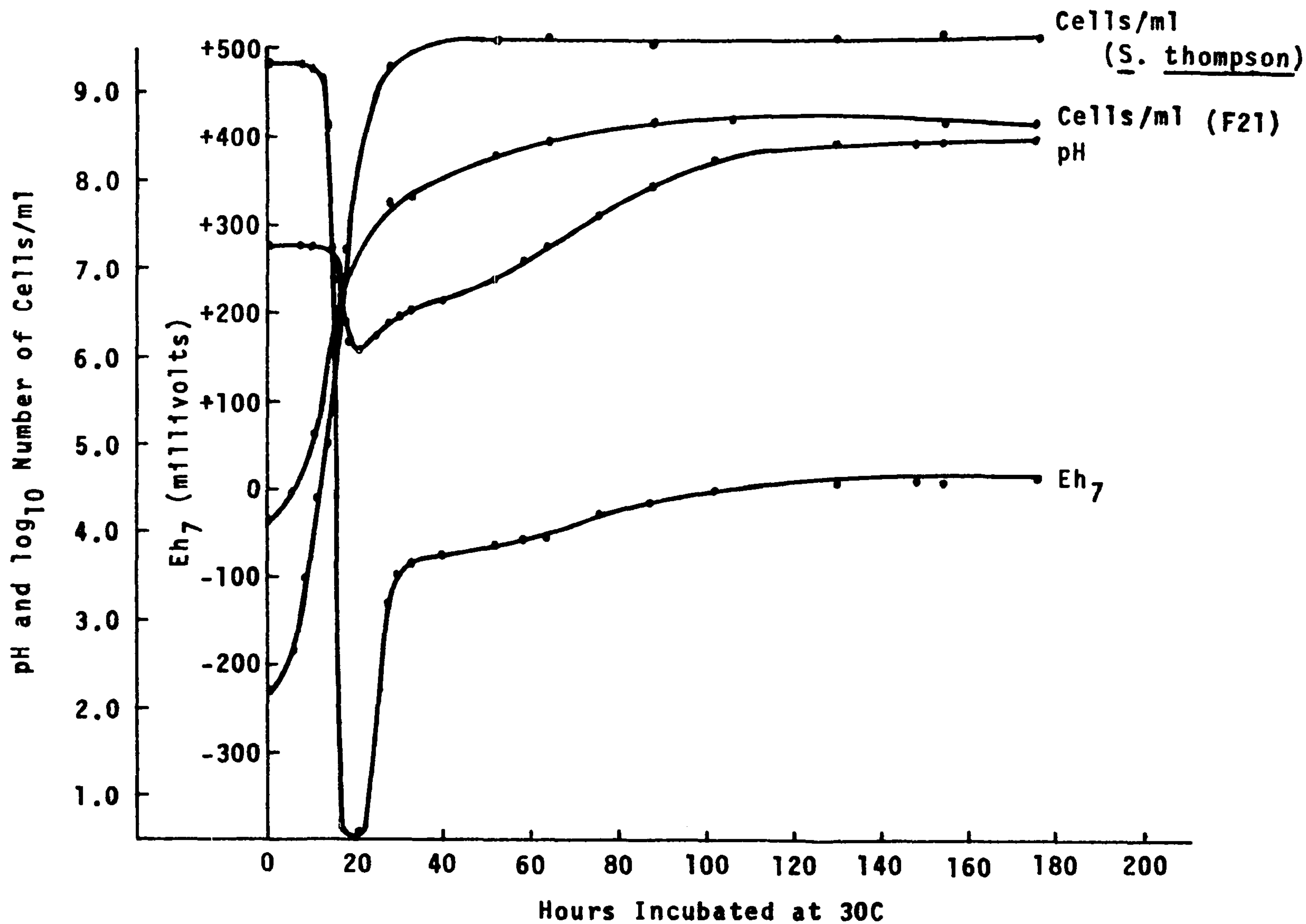


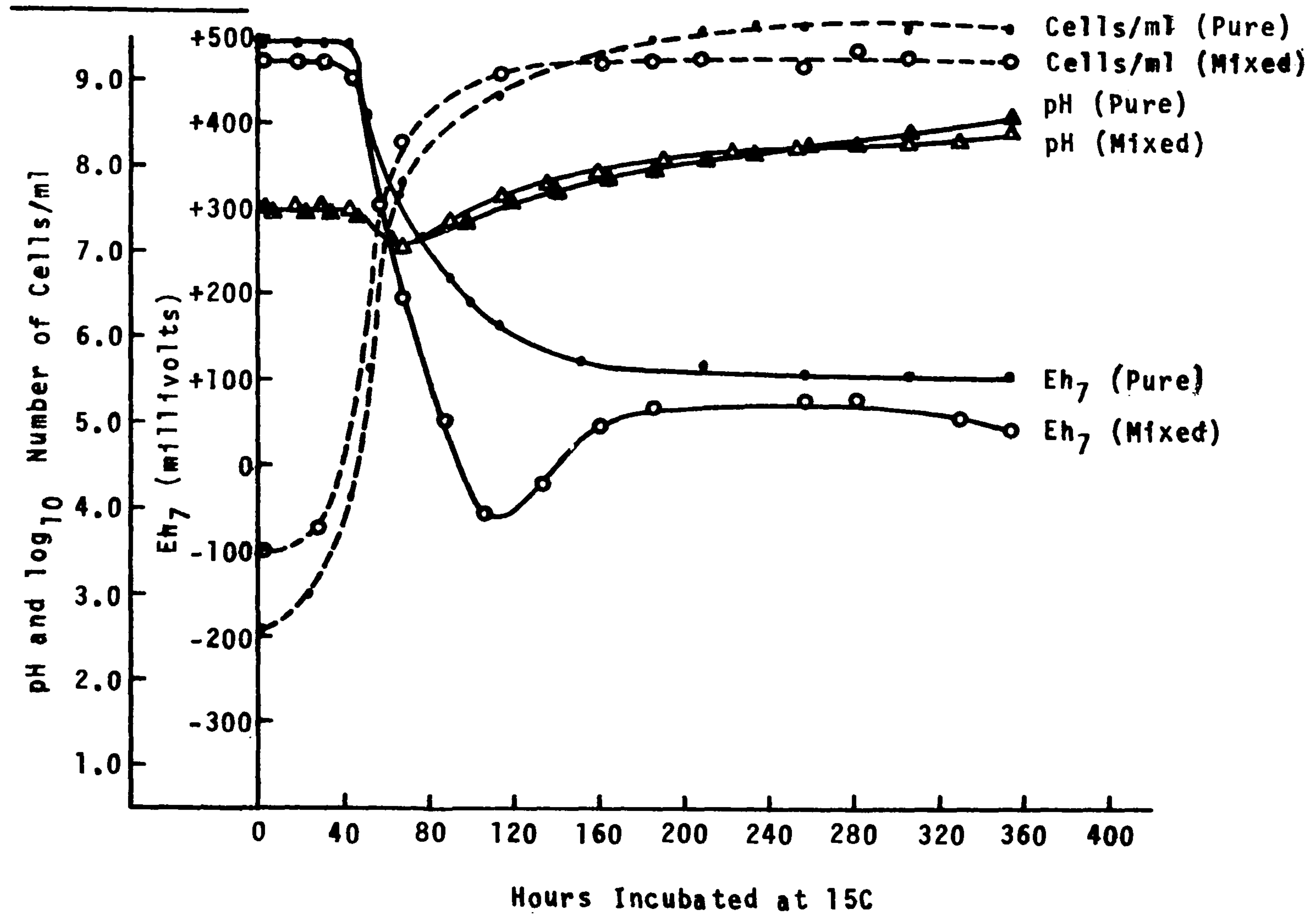

Figure 29. Growth, $Eh_7$, and pH curves of _Salmonella thompson_ and _Pseudomonas fluorescens_ F21 in TSB at 30C at an initial ratio of 1:100, respectively

approaching -400 mv at 20 hr, followed by the typical return to higher values, leveling off to a final value of 0 mv. The time that the Eh was at such low levels was not as long as was observed either for pure cultures or mixed cultures at a ratio of 1:1. The pH values that were noted varied only slightly during the first few hours of incubation, but eventually reached more alkaline values those of the 1:1 mixed cultures, attaining a final pH value of 8.50. Concentration of Salmonella became well above $10^9$ cells/ml and was maintained at this level for the duration of the experiment. Pseudomonas numbers and progression through the growth phase reflected the increased inoculum used. Maximal numbers were reached quicker but did not exceed counts recorded in either pure or mixed (1:1) cultures, attaining a final concentration of $5.6 \times 10^8$ cells/ml after 176 hr. It is possible that the higher final pH values that were recorded may be attributed to the effect of the Pseudomonas.

As a result of these and other mixed culture studies conducted at 30C, it was decided to conduct further investigations of this type at a lower temperature, i.e., 15C, in order to lessen any advantage that the Salmonella spp. may have had at the higher temperature.

Figures 30 and 31 present data obtained from experiments conducted at 15C where P. fluorescens F21 initially outnumbered S. typhimurium by a ratio of 30:1. Figure 30 shows

Figure 30. Effect of *Salmonella typhimurium* on changes in growth, $Eh_7$, and pH of *Pseudomonas fluorescens* F21 in TSB at 15C at an initial ratio of 1:30, respectively



Cells/ml (Pure)
Cells/ml (Mixed)
pH (Pure)
pH (Mixed)
Eh7 (Pure)
Eh7 (Mixed)
pH and log10 Number of Cells/ml
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Eh7 (millivolts)
+500
+400
+300
+200
+100
0
-100
-200
-300
0
40
80
120
160
200
240
280
320
360
400
Hours Incubated at 15C

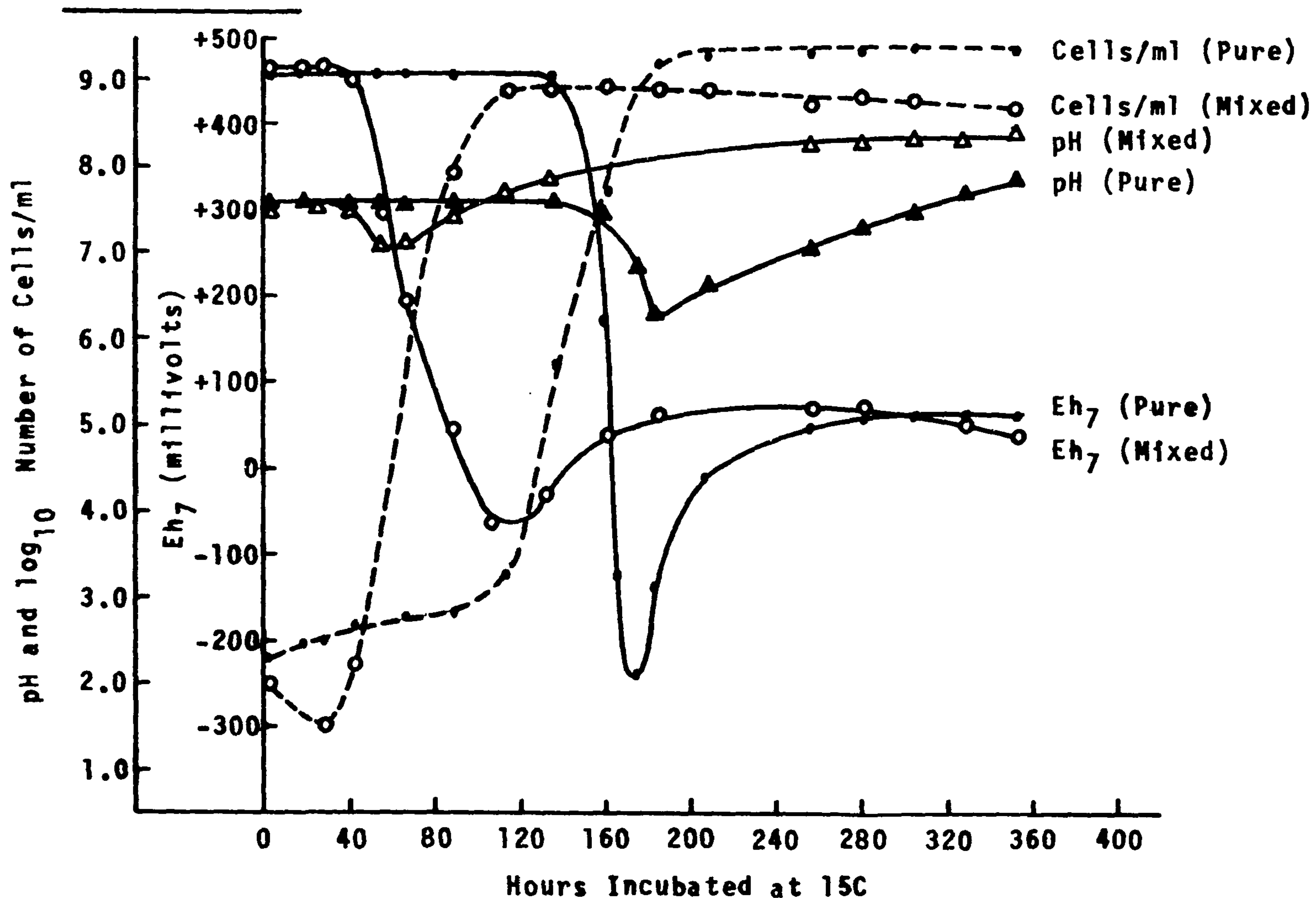



Figure 31. Effect of *Pseudomonas fluorescens* F21 on changes in growth, $Eh_7$, and pH of *Salmonella typhimurium* in TSB at 15C at an initial ratio of 30:1, respectively

the effect of *S. typhimurium* on changes in Eh, pH, and growth of *P. fluorescens* F21 at 15C. There was very little modification of the pH pattern observed in mixed as compared with pure culture, indicating that the growth of the *Salmonella* had little or no effect on the pH that was manifested. The growth of the pseudomonad in mixed culture was somewhat less than that in pure culture even though these bacteria entered both logarithmic and stationary phases more rapidly in mixed cultures. Maximal population in pure culture was $4.4 \times 10^9$ cells/ml vs. a maximal population of $2.0 \times 10^9$ cells/ml in mixed culture. Perhaps the effect of the *Salmonella* on the *Pseudomonas* was most pronounced on changes in Eh. For the mixed culture, there was a more intense reduction down to a level of about -60 mv at 112 hr and then a gradual rise in Eh values to a final Eh of +45 mv.

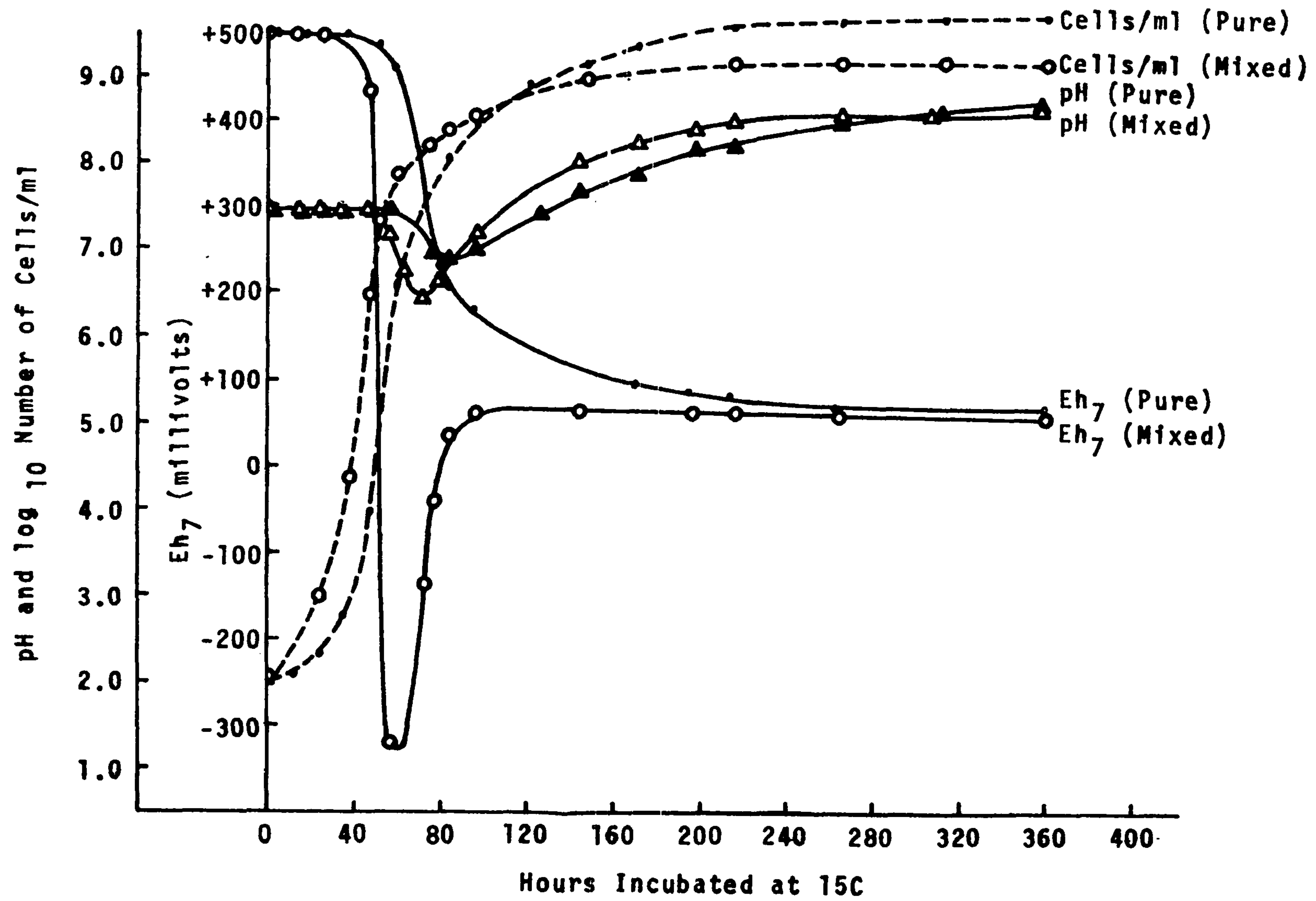
Figure 31 shows the effect of the pseudomonad on *S. typhimurium* in regard to changes in Eh, pH, and growth. In this instance, the influence of the pseudomonad is evident more graphically, especially for observed pH values. The curve for pH in these mixed cultures was essentially that of the pure culture of the *Pseudomonas*. As for the Eh observed, the reducing activity in the cultures began at approximately the time that the pseudomonad would have begun its reduction, approximately 140 hr before the negative drift of

the pure Salmonella cultures. In mixed cultures, the reducing capacity of the Salmonella was not as great as that in pure culture, yet the recovery of Eh towards the end of the experiment to a value of +63 mv is very near the final Eh value of +42 mv observed in pure culture.

The final two illustrations to be presented are concerned with the Eh, pH, and growth patterns of S. typhimurium and P. fluorescens F21 when the Salmonella initially outnumbered the Pseudomonas by a ratio of 100:1.

Figure 32 shows results that reflect the effect of the Salmonella on the Eh, pH, and growth of the Pseudomonas strain. The Eh and pH patterns that developed in mixed culture are quite similar to those of a pure culture of S. typhimurium. The Eh pattern that developed showed the usual characteristic intense reduction of the medium. In this case a minimum Eh of -315 mv was reached after 60 hr of incubation, and then more positive potentials were recorded with a final Eh of +70 mv after 358 hr. This Eh value is approximately the same as the final Eh of the pure cultures. Values of pH observed were more acidic, no doubt due to the influence of the Salmonella. A minimum pH value of 6.48 was reached at 72 hr; followed by a return to more alkaline values and a final value of 8.67. Pseudomonas entered the log phase sooner in mixed cultures than in pure cultures, but did not attain as high a concentration of cells in mixed cultures.

Figure 32. Effect of *Salmonella typhimurium* on changes in growth, $Eh_7$, and pH of *Pseudomonas fluorescens* F21 in TSB at 15C at an initial ratio of 100:1, respectively



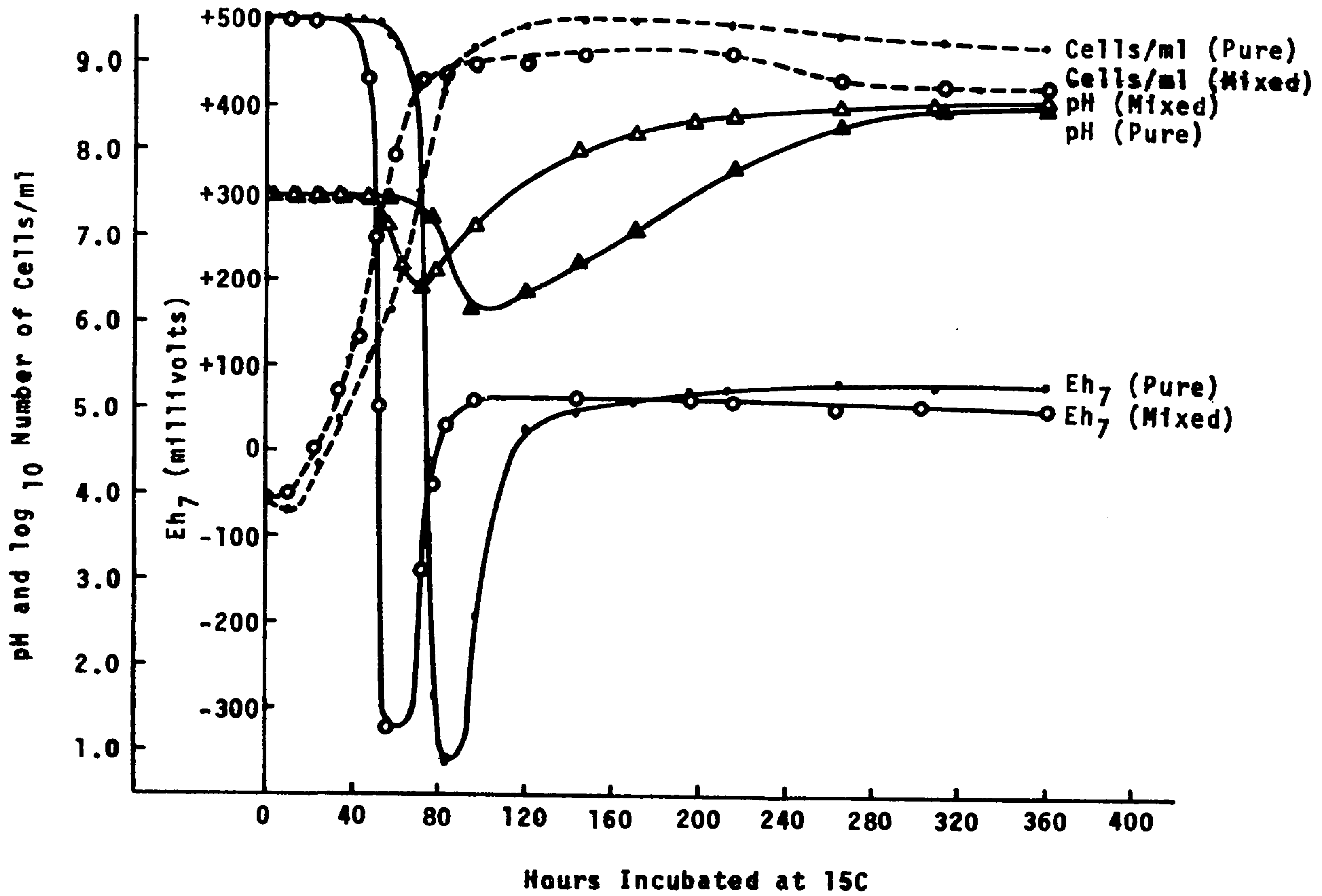
pH and log 10 Number of Cells/ml
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Eh7 (millivolts)
+500
+400
+300
+200
+100
0
-100
-200
-300
0
40
80
120
160
200
240
280
320
360
400
Hours Incubated at 15C
Cells/ml (Pure)
Cells/ml (Mixed)
pH (Pure)
pH (Mixed)
Eh7 (Pure)
Eh7 (Mixed)

Maximal cell concentration in mixed cultures was 1.0 x $10^9$ cells/ml vs. 3.9 x $10^9$ cells/ml in pure culture.

Figure 33 shows the effect of the pseudomonad on the growth, Eh, and pH trends of the S. typhimurium. The Salmonella entered log phase sooner than in pure culture, as was the case in the other mixed culture investigations reported, although lesser overall viable cells were produced. Rather than reaching populations above $10^9$ cells/ml, as observed for the pure culture, the Salmonella rapidly reached the $10^9$ level but then exhibited a marked decrease in cell numbers as the experiment progressed. The pH trend that was developed in mixed culture was not as acidic as observed for the pure culture, yet, at the same time, not as alkaline as was the pure culture of Pseudomonas. Rather, it might be said that the pH curve for the mixed cultures reflected a mixture of the influence of both organisms. Eh values showed intense reduction but not to the extent as that of the pure culture. The greater influence of the Salmonella on both the Eh and pH curves was probably due to the high inoculum employed which no doubt enabled the Salmonella to adjust more rapidly to the lower than optimum environmental temperature.

One facet of these mixed culture studies was that the Salmonella spp. entered the logarithmic phase of growth much quicker than it did in pure culture. The exact reason for this is not known, although it is entirely possible that

Figure 33. Effect of *Pseudomonas fluorescens* F21 on changes in growth, $Eh_7$, and pH of *Salmonella typhimurium* in TSB at 15C at an initial ratio of 1:100, respectively



Cells/ml (Pure)
Cells/ml (Mixed)
pH (Mixed)
pH (Pure)
Eh7 (Pure)
Eh7 (Mixed)
pH and log 10 Number of Cells/ml
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Eh7 (millivolts)
+500
+400
+300
+200
+100
0
-100
-200
-300
0
40
80
120
160
200
240
280
320
360
400
Hours Incubated at 15C

the pseudomonad, through its metabolic reactions, provided the *Salmonella* with more readily assimilable nutrients. The converse of this statement might also apply to the early entry into log phase by the *Pseudomonas*. It is also conceivable that the highly aerobic metabolism of the *Pseudomonas* strain consumed the available oxygen and thereby encouraged the growth of the *Salmonella*. Results that tend to support this reasoning have been presented in conjunction with work wherein oxygen tensions were lowered by eliminating aeration of *Salmonella* cultures. These experiments showed that under such conditions, *Salmonella* spp. were able to enter into log phase much sooner than with aeration.

As previously mentioned in the Literature Review, it has been suggested that oxidation-reduction potential measurements be considered as a potential aid to the classification of bacteria. It is the considered opinion of this author that such attempts would only add to the confusion and be of only limited value. The complexity of not only setting up appropriate experimental apparatus but also attempting to draw meaningful conclusions from data obtained in such experiments needs considerably more attention. The need for fixed, readily reproducible conditions cannot be overemphasized.

Finally, the author feels the need to mention another facet of this work for which there is little experimental

evidence, yet may be an area in which further experimentation might be particularly desirable.

From the results that have been obtained and reported herein in regard to oxidation-reduction potentials in cultures of *Salmonella* spp., and in view of the data that has been reported for *C. perfringens* (Tabatabai and Walker, 1970), as well as data pertaining to *Staphylococcus aureus* and other food-borne organisms, it is apparent that the pathogens and particularly the organisms important in food poisoning possess the capacity for bringing about a tremendous reduction of substrate, and thereby a pronounced change in Eh, in a very short period of time. This short period of time coincides very closely with the time that it takes to produce food poisoning symptoms in laboratory animals and human volunteers. It is possible that such drastic changes in Eh may in some way affect toxin production and its influence on nerve impulses. This in turn may bring about typical food poisoning symptoms such as diarrhea, nausea, and vomiting. This area is certainly worth further exploration in view of these considerations.

## SUMMARY

Through the use of the apparatus and techniques described, observations on Eh, pH, and growth of *S. heidelberg*, *S. typhimurium*, *S. infantis*, *S. tennessee*, *S. enteritidis*, *S. thompson*, *P. fluorescens* F21, *P. fluorescens* F17, and *P. fluorescens* 2 were readily reproducible. Several generalizations may be made concerning the investigations of these parameters in pure and mixed cultures under various experimental conditions. These conditions ranged from the use of three environmental temperatures (37, 30, and 15C) in the presence or absence of added aeration to the use of prepurified nitrogen to establish lower initial Eh levels. Perhaps the most obvious finding is that the *Salmonella* spp. examined in this study all possessed the capacity to bring about very intense reduction of the medium (TSB) within a very short period of time at 37 and 30C. At 15C, the lag phase was considerably extended but once the organisms had adapted to the somewhat adverse environment, definite reducing conditions were manifested in most cases.

The three strains of *P. fluorescens*, in contrast to the activity of the *Salmonella* spp., demonstrated gradual reducing activity at both 30 and 15C.

In all cases, greater growth occurred at 15C than at either 37 or 30C and the decrease in cell numbers over the experimental period was less at the lowest temperature

(15C).

Sinclair and Stokes (1963) have reported similar data for P. fluorescens and attributed the higher cell yields at the lower temperature (10C vs. 30C) to the increased solubility and, therefore, availability of oxygen.

Similarities in Eh, pH, and growth patterns within genera of Salmonella and Pseudomonas are evident and, at the same time slight but consistent differences are evident between species of Salmonella. This observation is compatible with that made by Burrows and Jordan in 1935. If, in fact, redox potentials are indicative of metabolic pathways and enzymes possessed as has been theorized, data of this type tend to support the close relationship between these organisms.

Minimal Eh and pH values and maximal cell concentrations were very nearly coincidental within the various Salmonella spp. and P. fluorescens strains studied. At temperatures of 37 and 30C, the attainment of a minimal Eh was followed shortly (usually with 1-2 hr) by the attainment of minimal pH values which were, in turn, followed by the attainment of maximal cell numbers. At 15C, the sequence of events was slightly slower. In the case of the Pseudomonas investigations, the Eh and growth curves were mirror images of one another.

From the results of work in which no added aeration of the cultures was used, it is evident that the Salmonella spp.

were favored by the reduced oxygen tension as evidenced by the shortening of lag phases in non-aerated cultures. On the other hand, strains of *P. fluorescens* were markedly hindered in growth and development as shown by the rather poor survival of these organisms in experiments similar to those just described.

From the work that has been done in the area of mixed cultures, it can be seen that fewer cells/ml were attained by both the *Salmonella* and *Pseudomonas* strains in mixed culture as compared with pure cultures at 15C. The *Pseudomonas* may have exerted a stimulatory effect on the *Salmonella* spp. initially as evidenced by the decreased length of the lag phase; but may have also produced an inhibitory effect toward the end of the experiments. Eh curves obtained from mixed cultures show a blending of the Eh trends of both organisms with the exception of the 30C work, where the Eh curves were essentially those of the *Salmonella*. On the other hand, pH values observed in mixed cultures show the influence of the *Pseudomonas* on this parameter.

There are definite values of Eh that determine whether or not a particular organism can initiate growth and(or) maintain its survival. Under the experimental conditions used, this limit was an Eh level of +80 mv (pH 7.00) for the *P. fluorescens* strains studied and +30 mv (pH 7.00) for the *Salmonella* spp. studied.

Differences in poising capacity of various bacteriological media have been demonstrated. These differences in poising capacity in turn affect the oxidation-reduction potentials that are observed when bacteria are cultured in a given medium. The poising factor must be given consideration when one is looking for slight differences in a particular system.

Problems with layering of Eh have been touched upon earlier. In our experiments, layering was not a problem. In experiments where there was no added aeration to provide agitation, Eh readings between swirled and stationary cultures showed agreement within $\pm$ 5 mv. This may be attributable to the fact that only motile organisms were used and(or) the central positioning of the platinum electrode in the culture vessel which would minimize zonal effects of Eh. These explanations are in line with those presented by Ward (1938) and Williams and Sullivan (1942).

In instances when very intense reducing conditions were established, e.g., for the *Salmonella* spp. examined, the oxidizing effect of molecular oxygen contained in the compressed air used for aeration was essentially undetectable as far as electrode potentials were concerned. This may be a result of the complete utilization of oxygen in the electron transfer process, resulting in an oxygen deficit in the cultures. This explanation would be compatible with results

reported by Lepper and Martin (1929), Longsworth and MacInnes (1936), and Hewitt (1950).

The potentiometric method used in these experiments may not be as sensitive to changes in culture Eh as has been suggested by others. In our experiments, concentrations of organisms approached $10^5$ to $10^6$ cells/ml before detectable changes in electrode potentials were observed.

Further work in the area of oxidation-reduction potentials and their relation to bacterial growth might be directed towards the measurement of Eh in selected food systems, e.g., ground meats, sausages, turkey rolls, etc. This type of effort would be helpful in determining any relationship that may exist between Eh and the microbial load of such products. Information of this nature would be beneficial from the standpoint of quality control and storage stability of a given product.

# CONCLUSIONS

The following conclusions may be made with regard to the study of oxidation-reduction potentials and growth of *Salmonella* spp. and *Pseudomonas fluorescens* used in this investigation.

1. Similarities in Eh, pH, and growth patterns within species of *Salmonella* and strains of *P. fluorescens* are evident. The six species of *Salmonella* examined in this study all possess the capacity to bring about very intense reduction of the medium (TSB) within a short period of time under favorable temperature conditions.

2. *P. fluorescens* does not possess as great a reducing capacity as the *Salmonella* spp. studied.

3. The attainment of maximal cell populations is nearly coincidental with attainment of minimal Eh and pH values.

4. Definite values of Eh exist that determine if a particular organism can initiate growth and(or) maintain its survival. Under the experimental conditions used, this limit was an Eh level of +80 mv (pH 7.00) for the *P. fluorescens* strains and +30 mv (pH 7.00) for the *Salmonella* spp.

5. There are differences in poising capacity of nutrient broth, brain heart infusion broth, and trypticase soy broth, which affect the electrode potentials that are observed in bacterial cultures.

6. For given cultures, inoculum size does not affect general patterns of changes in growth, Eh, or pH other than influencing the rate at which such changes occur. Cell populations approached $10^5$ to $10^6$ cells/ml before significant changes in Eh were observed.

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## ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. Allen A. Kraft for his helpful guidance and constructive criticism throughout my entire graduate career and, in particular, during the preparation of this thesis.

To Drs. Paul A. Hartman, John G. Holt, Harry E. Snyder, and Homer W. Walker, my thanks for their willingness to serve on my graduate committee.

To the graduate students, research associates, and technicians, my thanks for their meaningful discussions, advice, and cooperation.

To my parents, my thanks for their encouragement throughout my academic career.

And to my wife, Mary, and son, Andrew, I wish to express my appreciation for their patience and understanding that enabled me to complete this work.

Finally, appreciation is expressed to the Department of Food Technology and the U.S. Public Health Service for financial support.