A STUDY OF THE "PHOENIX PHENOMENON" IN CLOSTRIDIUM PERFRINGENS

by

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INTRODUCTION

Clostridium perfringens is of interest to the food scientist because it is one of the leading causative agents of food-borne illness in the United States (USPHS, 1972). This ubiquitous organism whose role as a food poisoning agent has been widely recognized only in recent years is thought to be the most widespread of all the pathogenic bacteria (Smith and Holdeman, 1968). *C. perfringens* food poisoning occurs in a variety of pre-cooked, pasteurized, or improperly cooled foods. The foods most commonly implicated are processed beef, poultry, stews, gravies, and meat pies.

Conditions suitable for food poisoning occur when spores of *C. perfringens* are present and are able to survive the heat treatment applied to certain foods, or when contamination occurs after the heating process such that *C. perfringens* is able to grow to a critical concentration of 10^6-10^8 cells per gram of food. Ingestion of 10^6-10^8 viable cells permits growth and sporulation in the small intestine. Enterotoxin produced in the sporulating cells is released upon cell lysis causing accumulation of fluid in the intestinal lumen. The principal symptoms of *C. perfringens* food poisoning are abdominal pain and diarrhea which result from this excess fluid accumulation (Hauschild, 1973). These symptoms appear eight to twenty hours after the ingestion of viable cells and last usually less than a day.

The important position of this organism as a leading cause of food poisoning is realized by its ability to grow at a rapid rate (10-15 minutes per generation) at relatively high temperatures (45-50 C). Above

these temperatures vegetative cells are inactivated (Nishida et al., 1969). In addition, there is a high probability of food becoming contaminated if prepared, especially in large quantities, the day before it is served and then improperly refrigerated. Thus, studies of this organism under various conditions at high temperatures would prove helpful in giving further understanding of its growth in food.

Sublethal heat treatments give much information on the mechanism of temperature inactivation and have been related to germination of spores, extended lag phase in the growth of bacteria, bacterial injury, death of old cells with continued growth of young cells, as well as the direct cause of other unexplained growth patterns. One such unusual growth pattern was observed for food poisoning strains of C. perfringens at 50 C (Collee et al., 1961). The apparent cell numbers decreased within the first few hours at 50 C, but later increased to a maximum count within about 6 hours. They named this curious pattern the "Phoenix phenomenon" but they were unable to elucidate the mechanism involved in this phenomenon. The purpose of the present investigation was to explain the "Phoenix phenomenon" and to further add to the knowledge on the behavior of C. perfringens at a sublethal temperature.

REVIEW OF LITERATURE

The ability of Clostridium perfringens to produce food poisoning in humans had not been considered until Klein (1895) and Andrews (1899) isolated it in significant numbers from patients suffering from mild but chronic diarrhea. The investigations made in these two outbreaks were not sufficient to establish with certainty the causative role of the organism. However, the involvement of this microorganism in human food poisoning was clearly established in England in 1943 (Knox and MacDonald) and in the United States in 1945 (McClung). Interest in C. perfringens food poisoning was stimulated by the classical investigation of Hobbs et al. (1953).

C. perfringens has been defined as an anaerobic blunt-ended rod, forming oval, subterminal spores, nonmotile, hydrolyzing gelatin, forming lecithinase, and fermenting lactose and sucrose (Smith and Holdeman, 1968). Heat resistance is increased with the formation of spores. Some strains of C. perfringens form heat resistant spores such as the Hobbs' type strains, while other strains form heat sensitive spores. Media that have been used to demonstrate the presence of spores of C. perfringens include Ellner's medium (Ellner, 1956), SEC medium (Angelotti et al., 1962), and DS medium (Duncan and Strong, 1968).

Heat was employed in many cases to provide the shock which stimulated spore germination (Barnes et al., 1963; Hobbs, 1965; Nishida et al., 1969). According to Barnes et al. (1963) only 3-20% of the spores of C. perfringens germinated without heat shock. Heat shock is commonly employed in estimating the number of spores present in a test medium. A time-temp-

erature relationship of 10 minutes at 80 C has been used to ensure the death of all vegetative cells and the germination of any spores of C. perfringens present in the test medium (Canada et al., 1964; Duncan and Strong, 1968; Duncan et al., 1972). However, some investigators (Nishida et al., 1969; Duncan and Strong, 1968) have demonstrated that the heat applied to spores induces mutagenic effects.

Duncan et al. (1972) successfully isolated sporulation negative mutants of Hobbs' serological type 9 (HT_9) as transparent colonies from either spontaneous mutants or after mutagenesis with a noninhibitory concentration of acridine orange or nitrosoguanidine. All mutants were checked for their ability to sporulate on Ellner's medium (Ellner, 1956), SEC medium (Angelotti et al., 1962), and DS medium (Duncan and Strong, 1968). Duncan (1972) found the cultural characteristics of each of these mutants to be stable after a limited number of transfers.

Several media have been employed for the enumeration of C. perfringens (Angelotti et al., 1962; Hall et al., 1969; Harmon et al., 1971b; Marshall et al., 1965; Mossel, 1959; Mossel et al., 1956; Shahidi and Ferguson, 1971). Most of the media used in the enumeration make use of C. perfringens' relative resistance to sulfite, polymyxin, sulfadiazine, neomycin, cycloserine, and kanamycin. Four media in particular have been designed specifically for the enumeration of C. perfringens: SPS (sulfite-polymyxin-sulfadiazine medium; Angelotti et al., 1962), TSN (tryptone-sulfite-neomycin medium; Marshall et al., 1965), SFP (Shahidi-Ferguson-perfringens medium; Shahidi and Ferguson, 1971), and TSC (tryptone-cycloserine medium; Harmon et al., 1971). TSN has been found to be the most inhibitory of the media to other organisms and somewhat to C. perfringens

as well, while SFP has been found to be the least inhibitory (Harmon et al., 1971a). Other media that have been used for the enumeration of C. perfringens are PYA (peptone-yeast extract agar; Ades, 1973) and SIA (sulfite-iron agar; Collee et al., 1961; Mossel et al., 1956).

Because of the interest in C. perfringens as a food poisoning organism many workers investigated factors relating to its growth (Ades, 1973; Barnes et al., 1963; Collee et al., 1961; Hobbs et al., 1953; Mead, 1969; Smith, 1972; Smith, 1970; Smith and Holdeman, 1968). Strict anaerobic conditions have been found to be unnecessary for its growth, for it is just barely considered to be an anaerobe (Smith and Holdeman, 1968). However, for any quantitation of C. perfringens, it has been found that strict anaerobic conditions must be maintained (Ades, 1973; Smith, 1972; Smith, 1970). The hydrogen ion concentration has been shown to have an effect on the growth of C. perfringens (Barnes et al., 1963; Smith, 1972). Barnes et al. (1963) showed that the growth of C. perfringens was variable at pH 5.7 but good at pH 7.2. Smith (1972) found for almost all strains that growth readily takes place between pH 6.0 and pH 7.5 but is very sharply limited at pH 5.0 and pH 8.3.

Much work has been carried out on the effects of temperature (Ades, 1973; Barnes et al., 1963; Boyd et al., 1948; Canada et al., 1964; Collee et al., 1961; Hobbs, 1965; Mead 1969; Smith, 1972; Smith, 1970; Smith and Holdeman, 1968; White and Hobbs, 1963). From these studies, it has been found that the temperature range over which most strains grow readily is from 20 C to 50 C with the most rapid growth occurring at 43-47 C. Barnes et al. (1963) demonstrated that the minimal growth temperature for C. perfringens was related to pH and medium, so that in meat

medium at pH 7.2, C. perfringens had a lower minimal growth temperature than in meat medium at pH 5.8. Furthermore, Barnes et al. (1963) showed in beef at pH 5.7-5.8 that growth was restricted at temperatures below 15 C and spores survived after freezing at -5 C and -20 C for 6 months. However, few vegetative cells were recovered after freezing in meat (Canada et al., 1964). Likewise, White and Hobbs (1963) observed the absence of growth in cooked meat after 7 days at 6.5 C. Ades (1973) recently demonstrated heat injury and recovery of vegetative cells of C. perfringens HT_2 and HT_{10} at 51.0 C and of HT_1 and HT_{13} at 50.5 C in a peptone-yeast extract growth medium. He observed essentially constant numbers at these temperatures when cells were assayed on peptone-yeast extract agar. However, he observed that as the heating time increased there was a decrease in cell numbers followed by an increase in cell numbers to the initial level when assayed on peptone-yeast extract agar + 1.5% salt.

Most strains of C. perfringens will grow at or near 50 C, although at this temperature an unusual growth pattern has been reported. Collee et al. (1961) first noted that at 50 C, strains HT_1, HT_{10}, and one strain untypable by Hobbs' sera 1-13, after being added as inoculum into a cooked meat medium fell to minimum counts at 4 hours followed by a sudden increase to maximum counts at 6 hours. They named this puzzling phenomenon the "Phoenix phenomenon" as it has since been referred to in the literature (Mead, 1969; Smith, 1972; Smith and Holdeman, 1968). The procedure of Collee et al. (1961) which gave the "Phoenix phenomenon" employed an 18 hour culture of C. perfringens which had undergone two successive 18 hour transfers before being inoculated into the cooked meat growth medium at 50 C. After inoculation of the cooked meat medium, samples were placed

in quarter strength Ringer's solution (Cruickshank, 1965) at 0, 1/2, 2, 4, 6, 8, 10, and 24 hours. Viable cells were enumerated using sulfite-iron agar (Mossel et al., 1956) in Astell roll tube bottles and incubating in an anaerobe jar under an atmosphere of hydrogen and carbon dioxide for 48 hours at 37 C. In addition, Collee et al. (1961) obtained spore counts after heating 2 ml of the cooked meat supernatant to 80 C for 5 minutes, and inoculating into sulfite-iron agar in roll tube bottles.

Attempts were made by Collee et al. (1961) to explain the "Phoenix phenomenon". First, they studied the possible role of spores. They found that the Phoenix type of growth curve could be obtained with C. perfringens HT_1 containing 250,000 spores and no viable vegetative organisms at 50 C. They concluded that spores may be necessary to ensure the survival of the initial heat shock when the inoculum was transferred to growth menstruum at 50 C. They further hypothesized that the prompt decrease in viable count may be attributed to this effect, with subsequent germination and selection of heat adapted variants.

Second, they investigated the resulting growth curve at 50 C when spores were excluded from the initial inoculum. The exclusion of spores was accomplished by the addition of 1% glucose to the medium. A detailed study of the growth of HT_1 in glucose-cooked meat medium at 50 C demonstrated a gradual decline to minimum counts in 8 hours followed by an even more gradual increase in counts to a maximum at 24 hours. They concluded that the possible participation of spores could not be excluded in light of this rather extended "Phoenix phenomenon" produced by an inoculum containing less than 2 spores/ml.

Third, they compared Gram stains of HT_1 in cooked meat medium at 37 C and 50 C, and observed filamentous forms and chains at the higher temperature. They concluded that some malfunction of cell division systems as illustrated by the presence of these abnormal forms might be responsible for the observation that normal maximum counts are never attained at 50 C.

Fourth, attempts were made by Collee et al. (1961) to replicate the "Phoenix phenomenon" by using C. perfringens HT_1 grown at 50 C to inoculate fresh medium at 50 C. Two methods were used. In one case, cooked meat broth at 50 C was inoculated with a culture of HT_1 which had been incubated for 24 hours at 50 C. In a second case, a cooked meat broth culture incubated at 50 C for 24 hours was subcultured on blood agar at 37 C for 24 hours. Then single colonies were picked into bottles of broth preheated at 37 C, incubated for 18 hours, and inoculated into fresh cooked meat medium at 50 C. In both of these experiments the Phoenix effect was reproduced and they concluded that the "Phoenix phenomenon" was not solely the result of selection of cells adapted to the higher temperature.

Fifth and finally, Collee et al. (1961) postulated the possibility that a temperature of 50 C might have a mutagenic effect on C. perfringens. Using this theory, the "Phoenix phenomenon" was explained in the following way. Introduction of the culture to medium at 50 C caused some of the cells to mutate while most of the vegetative cells died causing a decrease in viable count. Then after mutagenesis, the resulting mutants were able to adapt and grow at this temperature causing an increase in viable counts. However, maximum counts could not be attained because of the mutation. This hypothesis was supported by the appearance of abnormal

forms in the Gram stain at 50 C in studies done with a thermoduric micrococcus by Williams (1956) and studies using Escherichia coli (Zamenhoff, 1958).

These additional studies and observations on the "Phoenix phenomenon" made by Collee et al. (1961) suggested possible explanations for this unusual growth pattern but its mechanism remained obscure. Since the work of Collee et al. (1961) only one other study directly relating to the "Phoenix phenomenon" has been cited in the literature. Mead (1969) investigated the "Phoenix phenomenon" at 50.0 C. He found that the "Phoenix phenomenon" could be reproduced with a mixture of spores and vegetative cells of C. perfringens HT_1 in a cooked meat medium (pH 7.2) at 50.0 C. He observed that in chicken leg medium (pH 6.8) there was an initial increase followed by a decrease in viable cells before a sudden increase in cell numbers, and that in chicken breast medium (pH 5.8) there was a logarithmic increase in the viable cells without any detectable lag or erratic behaviour. He concluded that the growth pattern at 50.0 C was considerably influenced by the nature of the medium.

Since this study no further investigations on the "Phoenix phenomenon" have been reported in the literature and its mechanism has remained obscure. Furthermore, as Wood (1956) has been quoted as saying "It is not suprising that a great deal of controversy should have arisen as to how microorganisms are killed by both high and low temperatures . . . so few of the many studies on temperature inactivation of cells have been carried out to elucidate the mechanism of temperature action (Allwood and Russell, 1970)." Thus, the purpose of the present research was to further investigate the "Phoenix phenomenon" in the hopes of elucidating its mechanism.

MATERIALS AND METHODS

A. Preparation of Aerobic Media and Diluents

1. Cooked Meat Broth and Cooked Meat Medium

Cooked meat broth (CM) was used as a transfer medium and cooked meat medium (CMM) was used as a heating menstruum in agreement with the work of Collee et al. (1961). Cruickshank's (1965) procedure was modified for the preparation of these media. CM consisted of the following:

Ingredient	Amount
Ground veal	500.0 g
Bacto-peptone (Difco)	15.0 g
Sodium chloride	5.0 g
Distilled water	1000.0 ml

The ground veal was added to the distilled water and the mixture heated with frequent stirring until it reached a boil. After overnight refrigeration any fat on the surface was removed by skimming with a piece of filter paper. The mixture was then squeezed through 2 layers of cheesecloth, filtered through Whatman No. 4 filter paper, and the broth boiled for 15 minutes. The broth was filtered through Whatman No. 4 filter paper, peptone and salt added and dissolved by heating. The solution was filtered again and the pH adjusted to 7.0 ($\pm$0.1) with 8N NaOH. For the use of CM, 50 ml portions of the broth were added to 100 ml screw cap flat bottom bottles, and for the preparation of CMM, 50 ml portions of the broth and 10 g of extracted veal were added to 100 ml flat bottom bottles. The bottles were sterilized in an autoclave for 20 minutes at 121 C, and before use, the bottles were placed in boiling water for 15 minutes to drive out dissolved oxygen in the medium and cooled slowly to ambient temperature.

2. Hedley Wright's Broth

Hedley Wright's (W) broth was used as a transfer medium in agreement with the work of Collee et al. (1961). Cruickshank's procedure was modified for the preparation of this medium. W broth consisted of the following:

Ingredient	Amount
Ground veal	500.0 g
Bacto-peptone (Difco)	15.0 g
Sodium chloride	5.0 g
Distilled water	1000.0 ml

The ground veal, peptone, and salt were added to the distilled water and extracted for 24 hours at 3-5 C. Any fat remaining on the surface of the mixture was removed by skimming with a piece of filter paper. The mixture was squeezed and strained through 2 layers of cheesecloth, filtered through Whatman No. 4 filter paper, and boiled for 15 minutes. The resulting liquid was filtered through Whatman No. 4 filter paper, and the pH adjusted to 7.0 ($\pm$0.1) with 8N NaOH. Fifty milliliter portions of the broth were added to 100 ml screw cap flat bottom bottles. The bottles were sterilized in an autoclave for 20 minutes at 121 C. Before use, the bottles were placed in boiling water for 15 minutes and cooled slowly to ambient temperature.

3. Sulfite-Iron Agar

Sulfite-iron agar (SIA) was the assay medium used by Collee et al. (1961) and was described by Mossel et al. (1956). It was further modified to incorporate ferric citrate in the basal medium and to give a final concentration of agar at 2.5% (w/v). It was always prepared fresh on the day

of use. The basal medium consisted of the following:

Ingredient	Amount
Bacto-tryptone (Difco)	15.0 g
Yeast extract (Difco)	10.0 g
Bacto-agar (Difco)	25.0 g
Ferric citrate	0.5 g
Distilled water	1000.0 ml

The above compounds were combined and dissolved by boiling for 1 minute. The pH was adjusted to 7.0 (±0.1) with 8N NaOH, and the mixture was dispensed in 250 ml amounts into 1000 ml erlenmeyer flasks. The basal medium was sterilized in an autoclave for 15 minutes at 121 C. A sodium sulfite solution was prepared by dissolving 10 g of sodium sulfite in 100 ml of distilled water and sterilized by millipore filtration (Type GS, 0.22 μm pore size; Millipore Corporation, Bedford, Mass.). Then 2.5 ml of fresh sodium sulfite solution was aseptically added to 250 ml of the sterile basal medium. The resulting solution was mixed and aseptically dispensed in 10 ml amounts to sterile rubber stoppered 18 x 150 mm roll tubes. The SIA tubes were tempered in a water bath at 47 C.

4. Veal Agar

Veal agar (VA) was used as an assay medium, and it was always prepared fresh on the day of use. Twenty-five grams of Bacto-agar (Difco) were added to 1000 ml of CM. The agar was dissolved by boiling for 1 minute. The pH was adjusted to 7.0 (±0.1) with 8N NaOH and the solution was dispensed in 250 ml amounts into 1000 ml erlenmeyer flasks. The medium was sterilized in an autoclave for 20 minutes at 121 C. The sterile medium was then aseptically dispensed in 10 ml amounts to sterile rubber stoppered 18 x 150 mm roll tubes and tempered in a water bath at 47 C.

5. Ringer's Solution

Quarter strength Ringer's solution was used by Collee et al. (1961) as a diluent. It was prepared according to the procedure outlined by Cruickshank (1965). Stock solutions were first prepared with the following concentrations:

Stock Solution	Concentration
Sodium chloride	9.0 g/l
Potassium chloride	11.5 g/l
Calcium chloride (anhydrous)	12.2 g/l

Then 100 ml of NaCl solution was mixed with 4 ml of KCl solution and 3 ml of $CaCl_2$ solution. The resulting solution was diluted to quarter strength. Dilution blanks of 9.0 ml and 9.9 ml amounts were prepared using 13 x 150 mm screw cap test tubes. The dilution blanks were sterilized in an autoclave for 15 minutes at 121 C. Before use, the tubes were placed in boiling water for 15 minutes and cooled slowly to ambient temperature.

6. Veal Diluent

Veal diluent was prepared by dispensing 9.0 ml and 9.9 ml amounts of CM to 13 x 150 mm screw cap test tubes. The diluent was always prepared fresh on the day of use. The dilution blanks were sterilized in an autoclave for 15 minutes at 121 C.

B. Preparation of Pre-reduced Media and Diluents

All pre-reduced media were prepared in accord with the principles outlined by Holdeman and Moore (1972).

1. Pre-reduced Cooked Meat Broth and Pre-reduced Cooked Meat Medium

For the preparation of pre-reduced cooked meat broth (PCM) and

pre-reduced cooked meat medium (PCMM) both CM and CMM were prepared as outlined in section A-1 with the following modifications. After the final filtration step, 3 ml of resazurin solution (22 mg resazurin/88 ml water) was added to 750 ml of the broth. The broth was then boiled for 10 minutes until the solution turned from red to yellow indicating the absence of oxygen. The broth was cooled under oxygen-free CO_2, 0.375 g of cysteine hydrochloride added and the pH adjusted to 7.0 ($\pm$0.1) with 8N NaOH. Fifty milliliter portions of the broth were dispensed into 125 ml rubber stoppered erlenmeyer flasks which were being sparged with oxygen-free nitrogen. For PCMM, 10 g of extracted veal were added to the flask. The flasks were placed in a cushioned press and sterilized in an autoclave for 20 minutes at 121 C.

2. Pre-reduced Hedley Wright's Broth

Pre-reduced Hedley Wright's (PW) broth was prepared from the filtered broth described in section A-2 and pre-reduced in the same manner as described in B-1.

3. Pre-reduced Peptone-Yeast Extract Agar

Pre-reduced peptone-yeast extract agar (PYA) was used as an assay medium in the anaerobic studies, and has been described by Ades (1973). PYA consisted of the following:

Ingredient	Amount
Bacto-peptone (Difco)	15.0 g
Yeast extract (Difco)	7.5 g
Cysteine hydrochloride	0.375 g
[1]Resazurin solution	3.0 ml
[2]Salts solution	30.0 ml
Distilled water	750.0 ml
Bacto-agar (Difco)	2.5% (w/v)/ roll tube

[1]Resazurin solution consisted of 22 mg resazurin/88 ml water.
[2]Salts solution was prepared as described by Holdeman and Moore (1972).

The above ingredients except cysteine hydrochloride and Bacto-agar were combined and boiled for 10 minutes. The solution was cooled under CO_2, cysteine hydrochloride added, and the pH adjusted to 7.0 ($\pm$0.1) with 8N NaOH. Then under N_2, 10 ml amounts were dispensed into 18 x 150 mm roll tubes to which 0.25 g of Bacto-agar had been added. The tubes were placed in a cushioned press and sterilized in an autoclave for 15 minutes at 121 C. The racks were gently shaken while hot to evenly distribute and dissolve the agar. PYA roll tubes were stored on shelves at room temperature until use. On the day of use they were clamped, placed in flowing steam for 15 minutes to melt the medium, and tempered in a water bath at 47 C.

4. Pre-reduced Tryptose-Soytone-Yeast Extract Agar

Pre-reduced tryptose-soytone-yeast extract agar (TSY) was modified from Shahidi Ferguson perfringens medium (Shahidi and Ferguson, 1971) to exclude kanamycin sulfate, polymyxin B sulfate, and egg yolk emulsion. Sodium metabisulfite was also excluded since it was found to be somewhat inhibitory to our organism. TSY consisted of the following:

Ingredient	Amount
Bacto-tryptose (Difco)	15.0 g
Bacto-soytone (Difco)	3.75 g
Yeast extract (Difco)	3.75 g
Ferric ammonium citrate	0.75 g
[1]Resazurin solution	3.0 ml
Cysteine hydrochloride	0.375 g
Distilled water	1000.0 ml
Bacto-agar (Difco)	2.5% (w/v)/roll tube

[1]Resazurin solution consisted of 22 mg resazurin/88 ml water.

The procedure for preparing TSY was identical with the procedure already described for PYA roll tubes (section B-3).

5. Pre-reduced Veal Agar

Pre-reduced veal agar (PVA) was used as an assay medium. PVA was prepared by dispensing PCM under N_2 in 10 ml amounts to 18 x 150 mm roll tubes to which 0.25 g of Bacto-agar had been added. The rack of tubes was placed in a cushioned press, sterilized in an autoclave for 15 minutes, and inverted several times while hot to evenly distribute and dissolve the agar. Before use, the tubes were clamped, placed in flowing steam for 15 minutes, and tempered in a water bath at 47 C.

6. Chopped Meat Medium

A chopped meat medium as described by Holdeman and Moore (1972) was used as the maintenence medium for all cultures. It consisted of the following:

Ingredient	Amount
Ground top round beef	500.0 g
Distilled water	1000.0 ml
1N NaOH	25.0 ml
Trypticase (BBL)	22.5 g
Yeast extract (Difco)	3.75 g
Potassium phosphate	3.75 g
Cysteine hydrochloride	0.375 g
[1]Resazurin solution	3.0 ml

[1]Resazurin solution consisted of 22 mg resazurin/88 ml water.

The beef, water and NaOH were mixed and heated to boiling. After overnight refrigeration, any fat remaining on the surface of the mixture was removed by skimming with a piece of filter paper. This mixture was filtered first through 2 layers of cheesecloth and then through 2 pieces of

Whatman No. 4 filter paper. To 750 ml of the filtrate was added the remaining ingredients except the cysteine hydrochloride. The remaining procedure was identical with the procedure for PCMM except instead of veal being added to the roll tubes, 1 inch of extracted chopped round beef was added. The tubes were sterilized in an autoclave for 30 minutes at 121 C, cooled, and incubated for 24 hours at 37 C to check for any contamination.

7. Pre-reduced Peptone Dilution Blanks

0.1% (w/v) Bacto-peptone (Hauschild et al., 1967) was used as the diluent in anaerobic studies. It consisted of the following:

Ingredient	Amount
Bacto-peptone (Difco)	0.75 g
[1]Resazurin solution	3.0 ml
Cysteine hydrochloride	0.375 g
Distilled water	750.0 ml

[1]Resazurin solution consisted of 22 mg resazurin/88 ml water.

The above ingredients were mixed except cysteine hydrochloride and boiled for 10 minutes. The solution was cooled, cysteine hydrochloride added, and the pH adjusted to 7.0 ($\pm$0.1) with 8N NaOH. Then under N_2, 9.0 ml and 9.9 ml amounts were dispensed into 18 x 150 mm roll tubes. The tubes were placed in a cushioned press and sterilized in an autoclave for 15 minutes at 121 C.

8. Pre-reduced Veal Dilution Blanks

Pre-reduced veal dilution blanks were prepared by dispensing PCM to 18 x 150 mm roll tubes to give amounts of 9.0 ml and 9.9 ml, and sterilized in an autoclave for 15 minutes at 121 C.

C. General Methods

1. Aerobic Culturing Techniques

In order to reproduce the "Phoenix phenomenon", the procedures of Collee et al. (1961) were followed as closely as possible. Their transfers, inoculations, samplings, and dilutions were made aerobically, but the presence of oxygen was minimized by placing media and diluents in boiling water for 15 minutes and cooling slowly before use. The assay media, SIA and VA, were prepared on the day of use to minimize the aborption of oxygen into the media.

Mixing of samples in both diluents and assay media was performed using a Vortex-Genie mixer (Fisher Scientific Co., Pittsburgh, Pa.). Pipeting was done using only glass disposable pipets.

2. Anaerobic Culturing Techniques

In the experiments following the procedure of transfers of Collee et al. (1961), aluminum foil covered roll tubes were incubated in a cold catalyst anaerobe jar (BBL, Cockeysville, Maryland) for 18-24 hours at 37 C. Fresh catalyst (palladium coated aluminum pellets) was used in the jar. Anaerobiosis was acheived in the jar by the use of Gas Paks (BBL, Cockeysville, Maryland).

In addition, experiments employing the techniques described by Holdeman and Moore (1972) were performed to ensure quantitative enumeration of Clostridium perfringens (Ades, 1973; Smith, 1972). In all cases where pre-reduced media or diluents were used, all culture transfers, inoculations, samplings and dilutions were conducted as described by Ades (1973): "under a flow of oxygen free CO_2 supplied through either

the V.P.I. culture system (Bellco Glass, Inc., Vineland, N. J.) or through 16 gauge, stainless steel biomedical needles (Popper & Sons, Inc., New York, N. Y.)." Samples were mixed in the same manner as described for the aerobic culturing techniques (section C-1), and all pipetting was done with glass disposable pipettes.

The roll tube method of culturing essentially eliminated exposure of the organism to oxygen or oxidized media. The rubber stoppered roll tubes were incubated 18-24 hours at 37 C. The counting procedure was the same for the two methods of incubating roll tubes. The roll tubes were spirally marked using a red felt tip marker and the "streaker" unit of the anaerobic culture system. The colonies were then counted with the aid of a dissecting microscope (American Optical Co., Buffalo, N. Y.).

3. Culture Maintenance

The maintenance medium was a chopped meat medium (section B-6). Stock cultures were transferred monthly, incubated for 4 hours at 37 C and stored at room temperature.

4. Temperature Control

Temperature control was attained with the same apparatus as used by Ades (1973) in which a Haake Model E52 constant temperature circulator (Haake Instruments, Inc., Rochelle Park, N. J.) was placed in an insulated water bath to give the desired temperatures used in this investigation. The water bath temperature ranged from 37 C to 53 C and was controlled to $\pm$0.01 C. The medium used as the heating menstruum was always submerged below the level of water in the bath.

5. Sampling Techniques

The heating menstruum was always agitated just before each sampling in agreement with the procedure of Collee et al. (1961). The bottles or flasks containing the heating menstruum were gently shaken in two directions and the veal meat particles allowed to sediment for 15 seconds. Then samples were taken.

6. Direct Microscopic Counts

Direct microscopic counts (DMC's) were obtained by taking a 0.5 ml aliquot of the heating menstruum at each sampling and adding it to sterile screw cap test tubes which contained 0.5 ml of 4% (w/v) formalin. The contents of the tubes were mixed thoroughly and before the counts were made 0.5 ml of 40% (w/v) glycerin was added and the tubes mixed again. The DMC's were performed using a Petroff-Hausser counting chamber and phase contrast microscopy. In all cases, twenty fields were counted and the number of bacteria per ml were estimated by the following formula:

$$\#\ \text{bacteria/ml} \doteq (2 \times 10^7)(\text{dil. factor})(\text{avg. no./field}).$$

7. Spore Counts

Spore counts were conducted by taking 2 ml samples from the heating menstruum at hourly intervals for 10 hours. The samples were put into sterile 13 x 100 mm screw cap test tubes and heated in a water bath (Precision Scientific, Chicago, Ill.) for 10 minutes at 80 C (Canada et al., 1-64; Duncan et al., 1972). The tubes were then cooled in tap water for 5 minutes and 0.5 ml was transferred to each of two tubes of SIA.

8. Preparation of Nalidixic Acid

The desired concentration of nalidixic was sterilized by millipore filtration (Millipore Corporation, Bedford, Mass.). The solvent used was 1N NaOH since nalidixic acid has been shown to be soluble in it (Ades, 1973; Ryan et al., 1970).

9. Reproducibility of Results

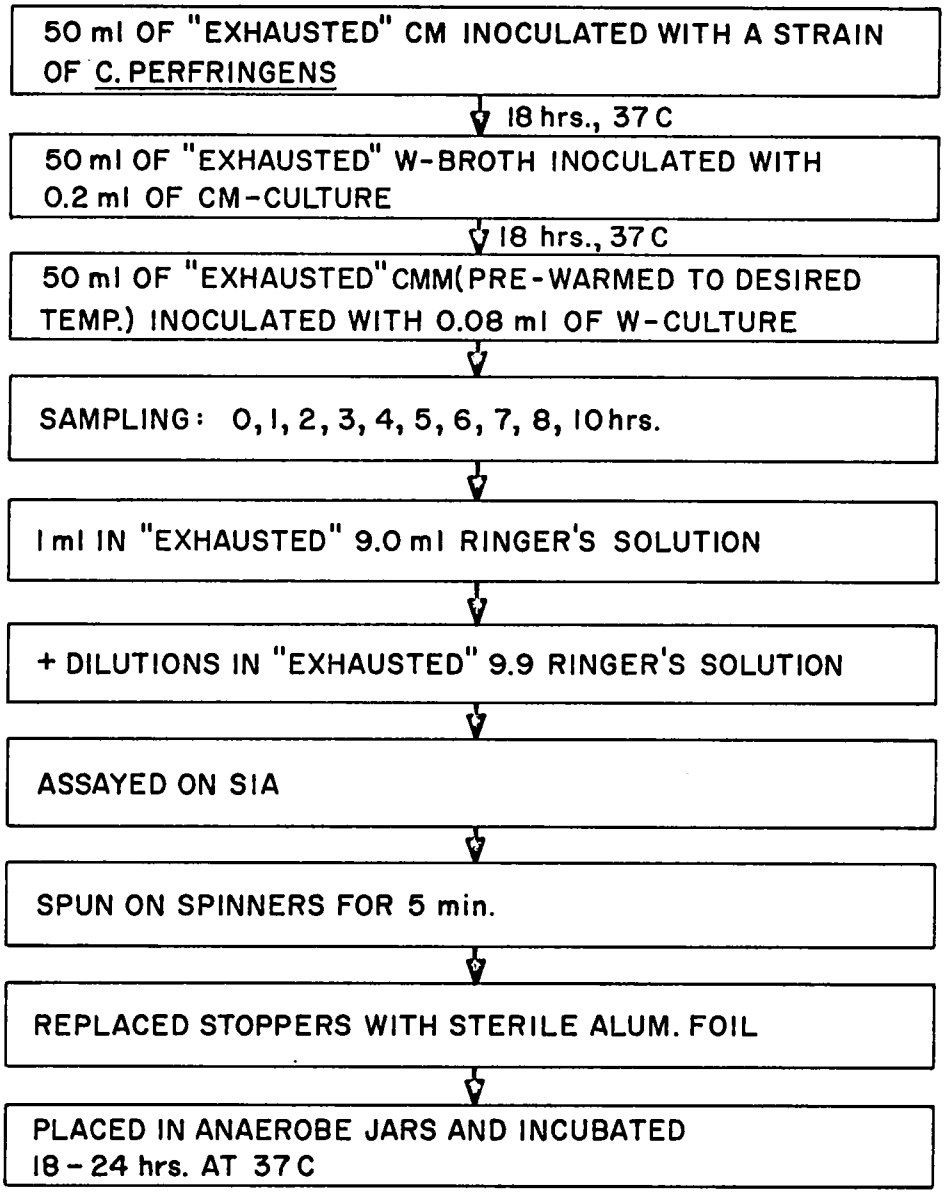
All counts were performed in duplicate and there was usually no greater than a 15% variation in counts between roll tubes. Important experiments were replicated when possible.

D. Test Organism

Cultures of Clostridium perfringens type A including Hobbs' serological type 9 (HT_9) and Hobbs' serological type 10 (HT_{10}) were obtained from the V.P.I. Anaerobe Laboratory. Strain HT_{10} was chosen as a test organism because this strain was used by Collee et al. (1961) in their work on the "Phoenix phenomenon". Strain HT_9 was chosen as a test organism because sporulation negative mutants of this strain could be obtained. Sporulation negative mutant strains 8-1, 8-5, 8-7, 8-16, and 8-17 of HT_9 as described by Duncan et al. (1972) were kindly furnished by C. L. Duncan (University of Wisconsin, Madison, Wisconsin).

E. Procedure of Collee et al. (1961)

The procedure of Collee et al. (1961) was followed closely in order to reproduce the "Phoenix phenomenon" and is outlined in a flow diagram (Fig. 1). Fifty milliliters of CM were inoculated with one of the C. perfringens strains that had been maintained in pre-reduced chopped



* SPORE COUNTS WERE OBTAINED BY HEATING 2 ml ALIQUOTS OF CM-CULTURE AT 80 C FOR 10 min., COOLING, AND ASSAYING 0.5 ml AMOUNTS ON SIA.

Fig. 1. Procedure of Collee et al. (1961).

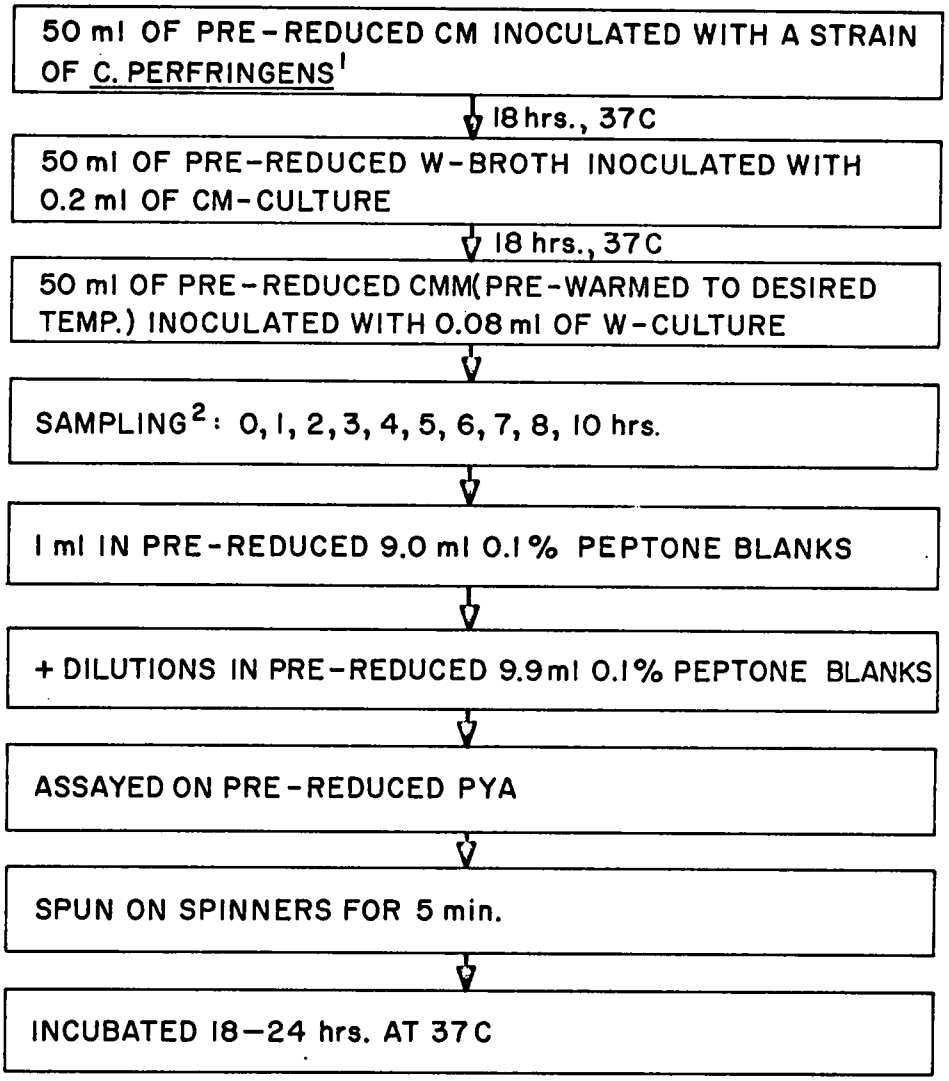
meat medium. This CM culture was incubated for 18 hours at 37 C and 0.2 ml inoculated into W broth followed by incubation for 18 hours at 37 C. After the CMM heating menstruum had been prewarmed to the desired temperature for 30 minutes, 0.08 ml of the W broth culture was added. Samples were taken at hourly intervals into 9.0 ml Ringer's solution. The desired dilution was assayed on SIA medium. The tubes were spun horizonally on spinners for 5 minutes to solidify the agar, the rubber stoppers replaced with sterile aluminum foil, and the tubes incubated in anaerobe jars for 18-24 hours at 37 C. Spore counts were taken at the time of sampling. DMC's were attempted but the number of cells present were too low to be detected by this method which needed at least 10^7 cells/ml. A series of experiments were run in menstrua at various temperatures until a temperature was found where the "Phoenix phenomenon" was observed.

F. Procedure For Pre-reduced Media Experiments

Strict anaerobic conditions were followed in this series of experiments, using only pre-reduced media and diluents and at all times maintaining the culture under an atmosphere of oxygen-free CO_2. Otherwise, this investigation (Fig. 2) was identical with the experiments described in section E.

G. Procedure For Comparison of Assay Media

Five different types of assay media were compared: SIA, VA, PYA, TSY, and PVA, using the methods outlined in Fig. 3. At the time of sampling, 1 ml aliquots were taken into both Ringer's solution and 0.1% peptone and assayed on aerobic and pre-reduced media, respectively. However, in the experiment comparing PVA to PYA, strict anaerobic conditions were followed throughout the experiment.



[1] ALL TRANFERS, INOCULATIONS, SAMPLINGS, AND DILUTIONS WERE CONDUCTED UNDER A FLOW OF OXYGEN-FREE CO_2.

[2] SPORE COUNTS WERE OBTAINED BY HEATING 2 ml ALIQUOTS OF CM-CULTURE AT 80C FOR 10 min., COOLING, AND ASSAYING 0.5 ml AMOUNTS ON PYA.

Fig. 2. Procedure for pre-reduced media experiments.

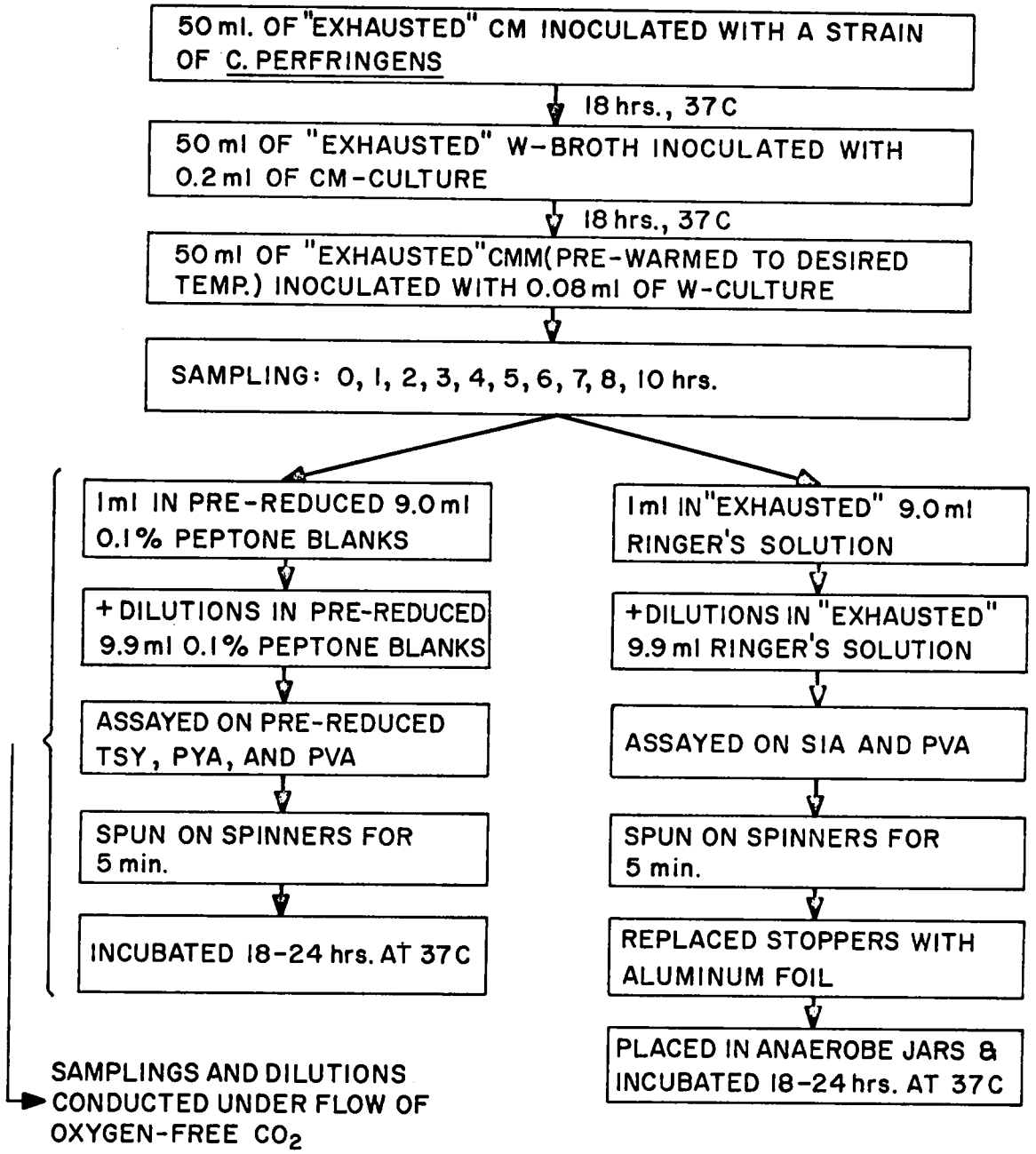


Fig. 3. Procedure for comparison of assay media.

H. Determination of the Concentration of Nalidixic Acid Used to Inhibit Growth

Nalidixic acid was used as a growth inhibitor for a period of no longer than 6 hours in studying the "Phoenix phenomenon". Therefore, the minimal inhibitory concentration(MIC) used in this study was defined as the lowest concentration of inhibitor in which there was no change in number of cells present over a period of 6 hours. The procedure used in this study (Fig. 4) was to add the desired concentration of nalidixic acid to CMM and heat the medium for 6 hours at the temperature where the inhibitor would be used. This heating was done to simulate the temperature to which the inhibitor would be exposed to in studies where it was used. Immediately after heating, the CMM-nalidixic acid medium was cooled to room temperature and the vessel was inoculated with 0.08 ml of an 18 hour culture of C. perfringens HT_9 or HT_{10}. The vessel was placed in a water bath at 37 C and samples were taken, via the procedure of Collee et al. (1961) at hourly intervals for 6 hours.

I. Procedure For Experiments Using Nalidixic Acid

The effect of the addition of the MIC of nalidixic acid on the growth curves of C. perfringens HT_9 and HT_{10} was tested under both aerobic and anaerobic conditions. Duplicate CMM vessels were inoculated with HT_9 or HT_{10}. One vessel served as the control in which there was no deviation from the procedure described in section E (aerobic studies) or section F (anaerobic studies). To the other vessel was added a MIC of nalidixic acid at the time where minimum counts had been observed. Otherwise, the procedure followed in this vessel was the same as the control.

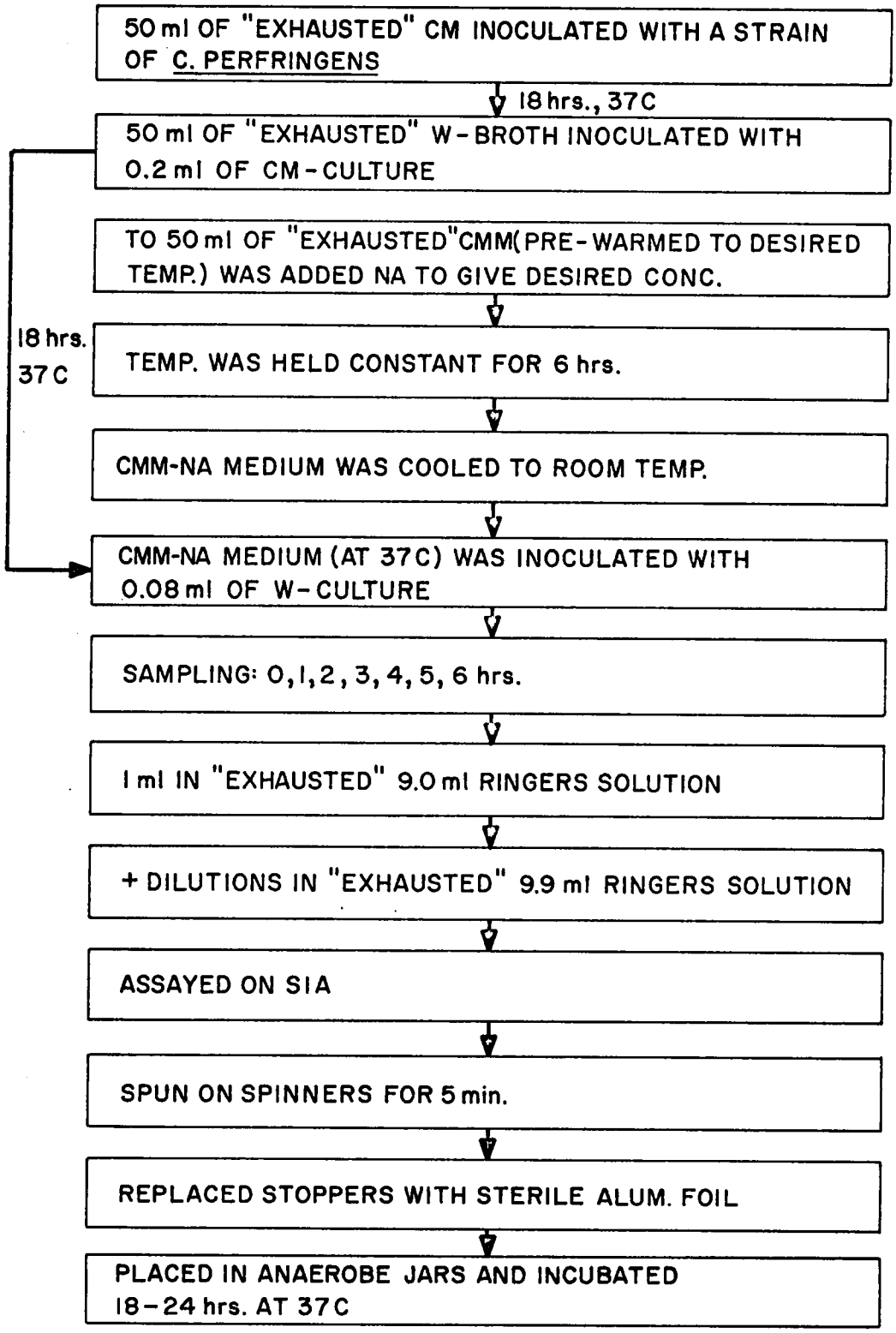


Fig. 4. Procedure for determination of minimal inhibitory concentration for nalidixic acid (NA).

J. Procedure For Diluent Study

The temperature and choice of diluent was investigated for C. perfringens HT_9 and HT_{10} under both aerobic and anaerobic conditions. In the aerobic studies (section E), Ringer's solution was compared to veal diluent both being tempered at 25 C and 47 C, and in the anaerobic studies (section F) 0.1% peptone diluent was compared to pre-reduced veal diluent both being tempered at 25 C and 47 C. In each study, samples were taken from the same growth vessel and assayed on veal agar within 4-6 minutes after sampling.

RESULTS AND DISCUSSION

A. Reproduction of the "Phoenix Phenomenon"

Type A Clostridium perfringens Hobbs' serotypes 9 (HT_9) and 10 (HT_{10}) were used in this study. Since Collee et al. (1961) and Mead (1969) had observed the "Phoenix phenomenon" for HT_{10} at 50 C, this was the first temperature investigated. However, in this study, strains HT_9 and HT_{10} did not exhibit the "Phoenix phenomenon" but readily exhibited growth (Figs. 5 and 6). Generation times at 50 C for HT_9 and HT_{10} were found to be 15.0 minutes and 12.0 minutes, respectively. Late log stage in growth was reached within 4 hours for both strains; however, a more extended lag phase was observed for HT_{10}.

The temperature was raised in subsequent experiments until the "Phoenix phenomenon" was observed. As the temperature was raised from 50 C to 52 C it was found that the lag phase increased, maximum counts decreased, and the Phoenix effect became more pronounced. However, apparent generation times for both strains remained fairly constant between 50 C and 52 C. Differences in the pattern of growth of C. perfringens for small temperature changes in this temperature range have been noted by Ades (1973).

From this study the most typical "Phoenix phenomenon" was observed for HT_9 and HT_{10} at 51.7 C and 51.5 C, respectively. The growth pattern observed at these temperatures was compared to the growth pattern observed by Collee et al. (1961) at 50 C (Table 1) and it can be seen that the results are in general agreement. Reasons for the observation of the "Phoenix phenomenon" at this higher temperature probably lie in small unavoid-

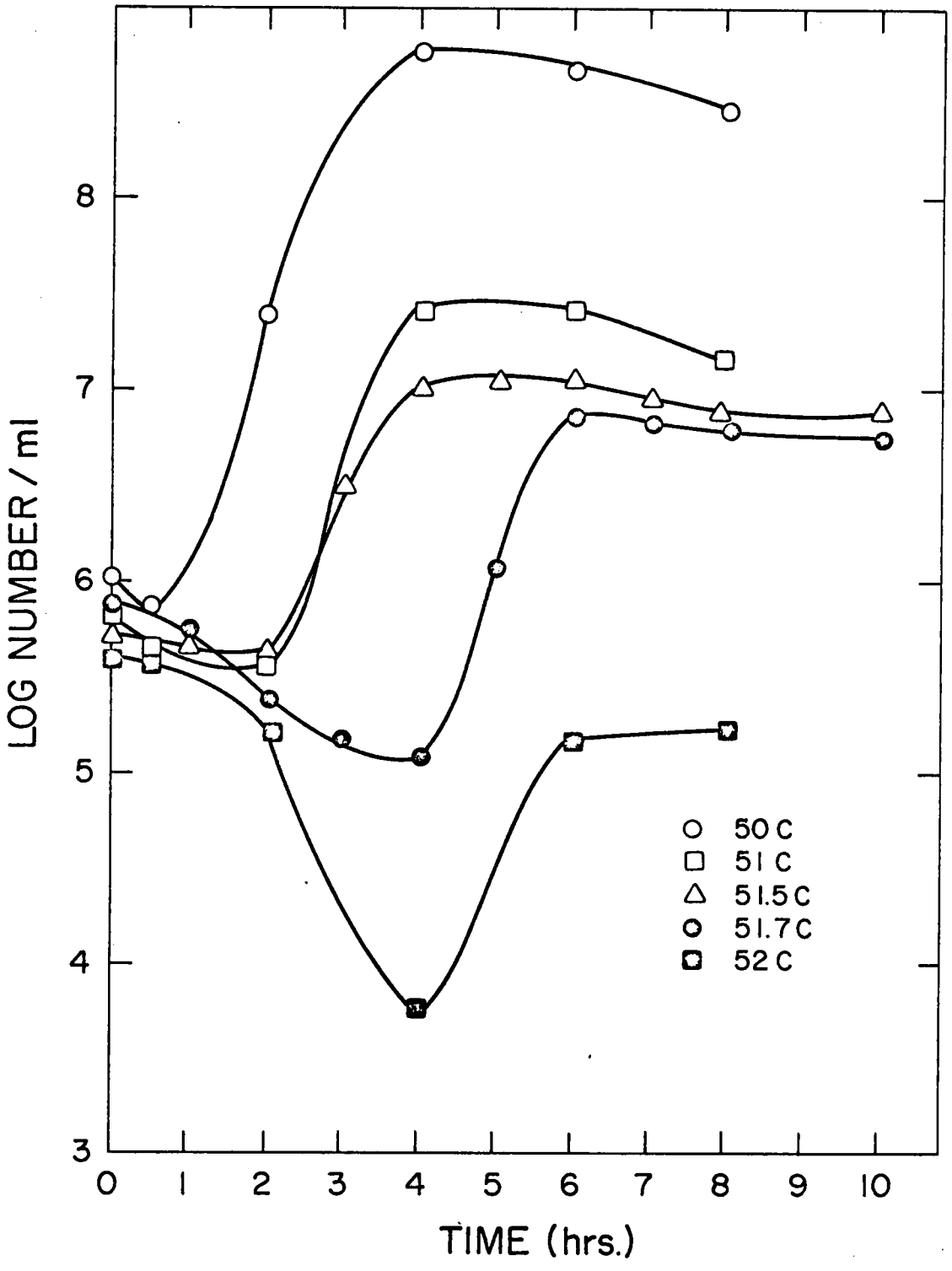


Fig. 5. Growth curves for C. perfringens, Hobbs' type 9 at various temperatures in cooked meat medium.

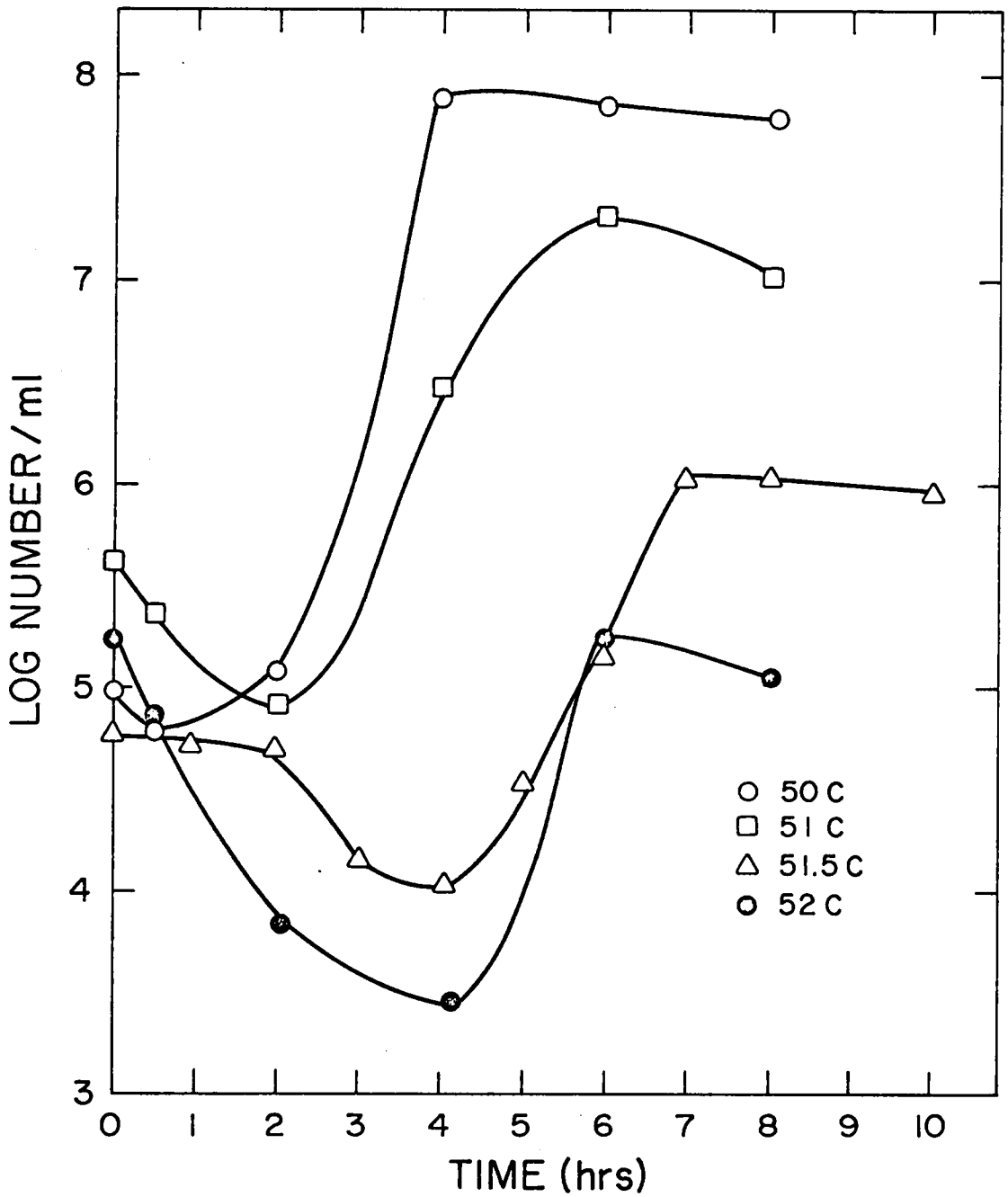


Fig. 6. Growth curves for C. perfringens, Hobbs' type 10 at various temperatures in cooked meat medium.

Table 1. Comparison of results obtained by Collee *et al*. (1961) to results obtained from the present study.

	Results of Collee *et al*.	Results of Present Study	
Strains tested	HT_{10}	HT_9	HT_{10}
Temperature observed "Phoenix phenomenon"	50 C	51.7 C	51.5 C
Time of m_c*	4-5 hrs.	4 hrs.	4-5 hrs.
Time of M_c*	6 hrs.	6 hrs.	6-7 hrs.
Log (I_c)*	4.0	5.9	4.8
Log (m_c)*	2.8	5.0	4.0
Log (M_c)*	6.0	7.0	6.0
Log ($I_c - m_c$)	1.2	0.9	0.8
Log ($M_c - I_c$)	2.0	1.1	1.2

* Where m_c = minimum counts

M_c = maximum counts

I_c = initial counts

able deviations in procedure, in the quality of veal and other ingredients used in the media, and in the much better method of temperature control used in our experiments. In addition, all media and diluents were adjusted to a pH of 7.0 (±0.1) as compared to a pH of 7.2-7.4 used by Collee et al. (1961) and Mead (1969), and as Mead has pointed out, the " 'Phoenix phenomenon' is considerably influenced by the nature of the medium." Spore counts on both strains were performed throughout this study, and in all cases, counts of no greater than 1×10^1 were observed.

The differences observed in this study between strains HT_9 and HT_{10}, and in subsequent studies which included strains of sporulation negative mutants, has been explained admirably by Smith (1972): "it seems to be a general biological law that individuals or strains within one species have the same optimal conditions for growth, although they will vary considerably in withstanding adverse conditions" (i.e. extremes in temperatures). In any case, the Phoenix phenomenon" was observed for HT_9 at 51.7 C and for HT_{10} at 51.5 C.

B. Effect of Sporulation Negative Mutants on the "Phoenix Phenomenon"

One hypothesis suggested by Collee et al. (1961) to explain the "Phoenix phenomenon" was based on the presence of spores. They postulated that spores present in the medium survive the initial heat shock when transferred as inoculum to the heating menstruum at 50 C while vegetative cells die, causing the initial decrease in counts. According to this theory germination of spores and selective outgrowth of heat adapted variants then occurs causing an increase in counts. They tested this theory to some extent using both an inoculum of spores and an inoculum

in which glucose was added to eliminate spores from the medium. In both cases, the "Phoenix phenomenon" was reproduced and the question of the role of spores remained unanswered.

It was the purpose of this experiment to prove whether the presence of spores was necessary for the observation of the "Phoenix phenomenon". To accomplish this, spores were excluded from the initial inoculum using sporulation negative mutant strains (8-1, 8-5, 8-7, 8-16, and 8-17 derived from HT_9). This method of excluding spores from the inoculum is much more accurate than the method employed by Collee et al. (1961), and the results should prove whether the presence of spores is necessary for the "Phoenix phenomenon". If they are then the "Phoenix phenomenon" should not be observed by these mutant strains.

At the temperature initially used, 51.5 C, 8-5 and 8-17 exhibited an initial decrease in counts followed by an increase to give maximum counts in five hours (Fig. 7). However, after 8 hours counts decreased again. At 51.5 C strains 8-1, 8-7, and 8-16 did not survive. The temperature was then lowered to provide a sublethal temperature for these strains. The characteristic dip occurred at 51.0 C for 8-16 and 50.5 C for 8-1 and 8-7 (Fig. 8). A summary of the results from this study are given below:

Strain	Temperature "Phoenix Phenomenon" Observed	Time of Minimum Count
8-1	50.5 C	6 hrs.
8-5	51.5 C	3 hrs.
8-7	50.5 C	6 hrs.
8-16	51.0 C	3 hrs.
8-17	51.5 C	3 hrs.

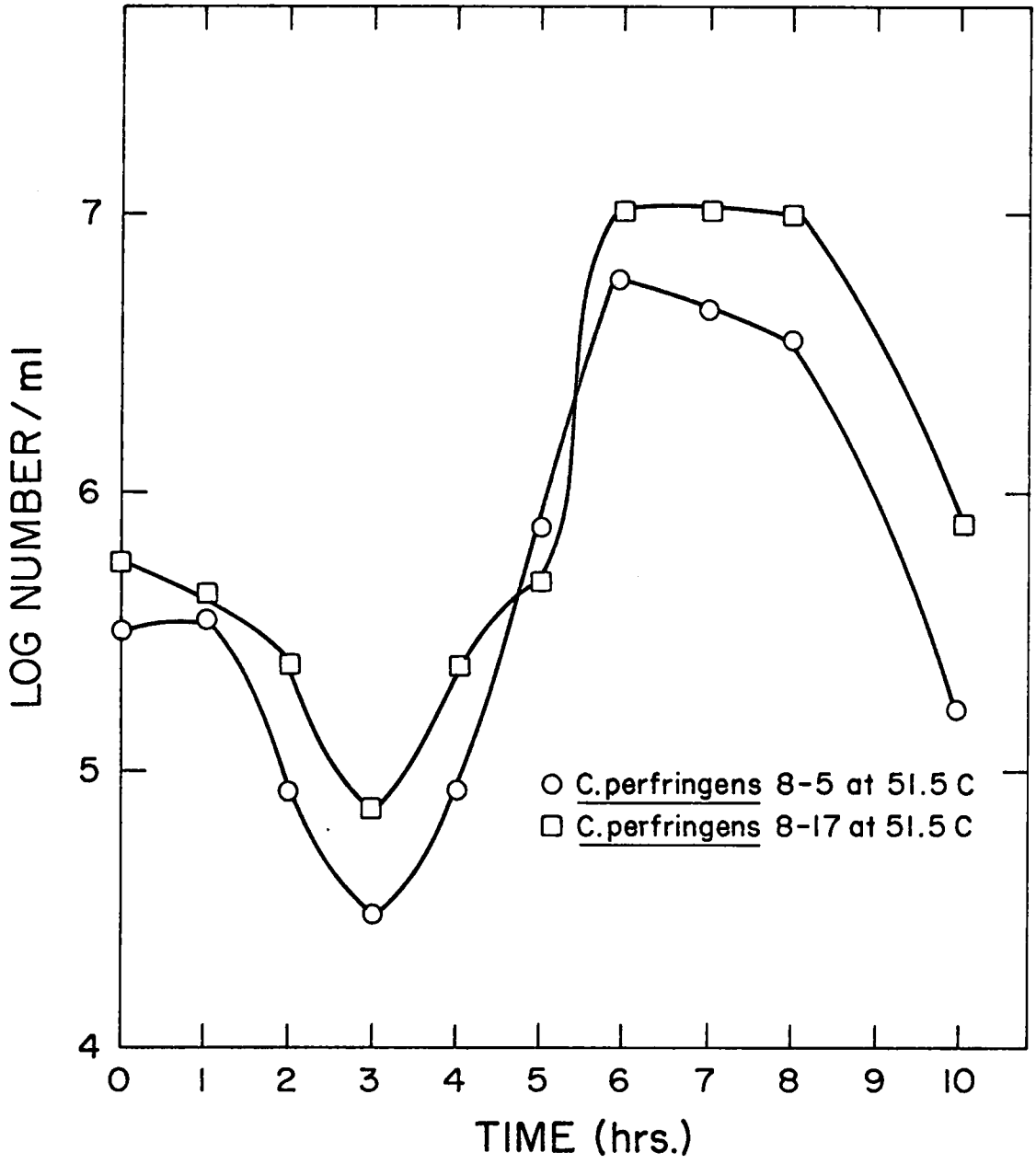


Fig. 7. Growth curves for C. perfringens sporulation negative mutant strains 8-5 and 8-17 at 51.5 C.

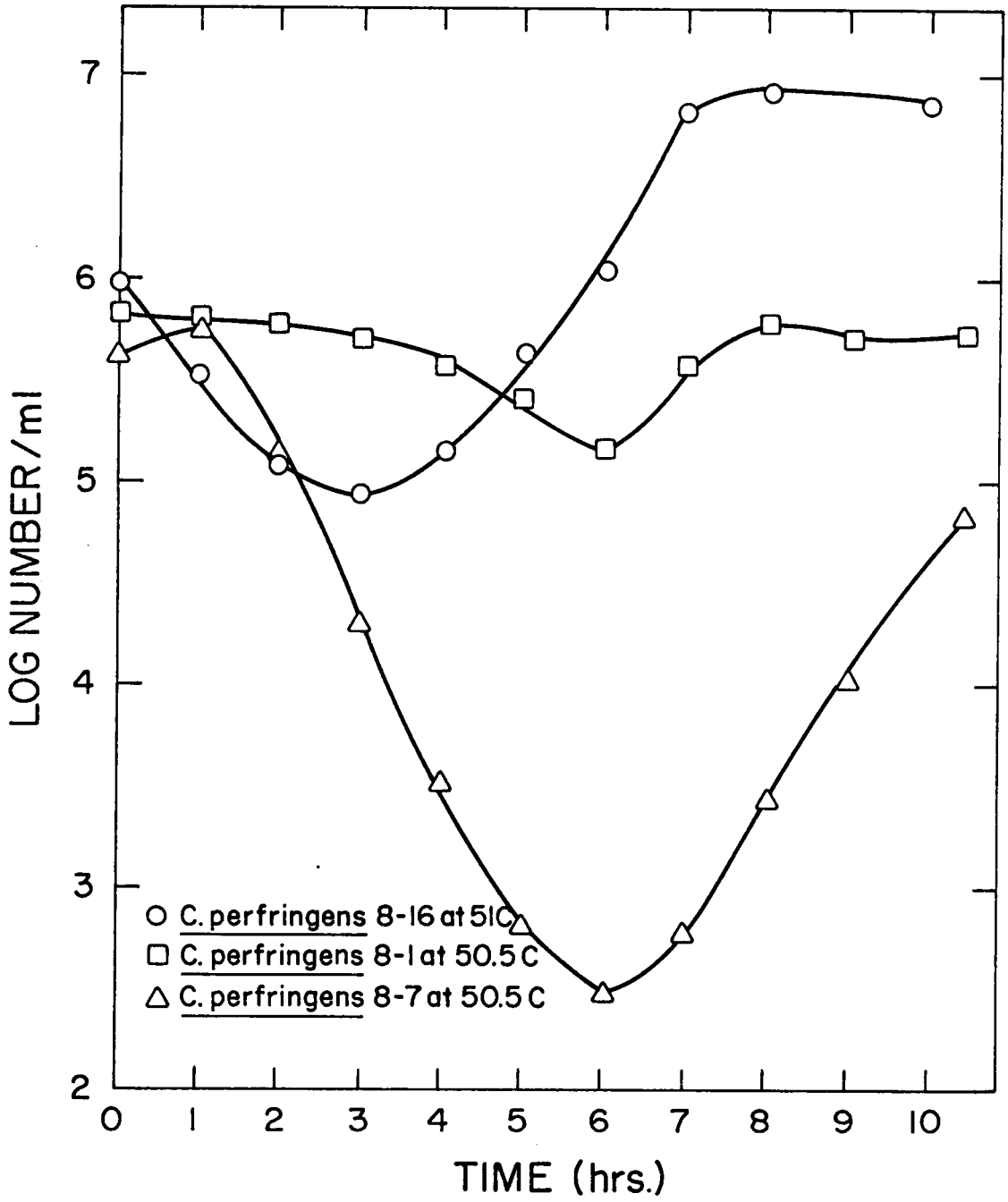


Fig. 8. Growth curves for C. perfringens sporulation negative mutant strains 8-16 at 51.0 C and 8-1 and 8-7 at 50.5 C.

A minimum count at 6 hours was observed for strains exhibiting the Phoenix effect at 50.5 C, whereas a minimum count at 3 hours was observed for strains exhibiting the Phoenix effect at a higher temperature of 51.0 C or 51.5 C. The extent of the dip varied with strains and ranged from a 3/4 lag cycle to a 3 log cycle difference between initial and minimum counts. Maximum counts also varied with the strains tested. But again, these variations between strains are not unusual in this temperature range (Smith, 1972). Spore counts were performed throughout this study and in no case were spores detected with these mutant strains. Since the "Phoenix phenomenon" was observed for these mutants it can be concluded that the survival of organisms at this temperature is not dependent on spores surviving the heat treatment with subsequent germination and outgrowth of heat adapted mutants. However, it was observed that these sporulation negative mutants were more sensitive to the effects of temperature. Maximum counts could not be maintained after 8 hours at 51.0 C for strains 8-1 and 8-17 and the temperature had to be lowered to observe the "Phoenix phenomenon" for strains 8-5, 8-7, and 8-16.

C. Effect of Age of Inoculum on the "Phoenix Phenomenon"

It was the purpose of this experiment to determine whether the age of inoculum had any effect on the occurrence of the "Phoenix phenomenon"; that is, in a culture containing younger more active cells instead of old less active cells, would the "Phoenix phenomenon" be observed?

It was found for HT_9 at 51.7 C that as the age of inoculum was increased from 4 to 8 to 12 or 18 hours, the "Phoenix phenomenon" became more pronounced, although maximum counts decreased as the age of inoculum

increased (Fig. 9). Also the time taken to reach minimum counts increased in most cases with the age of inoculum. The "Phoenix phenomenon" was observed for the 2 hour inoculum, although the initial count was 3 log cycles less than the initial counts for the other ages of inoculum tested, and thus it is difficult to compare the results of the 2 hour inoculum with the results of other ages of inoculum. The same findings were not as evident using HT_{10} (Fig. 10). Because the initial counts for inoculum of different ages varied it was difficult to draw any conclusions. The 2 and 4 hour inoculum did not survive at this temperature; probably because of their lower concentration of cells, and their high sensitivity (Jay, 1970) as a result of being in a log phase of growth when introduced to such a high temperature. It appeared that HT_{10} was more sensitive to the effects of temperature than HT_9 since with HT_{10} the 4 hour inoculum died, lower maximum counts were obtained, and a lower temperature was necessary to observe the "Phoenix phenomenon".

Spore counts were performed on all samples and in all cases spores could not be detected. It can be concluded from this study that age of inoculum has an effect, at least with HT_9, on the occurrence of the "Phoenix phenomenon". Although the "Phoenix phenomenon" was observed for nearly all ages of inoculum, it was found to be more pronounced with HT_9 as age of inoculum was increased.

D. Effect of Anaerobiosis on the "Phoenix Phenomenon"

Since the work of Collee et al. (1961) was done with media prepared under aerobic conditions, oxygen or oxidized media might have served as an initial stress factor, giving rise to the initial decrease in viable organ-

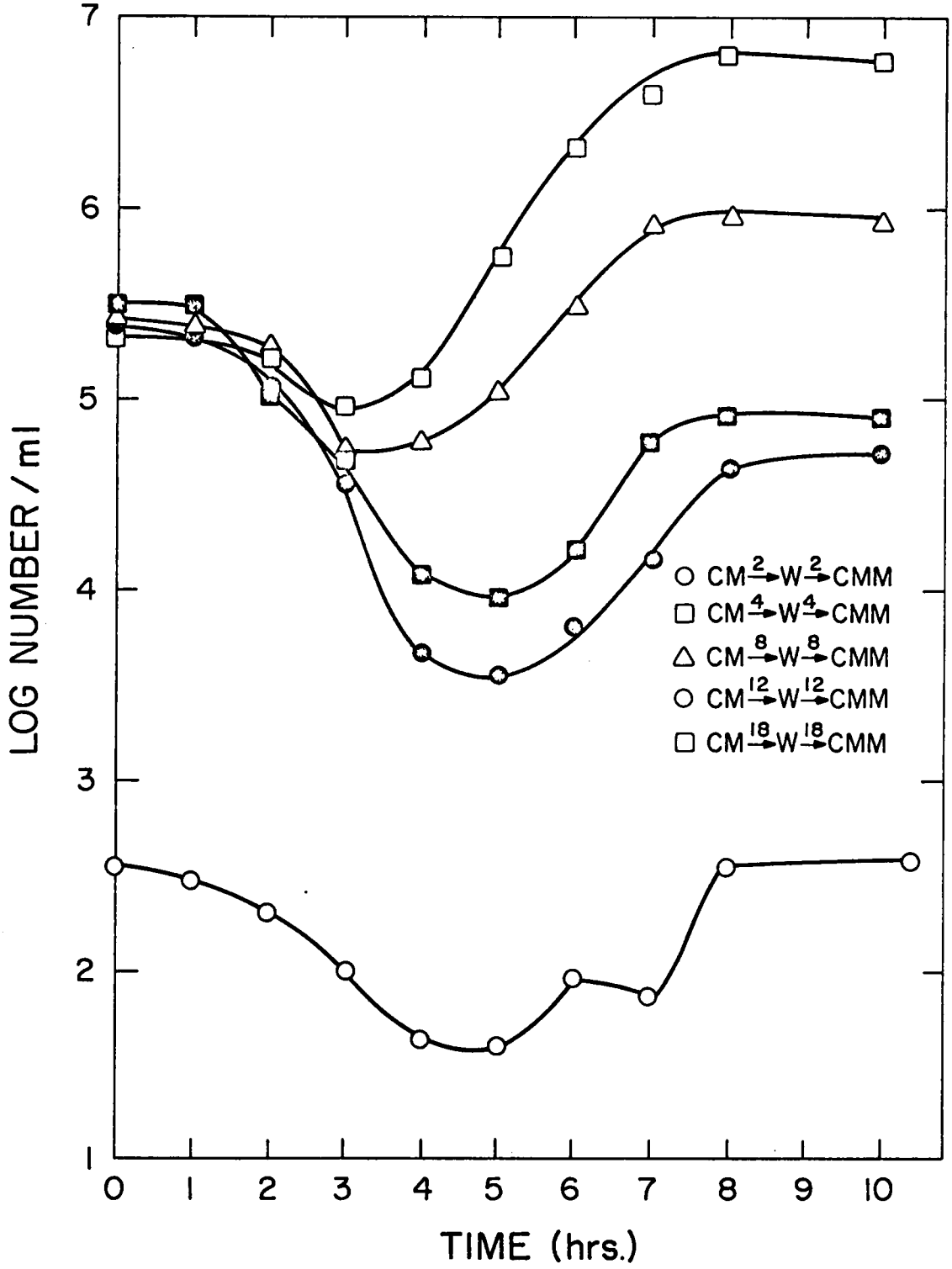


Fig. 9. Growth curves for C. perfringens, Hobbs' type 9 using different ages of inoculum at 51.7 C.

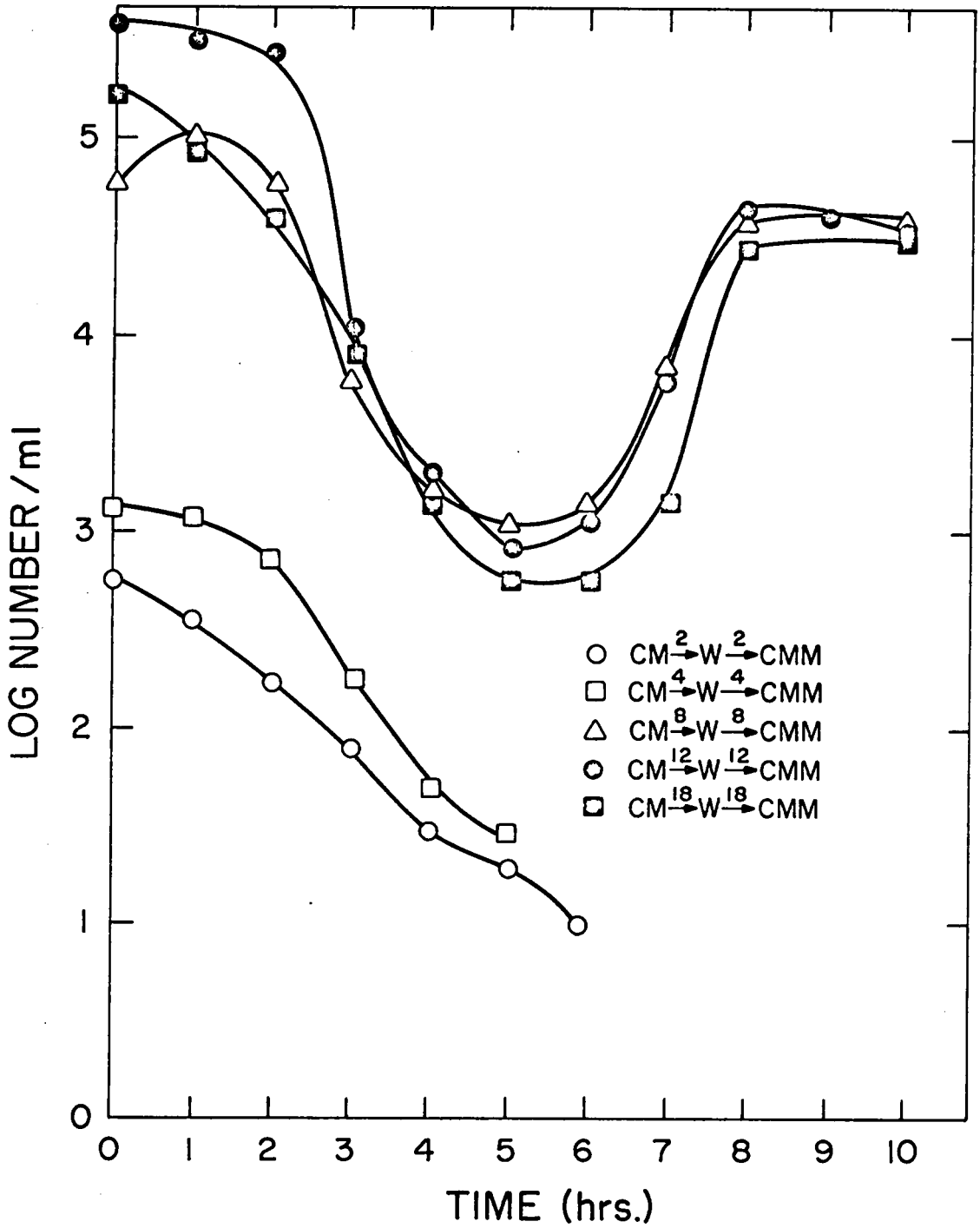


Fig. 10. Growth curves for C. perfringens, Hobbs' type 10 using different ages of inoculum at 51.5 C.

isms. With the combined effects of oxygen and oxidized media the cells may die until they reduce the oxidation-reduction potential sufficiently. At this point they may then be capable of growing to a limited extent. This theory was tested by eliminating the effects of oxygen and oxidized media using pre-reduced media and diluents, and by performing all transfers, inoculations, dilutions, and samplings under an atmosphere of oxygen-free CO_2 (Holdeman and Moore, 1972).

Results from this study on an 18 hour inoculum of HT_9 and HT_{10} are illustrated in Figs. 11 and 12. The temperature first investigated for HT_9 and HT_{10} was 51.7 C and 51.5 C, respectively since these were the temperatures where the "Phoenix phenomenon" occurred in media prepared under aerobic conditions. However, with strict anaerobic conditions prevailing, HT_9 and HT_{10} grew readily at 51.7 C and 51.5 C, respectively after a 2-3 hour lag phase to give maximum counts of about 10^7 organisms/ml. The generation times were calculated for HT_9 and HT_{10} and found to be 18.2 minutes and 16.9 minutes, respectively.

These results indicate that with the elimination of oxygen and oxidized media, C. perfringens HT_9 and HT_{10} were able to grow readily at 51.7 C and 51.5 C, respectively. But before it could be concluded that it was oxygen or oxidized media that was causing the Phoenix effect the temperature had to be raised in small increments until death was observed. If the characteristic dip in counts was not observed then it could be concluded that the aerobic techniques employed by Collee et al. (1961) gave rise to the "Phoenix phenomenon".

As the temperature was increased however, the "Phoenix phenomenon" was observed. The most typical Phoenix effect was observed for HT_9 and

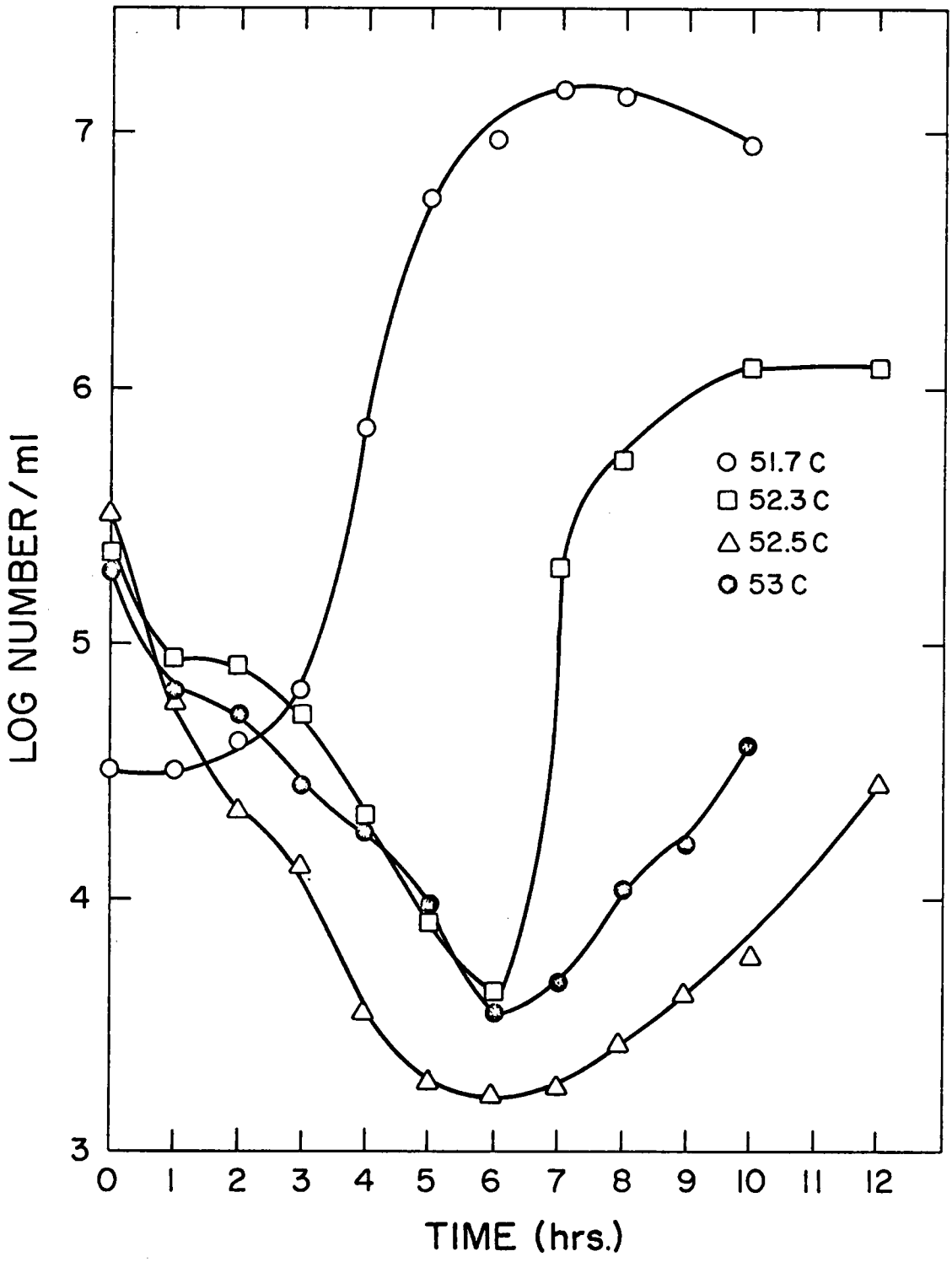


Fig. 11. Growth curves for C. perfringens, Hobbs' type 9 at various temperatures in pre-reduced cooked meat medium.

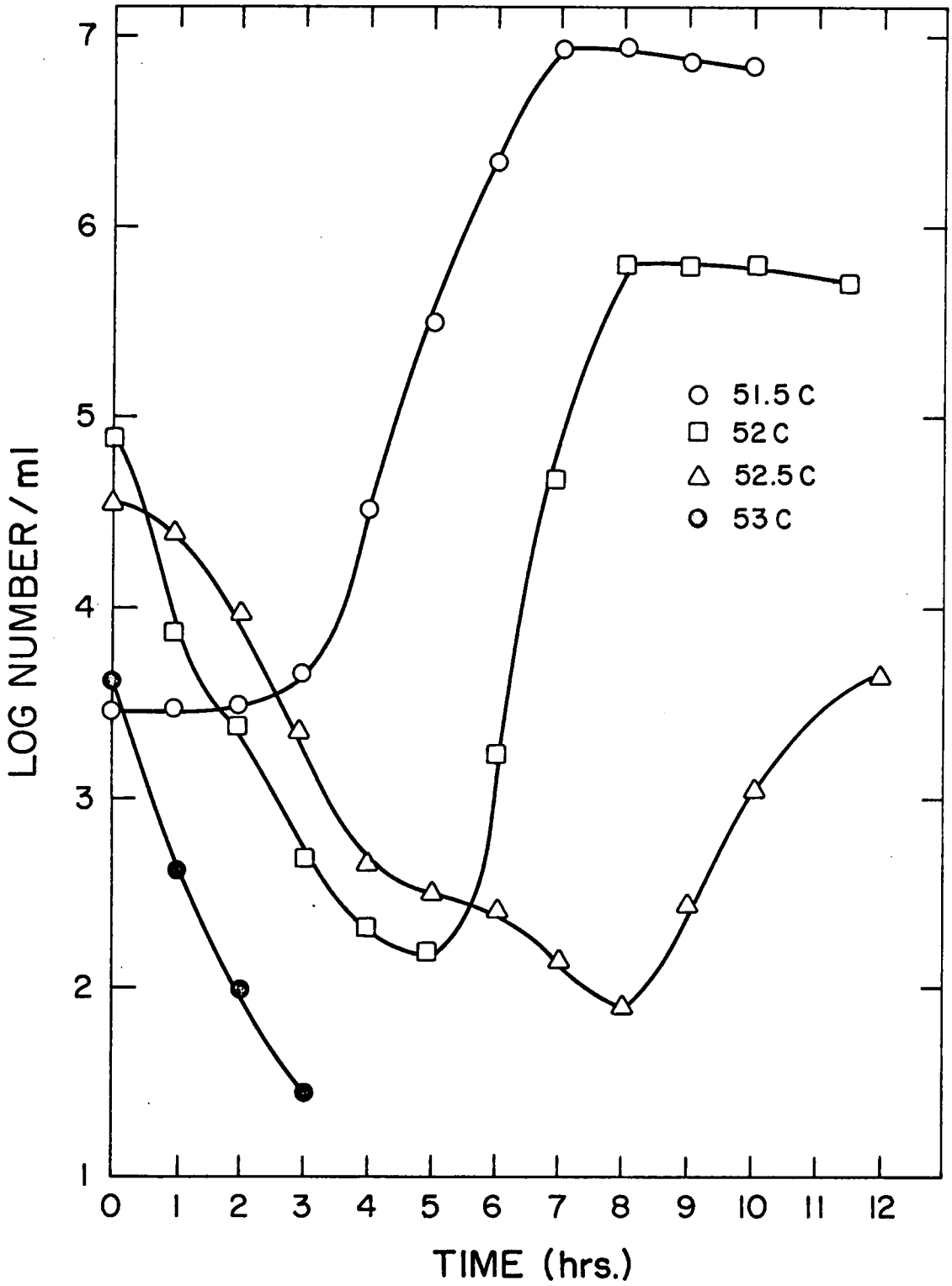


Fig. 12. Growth curves for C. perfringens, Hobbs' type 10 at various temperatures in pre-reduced cooked meat medium.

HT_{10} at 52.2 C and 52.0 C, respectively; only 0.5 C higher than it was observed in aerobically prepared media. A comparison between the pattern observed for the "Phoenix phenomenon" under aerobic and anaerobic conditions is given in Table 2. The time taken to reach minimum and maximum counts was increased under anaerobic conditions and overall, the "Phoenix phenomenon" was observed to a greater degree in pre-reduced media under strict anaerobic conditions.

Spore counts were performed throughout this study and were found to be less than 0.5/ml which is in agreement with previous results. From this study it can be concluded that by employing strict anaerobic techniques and thereby eliminating all exposure of oxygen and oxidized media to the organism, C. perfringens HT_9 and HT_{10} became a little more resistant to the effects of temperature, but the answer to the "Phoenix phenomenon" did not lie in this theory. Again in this study it was observed that HT_{10} was more sensitive to the effects of temperature since death was observed for that organism at 53.0 C.

E. Effect of Assay Medium on the "Phoenix Phenomenon"

To determine the effect of the assay medium on counts, five assay media (SIA, PYA, TSY, VA, and PVA) were investigated with an 18 hour culture of C. perfringens HT_9 and HT_{10}. No difference in counts was found for HT_9 and HT_{10} inoculated into "exhausted" growth menstruum at 51.7 C and 51.5 C, respectively and assayed on SIA, PYA, and TSY (Figs. 13 and 14) or assayed on SIA and VA (Figs. 15 and 16). Similarly, no difference in counts was found for HT_9 and HT_{10} inoculated into pre-reduced CMM at 52.2 C and 52.0 C, respectively and assayed on PYA and PVA (Figs. 17 and

Table 2. Comparison of results obtained under aerobic conditions to results obtained under anaerobic conditions.

	Results of Aerobic Study		Results of Anaerobic Study	
Strains tested	HT_9	HT_{10}	HT_9	HT_{10}
Temperature observed "Phoenix Phenomenon"	51.7 C	51.5 C	52.2 C	52.0 C
Time of m_c*	4 hrs.	4-5 hrs.	6 hrs.	5 hrs.
Time of M_c*	6 hrs.	6-7 hrs.	9-10 hrs.	8 hrs.
Log (I_c)*	5.9	4.8	5.4	4.9
Log (m_c)*	5.0	4.0	3.6	2.2
Log (M_c)*	7.0	6.0	6.1	5.8
Log ($I_c - m_c$)	0.9	0.8	1.8	2.7
Log ($M_c - I_c$)	1.1	1.2	0.7	0.9

* Where m_c = minimum counts

M_c = maximum counts

I_c = initial counts

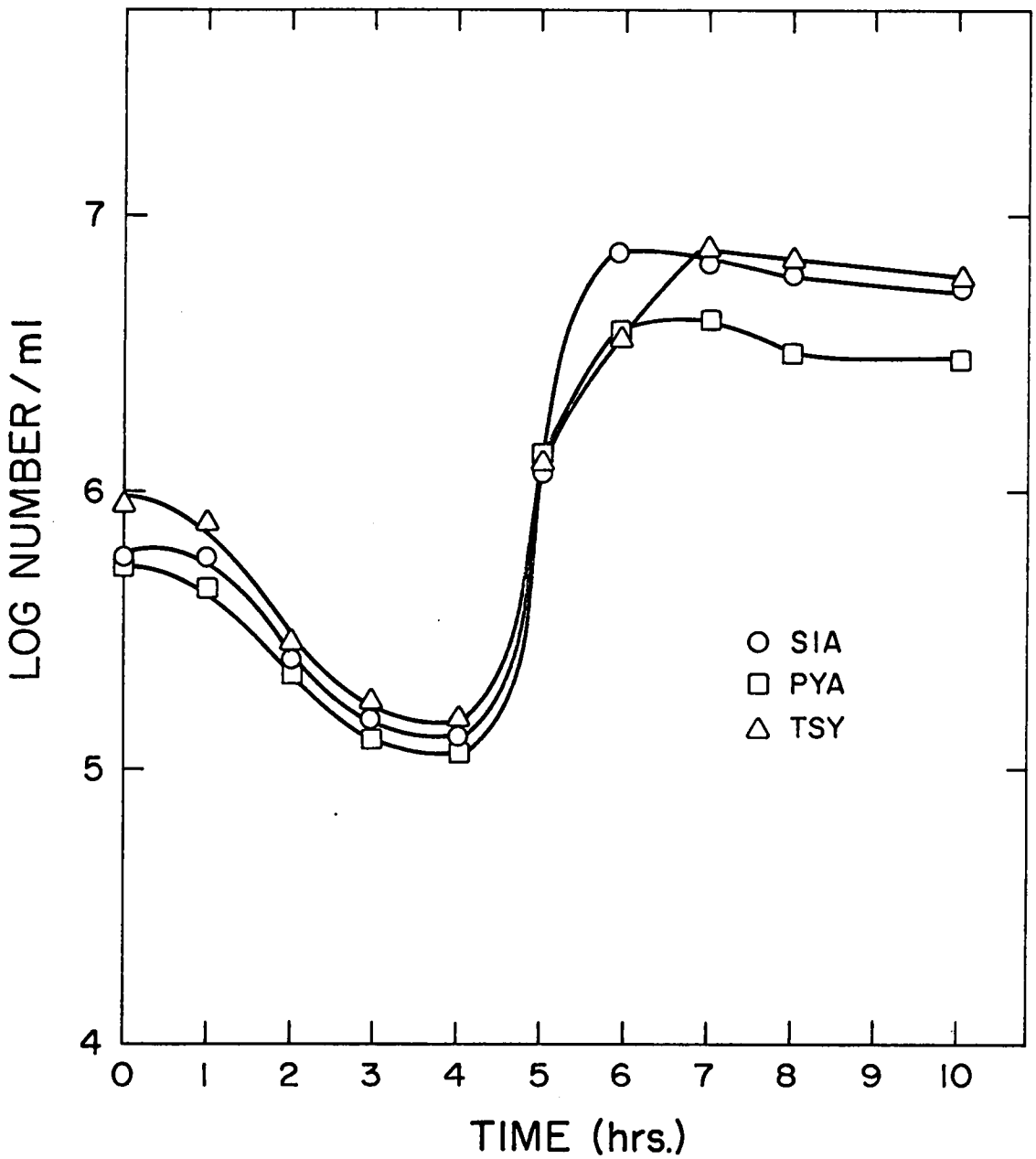


Fig. 13. Growth curve for C. perfringens, Hobbs' type 9 at 51.7 C in cooked meat medium and assayed on sulfite-iron agar (SIA), peptone-yeast extract agar (PYA), and tryptose-soytone-yeast extract agar (TSY).

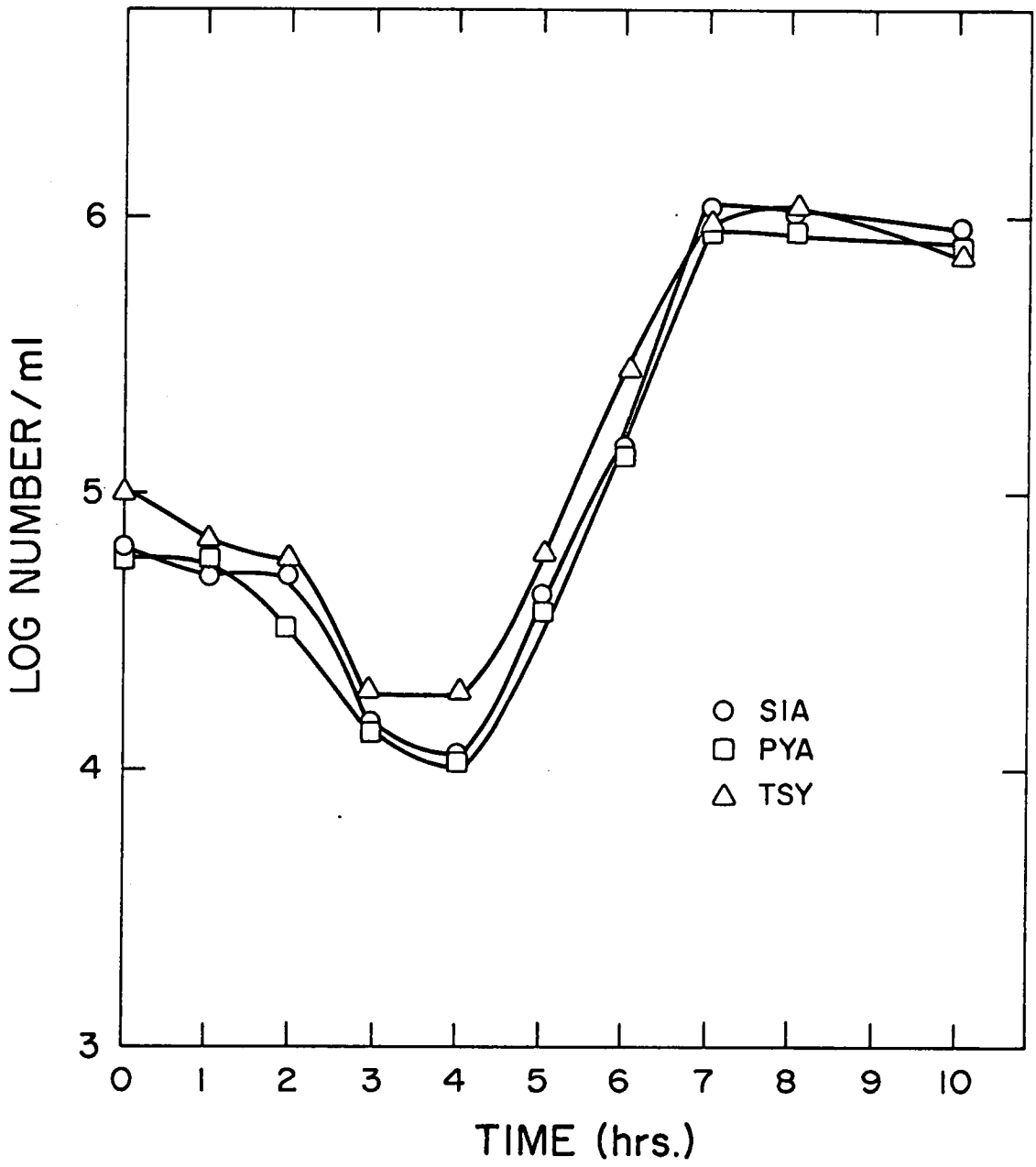


Fig. 14. Growth curve for C. perfringens, Hobbs' type 10 at 51.5 C in cooked meat medium and assayed on sulfite-iron agar (SIA), peptone-yeast extract agar (PYA), and tryptose-soytone-yeast extract agar (TSY).

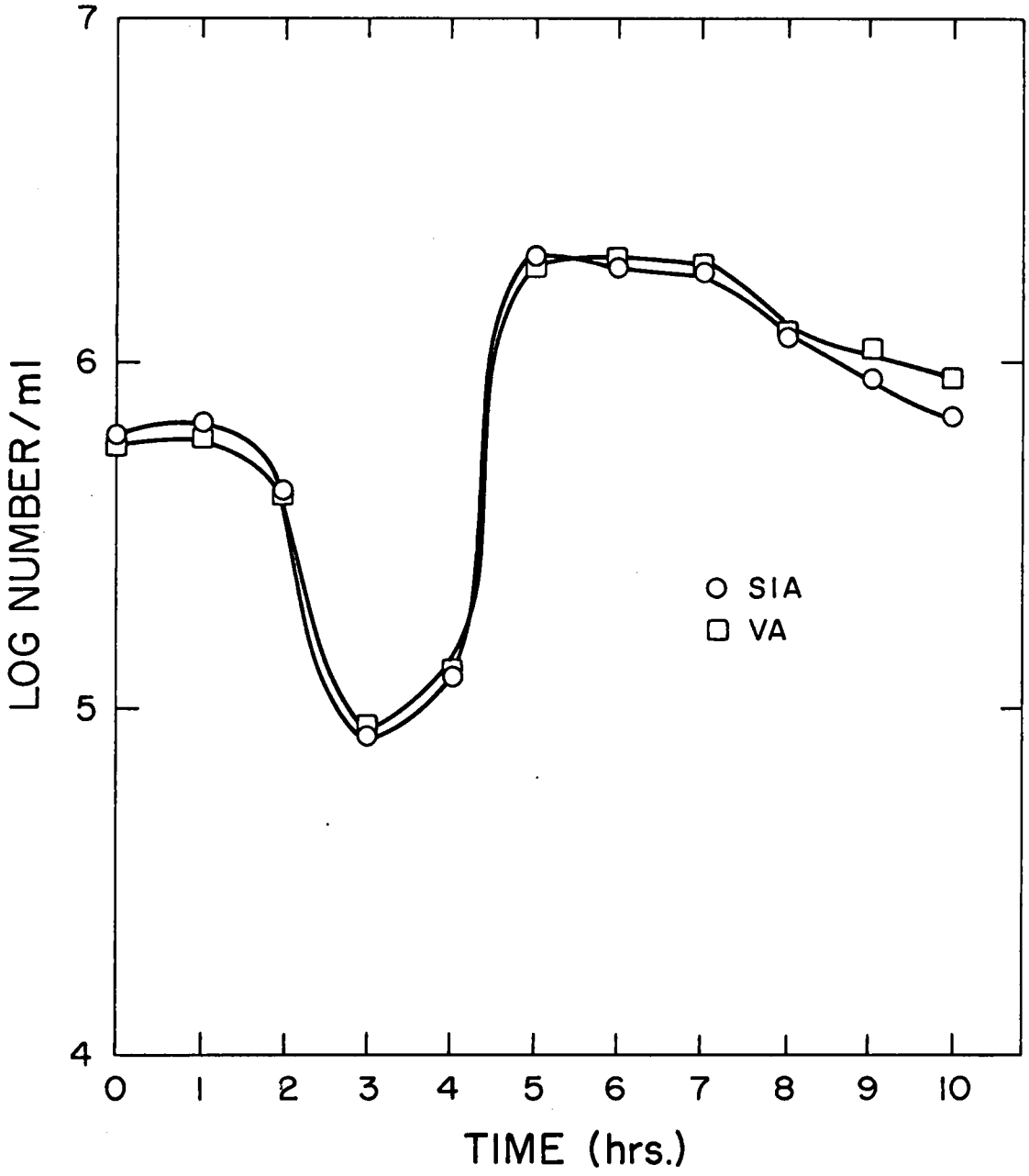


Fig. 15. Growth curve for C. perfringens, Hobbs' type 9 at 51.7 C in cooked meat medium and assayed on sulfite-iron agar (SIA), and veal agar (VA).

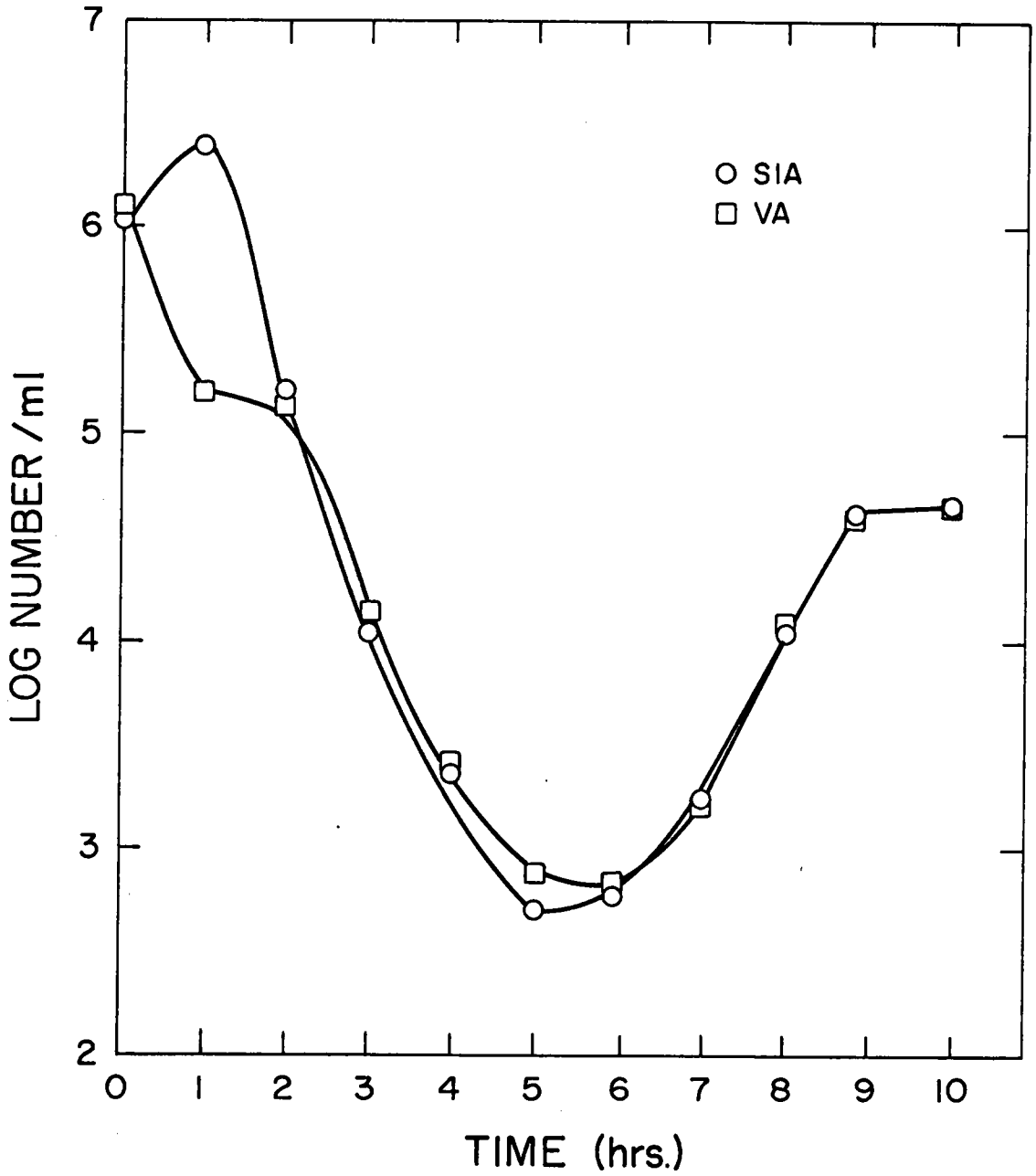


Fig. 16. Growth curve for C. perfringens, Hobbs' type 10 at 51.5 C in cooked meat medium and assayed on sulfite-iron agar (SIA), and veal agar (VA).

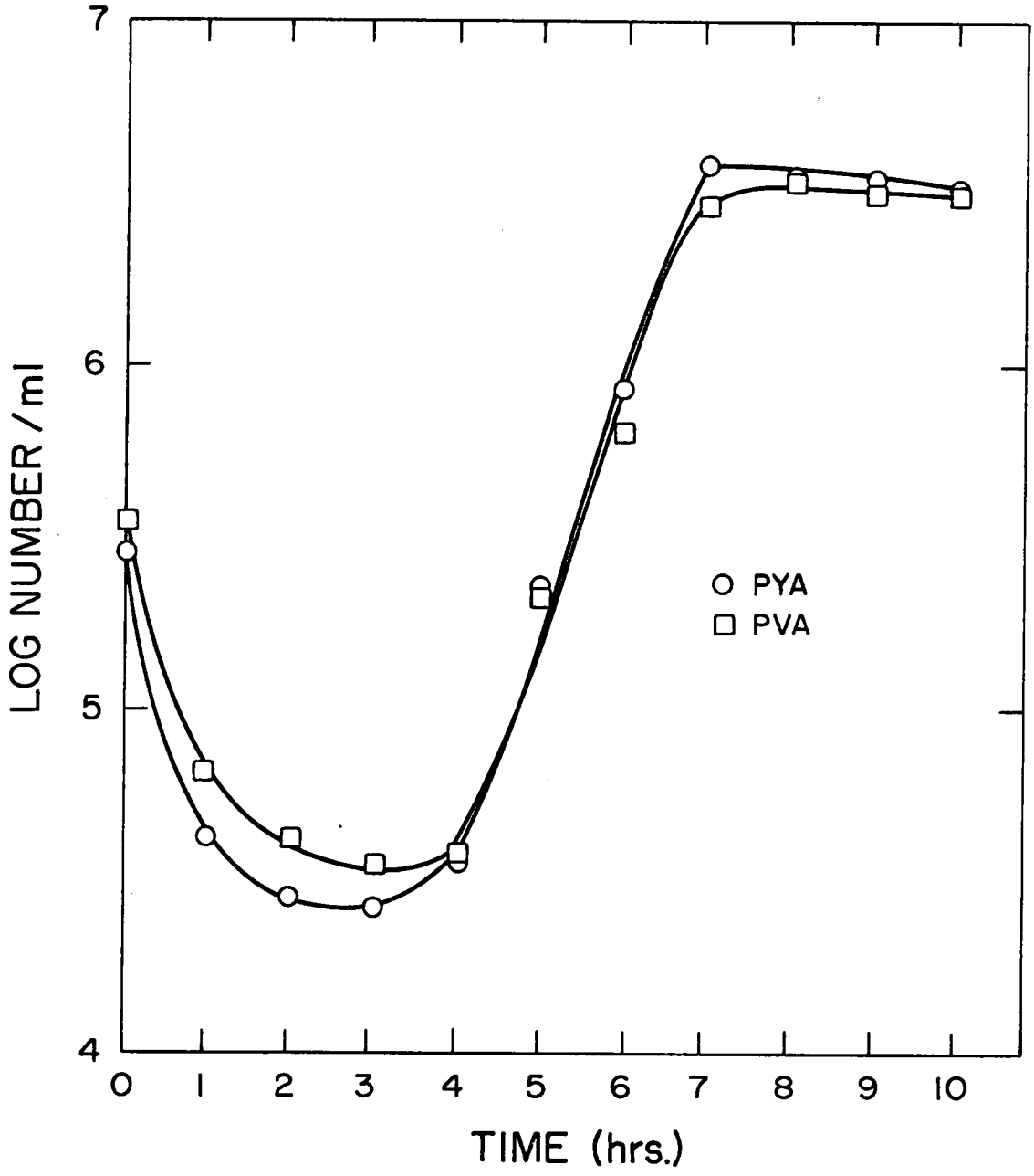


Fig. 17. Growth curve for C. perfringens, Hobbs' type 9 at 52.2 C in pre-reduced cooked meat medium and assayed on pre-reduced peptone-yeast extract agar (PYA) and pre-reduced veal agar (PVA).

18). The "Phoenix phenomenon" was observed on all the assay media tested and thus, the assay medium appeared to have no effect on the "Phoenix phenomenon".

F. Effect of Nalidixic Acid on the "Phoenix Phenomenon"

The "Phoenix phenomenon" appeared to represent three phases. Phase I represented an apparent decrease in the number of viable cells, Phase II represented an apparent increase in viable cells to the initial level of cells, and Phase III represented an increase in cell numbers above that of the initial inoculum. However, Phase III appeared to be very sensitive because it was not consistantly observed. All the possible explanations postulated by Collee et al. (1961) represented Phase II as the growth of cells, either as a result of outgrowth of germinated spores, or after mutagenesis to give heat adapted variants. Also it was possible that Phase I was the dying off of old cells and Phase II was the growth of younger cells which may be present in low numbers in the initial inoculum. The age of inoculum study neither proved or disproved this theory.

On the other hand, Phase II of the "Phoenix phenomenon" could represent an injury and recovery phenomenon whereby there is no change in cell numbers during Phase I and II. The viable cells could because of the initial temperature shock become sensitive to assay conditions (Phase I), but after a period of time these cells could recover their tolerance to assay conditions (Phase II) and finally grow (Phase III). Injury of bacteria has been interpreted as the increased sensitivity to otherwise uninhibitory compounds in the growth media. Normally, bacterial cells are injured at a sublethal temperature in a buffer, removed, washed,

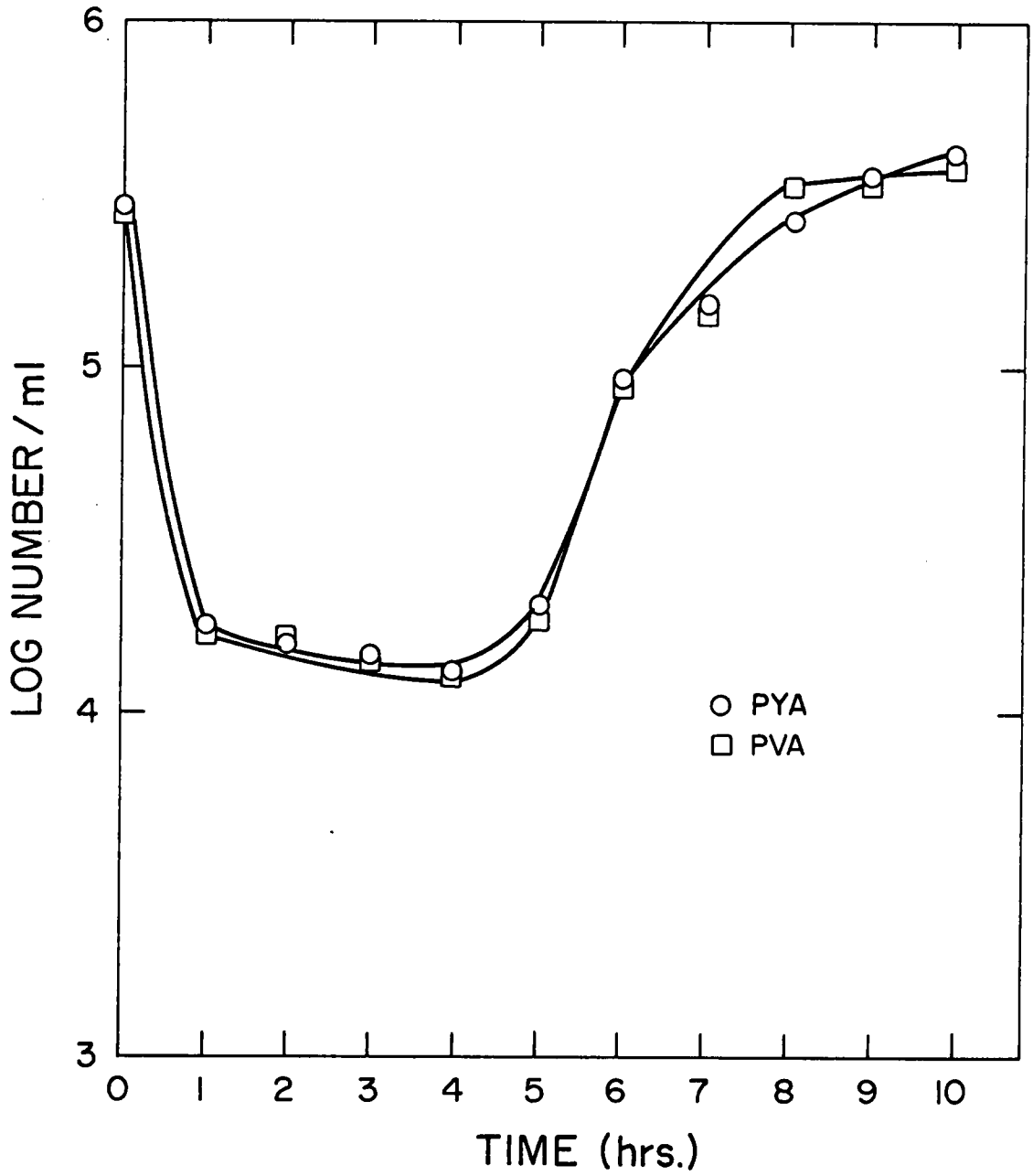


Fig. 18. Growth curve for C. perfringens, Hobbs' type 10 at 52.0 C in pre-reduced cooked meat medium and assayed on pre-reduced peptone-yeast extract agar (PYA) and pre-reduced veal agar (PVA).

and recovered in a "recovery vessel" containing fresh medium in which the cells can grow (usually at a lower temperature). Heat injured bacteria are normally detected by comparing counts obtained on a non-stress medium to counts obtained on a stress medium. The difference in counts represents the degree of injury (Iandolo and Ordal, 1966).

Ades (1973) demonstrated heat injury and recovery of cells of C. perfringens HT_{10} in the same growth vessel, at the same temperature (51.0 C), and in a medium which was suitable for growth (peptone-yeast extract). The extent of injury was detected by comparing the counts obtained on peptone-yeast extract agar (PYA) to counts obtained on peptone-yeast extract agar + 1.5% NaCl (PYAS). The addition of the salt served as the stress factor to the sensitive cells.

To determine whether growth or recovery of injured cells was taking place in Phase II of the "Phoenix phenomenon", a growth inhibitor was added at the start of Phase II. If Phase II was observed, then recovery of injured cells was what was taking place in the "Phoenix phenomenon", whereas if Phase II was not observed, then growth of bacterial cells was what was taking place in the "Phoenix phenomenon". The inhibitor chosen for this investigation was nalidixic acid (NA). NA has been found to have a specific mode of action against bacterial DNA synthesis (Bauernfeind, 1971; Franklin and Snow, 1971). Its ability to inhibit DNA synthesis has been readily demonstrated in intact bacteria by incorporation experiments with Escherichia coli (Cook et al., 1966b,c; Deitz et al., 1966; Goss et al., 1964, 1965) and with Bacillus subtilis (Cook et al., 1966a). Protein and RNA synthesis continue almost uninfluenced for some time after exposure to the minimal inhibitory concentration (MIC) of the drug (Cook et al.,

1966a). Miller and Ordal (1972) found that upon addition of a MIC of NA to the recovery medium, heat injured Bacillus subtilus cells were able to recover but subsequent multiplication was completely arrested. Similarly, Ades (1973) found when NA was added to heat injured C. perfringens HT_{13} cells they were able to recover, and he concluded that DNA synthesis was not required during recovery of C. perfringens HT_{13} cells.

Using a concentration of 35 µg/ml of NA, it was found from the present study for C. perfringens HT_9 and HT_{10} under both aerobic and anaerobic conditions that Phase II was observed and Phase III was not (Figs. 19-22). However, Phase III was observed in the control vessel. Thus, it can be concluded that Phase II of the "Phoenix phenomenon" is due to recovery of bacterial cells and not do to cell growth. The fact that growth is not occurring is consistant with previous results where theories that depended on growth in Phase II have been shown not to have an effect on the "Phoenix phenomenon" (i.e. the effect of spores and age of inoculum). The findings of the present study are similar to the findings of Ades (1973) except that the injured cells were observed on all the assay media tested. Ades (1973) using a peptone-yeast extract heating menstruum found a non-stress assay medium that consisted of the same ingredients as the heating menstruum (PYA). In an analogous way, VA and PVA were tested as possible non-stress assay media (section E) with a heating menstruum of CMM and PCMM, respectively. However, under both aerobic and anaerobic conditions the "Phoenix phenomenon" was reproduced. From this study the existence of a non-stress medium cannot be ruled out, but that may be the case. The cells of C. perfringens could be so sensitized in CMM or PCMM, that any assay medium chosen could, in a sense, be a stress medium.

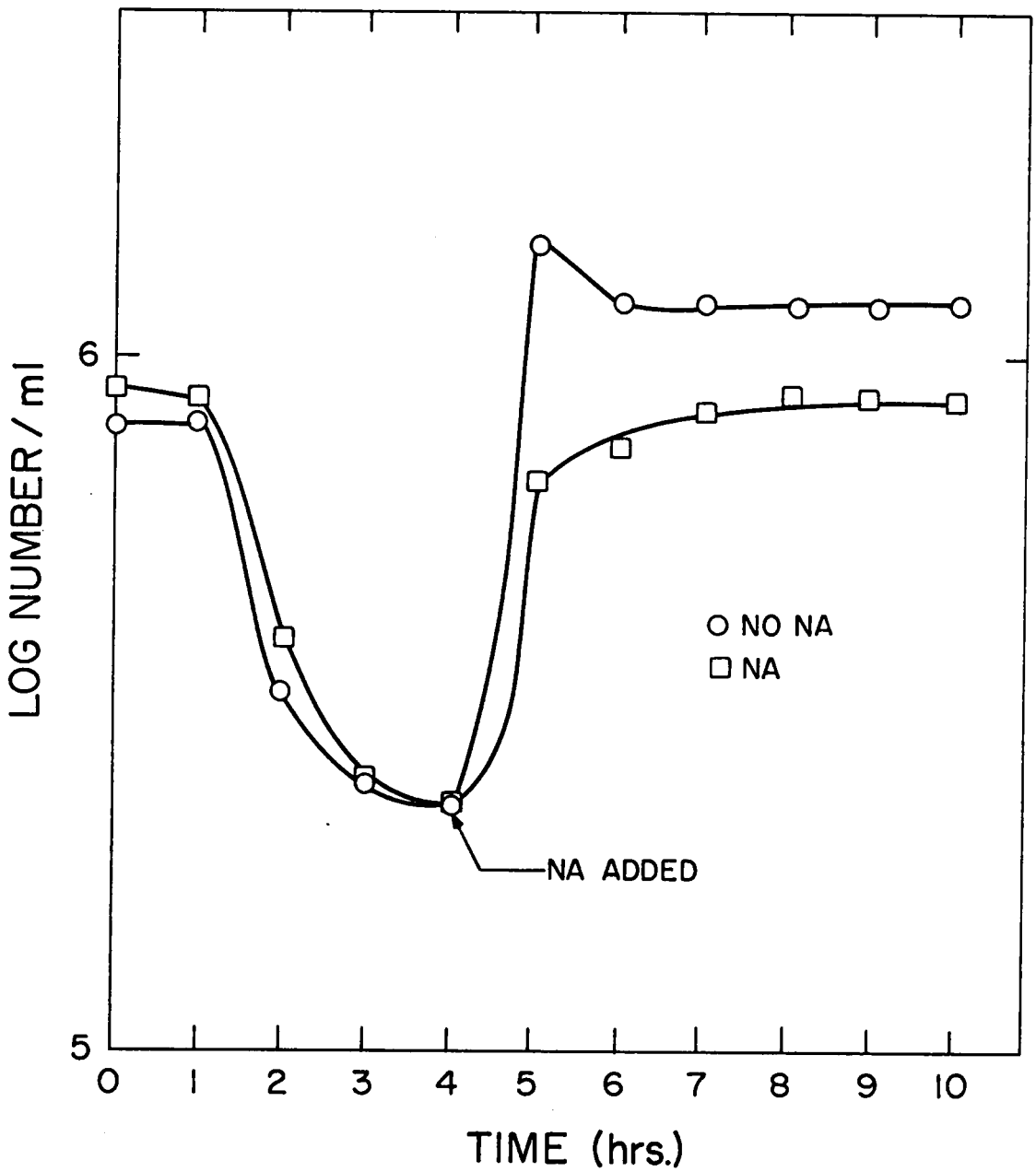


Fig. 19. Growth and recovery curves for C. perfringens, Hobbs' type 9, heated at 51.7 C in cooked meat medium. Two growth vessels were employed; one served as the control, and to the other was added enough nalidixic acid (NA) at 4 hours to give a final concentration of 35 µg/ml. SIA served as the assay medium.

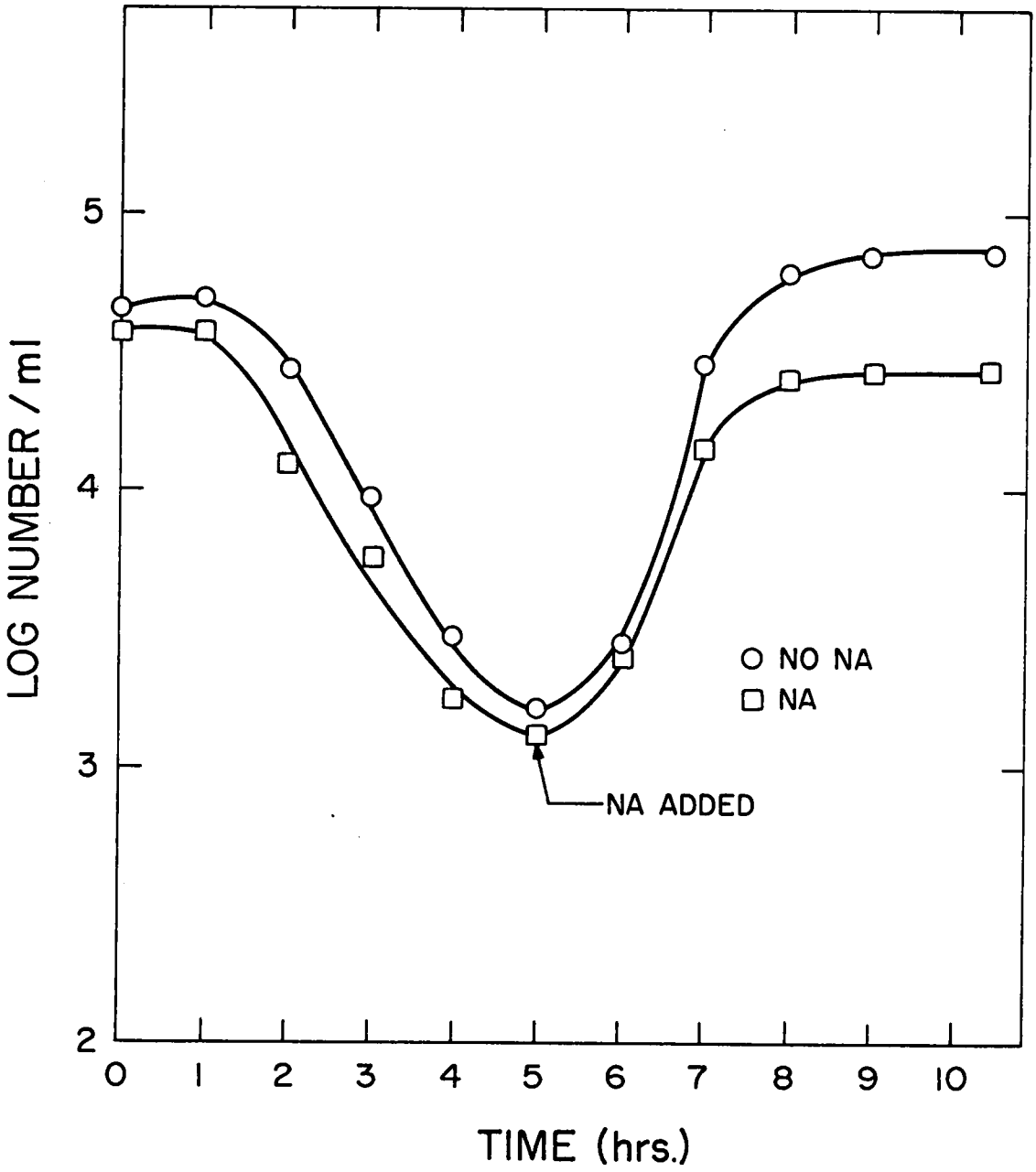


Fig. 20. Growth and recovery curves for C. perfringens, Hobbs' type 10, heated at 51.5 C in cooked meat medium. Two growth vessels were employed; one served as the control, and to the other was added enough nalidixic acid (NA) at 5 hours to give a final concentration of 35 µg/ml. SIA served as the assay medium.

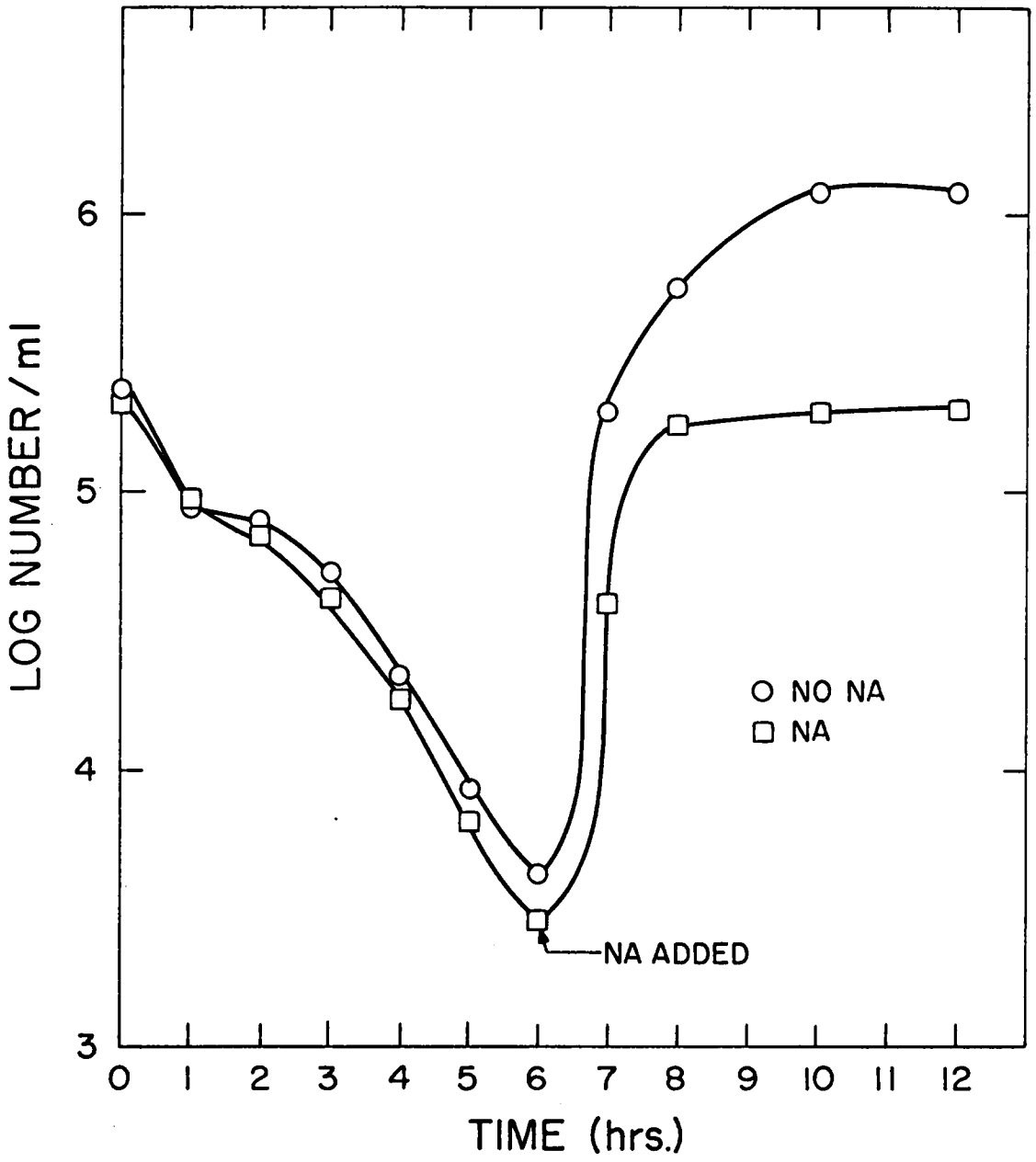


Fig. 21. Growth and recovery curves for C. perfringens, Hobbs' type 9, heated at 52.2 C in pre-reduced cooked meat medium. Two growth vessels were employed; one served as the control, and to the other was added enough nalidixic acid (NA) at 6 hours to give a final concentration of 35 µg/ml. PYA served as the assay medium.

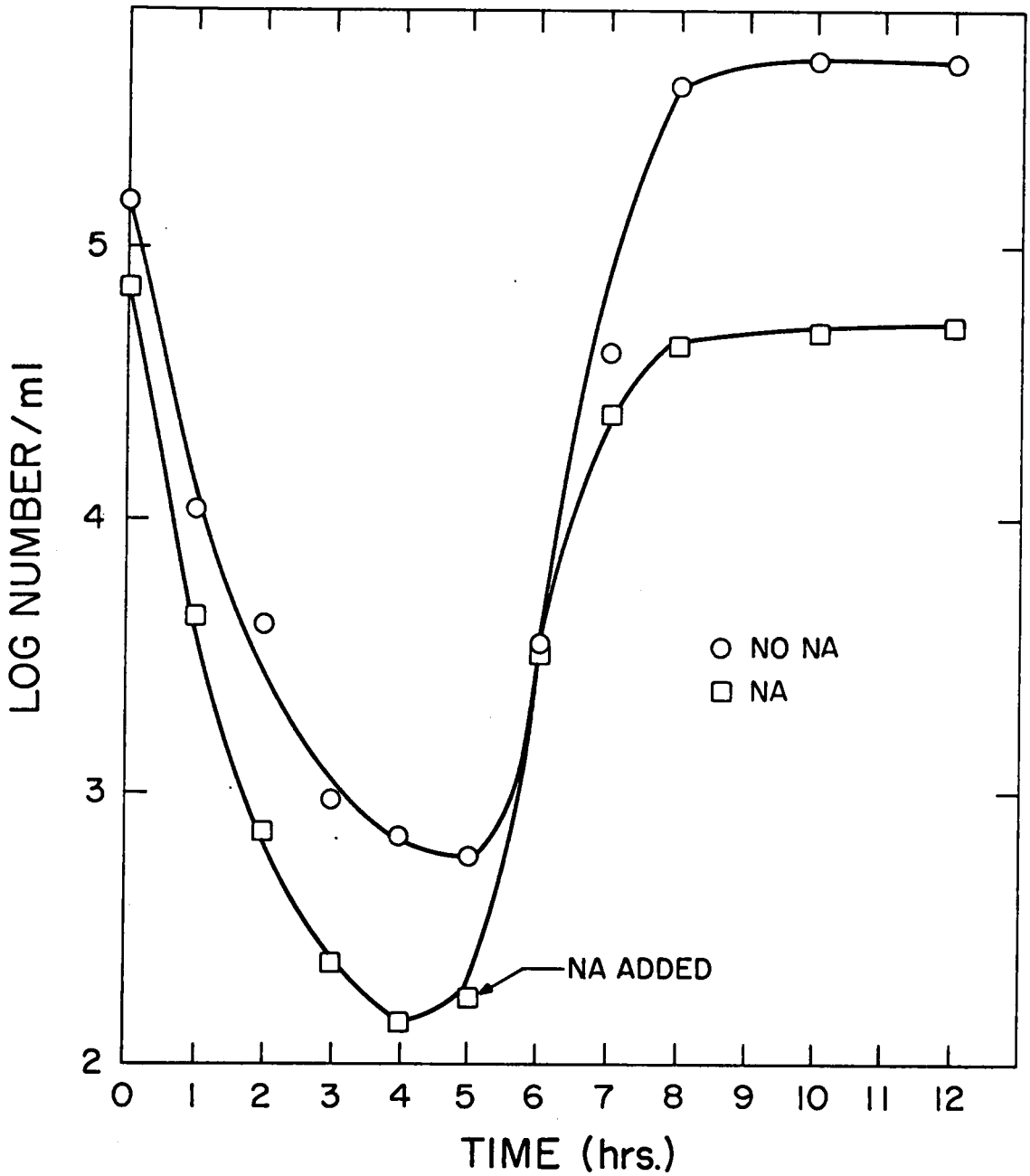


Fig. 22. Growth and recovery curves for C. perfringens, Hobbs' type 10, heated at 52.0 C in pre-reduced cooked meat medium. Two growth vessels were employed; one served as the control, and to the other was added enough nalidixic acid (NA) at 5 hours to give a final concentration of 35 µg/ml. PYA served as the assay medium.

In any case, it was shown by the use of nalidixic acid (NA) that Phase I and II of the "Phoenix phenomenon" was an injury and recovery phenomenon. Moreover, it has been proven in the control vessel that after cells are recovered, growth occurs until maximum counts are reached (Phase III). Throughout this study it was this growth phase ($M_c - I_c$) that was somewhat variable between experiments. Sometimes the cells were able to grow to maximum counts, while at other times, only recovery took place. This observation illustrates the extreme sensitivity and vulnerability of C. perfringens to any variation at this sublethal temperature.

G. Effect of Diluent on the "Phoenix Phenomenon"

Phase I and II of the "Phoenix phenomenon" were found to represent an injury and recovery system, but a non-stress assay medium had not been found. Gray (1973) studied the effect of temperature shifts on the growth of Pseudomonas aeruginosa. He found an apparent decrease in viable cell numbers followed by an unusually rapid increase in cell numbers when the temperature was shifted from 25 C to 36 C and back to 25 C. This phenomenon was negated when a diluent of the same composition as the growth medium was used. In the present research the diluent used was composed of a different medium than the heating menstruum, and thus this could be the source of stress to the organism. Also the temperature of the diluent might be significant, in that some stress might have been exerted on the cells when they were sampled from a heating menstruum held between 51.5 C and 52.2 C into a diluent tempered at 25 C. Both these possibilities were then investigated under aerobic (Figs. 23 and 24) and anaerobic (Figs. 25 and 26) conditions. The length of time the cells

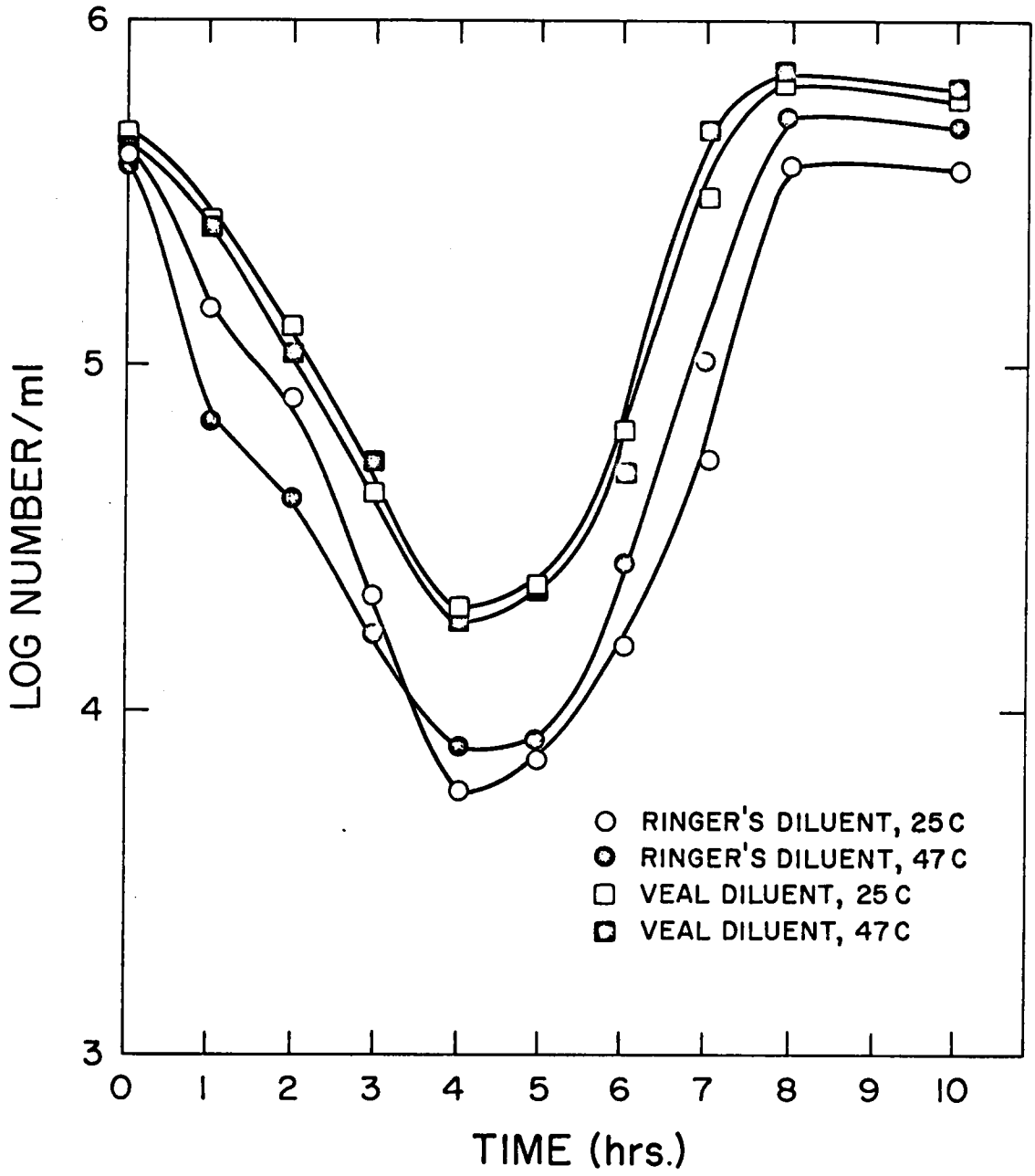


Fig. 23. Growth curves for C. perfringens, Hobbs' type 9 at 51.7 C in cooked meat medium using a Ringer's diluent at 25 C and 47 C and a veal diluent at 25 C and 47 C and assayed on veal agar (VA).

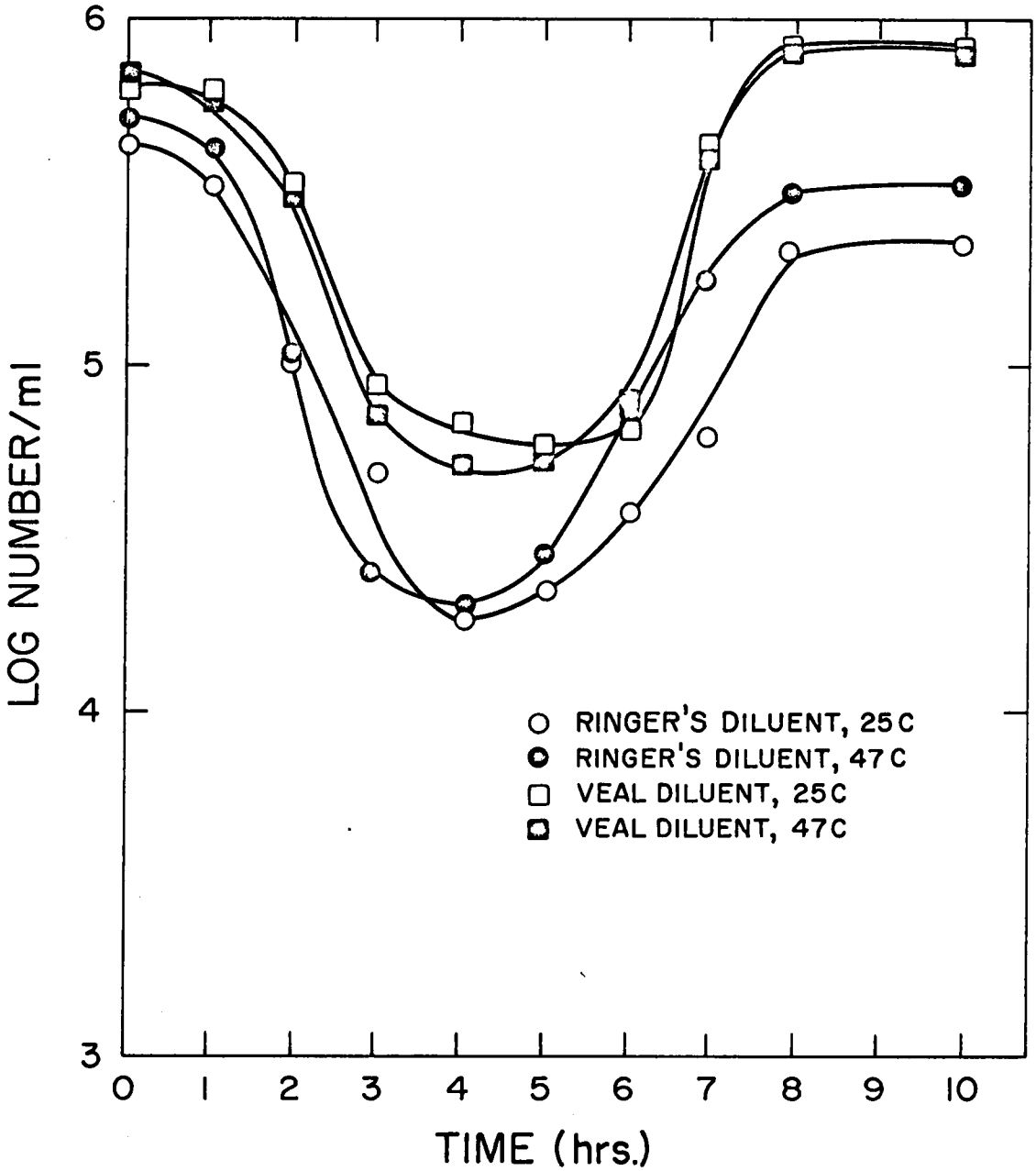


Fig. 24. Growth curves for C. perfringens, Hobbs' type 10 at 51.5 C in cooked meat medium using a Ringer's diluent at 25 C and 47 C and a veal diluent at 25 C and 47 C and assayed on veal agar (VA).

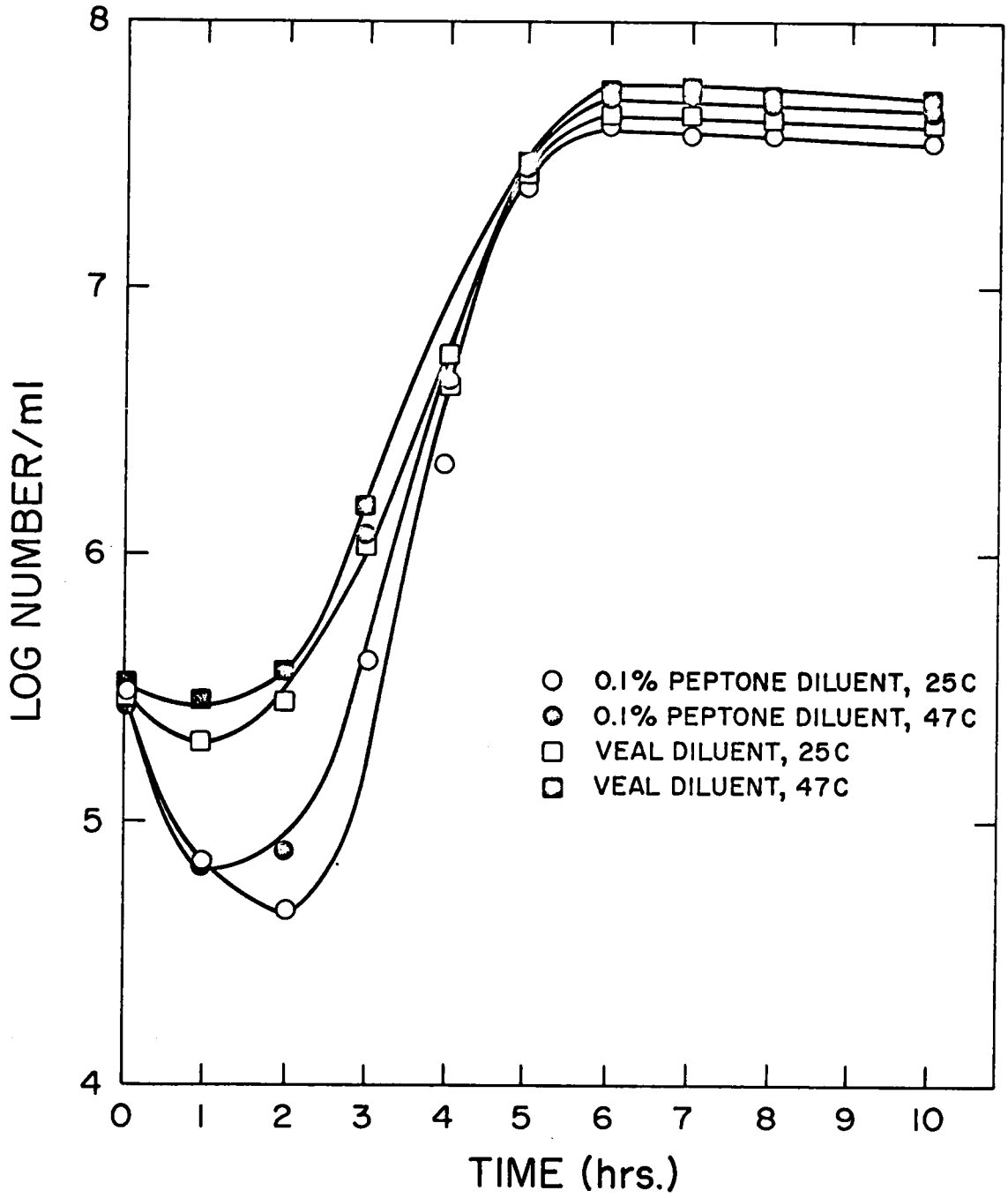


Fig. 25. Growth curves for C. perfringens, Hobbs' type 9 at 52.2 C in pre-reduced cooked meat medium using a 0.1% peptone diluent at 25 C and 47 C and a veal diluent at 25 C and 47 C and assayed on pre-reduced veal agar (PVA).

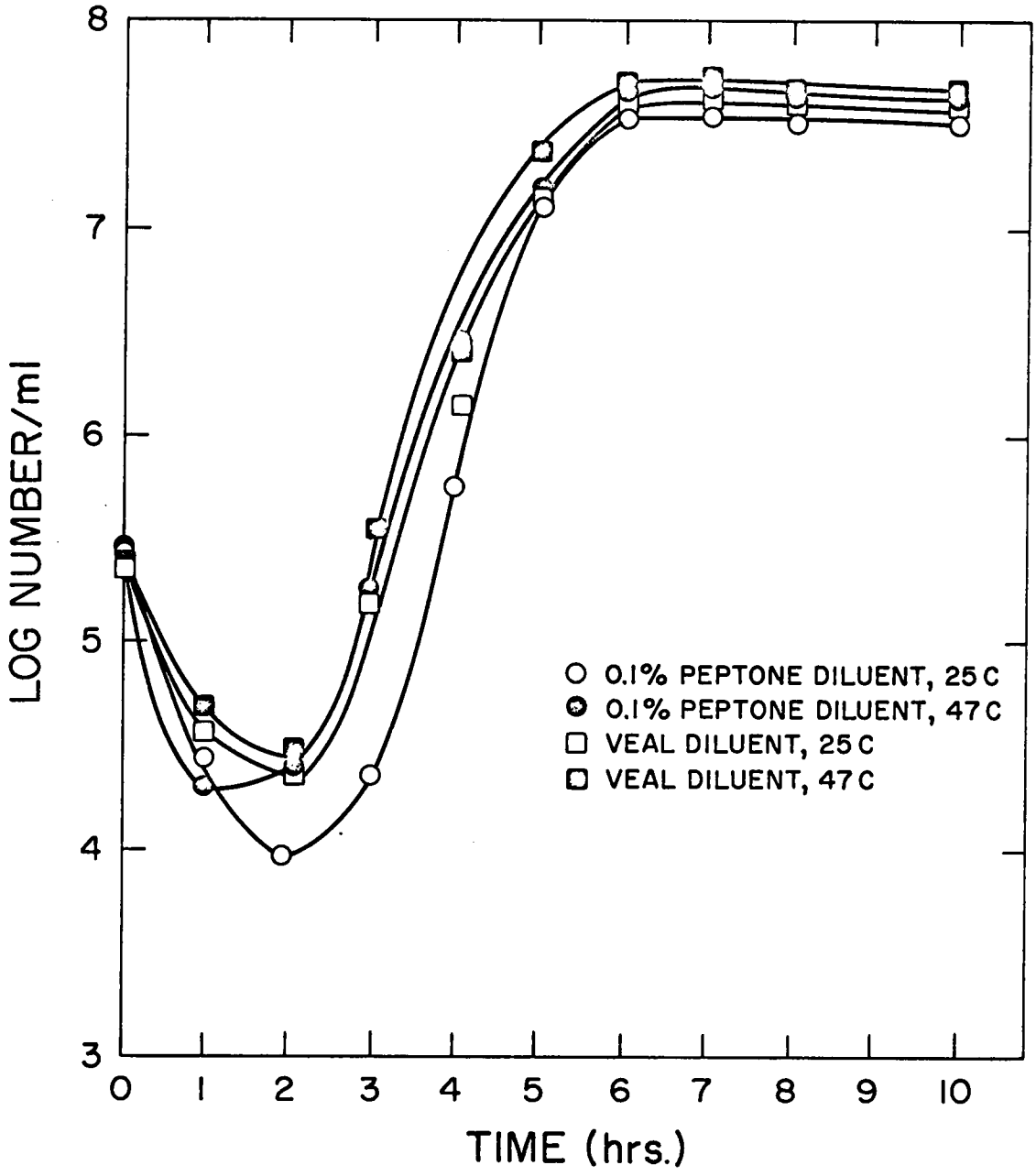


Fig. 26. Growth curves for C. perfringens, Hobbs' type 10 at 52.0 C in pre-reduced cooked meat medium using a 0.1% peptone diluent at 25 C and 47 C and a veal diluent at 25 C and 47 C and assayed on pre-reduced veal agar (PVA).

were maintained in the diluent was important since growth could occur in the veal diluent. This time interum was minimized as much as possible and at no time was it greater than 5-6 minutes.

The results from the studies using both aerobic and anaerobic conditions indicated that some of the stress to strains HT_9 and HT_{10} was relieved by the use of a veal diluent. In all cases, the counts decreased to a lesser extent using the veal diluent. However, the temperature of the diluent was found to have no effect on the "Phoenix phenomenon". Thus, the results from this study are similar to the results obtained by Gray (1973).

SUMMARY AND CONCLUSIONS

The "Phoenix phenomenon" as first observed by Collee et al. (1961) was investigated. This unusual growth phenomenon was observed for an 18 hour inoculum of C. perfringens, Hobbs' type 9 (HT_9) and Hobbs' type 10 (HT_{10}) at 51.7 C and 51.5 C, respectively in a CM medium at a pH of 7.0 ($\pm$0.1). The diluent used was quarter strength Ringer's solution and the assay medium was sulfite-iron agar (SIA). The entire procedure except for the incubation of the SIA tubes was performed in "exhausted" broth media. The SIA tubes were incubated in an anaerobe jar. Spore counts were performed on all samples by heating them for 10 minutes at 80 C, and they were found to be at a very low level at this temperature. The growth curves exhibited by C. perfringens HT_9 and HT_{10} at these temperatures were in good agreement with the growth curves observed by Collee et al. (1961) at 50 C for C. perfringens HT_{10}. Possible explanations for this unusual dip in counts followed by growth were then investigated.

The role of spores was examined using sporulation negative mutant strains of C. perfringens (8-1, 8-5, 8-7, 8-16, and 8-17) derived from HT_9. It was thought by Collee et al. (1961) that the "Phoenix phenomenon" might depend on survival of spores followed by germination and outgrowth. However, the "Phoenix phenomenon" was observed by each of the mutant strains although they did seem to be more sensitive to the effects of temperature. It therefore was concluded from this study that spores of C. perfringens did not have an effect on the "Phoenix phenomenon".

The effect of age of inoculum was studied for C. perfringens HT_9 and HT_{10}. In general, the "Phoenix phenomenon" became more pronounced as

the age of inoculum (2, 4, 8, 12, and 18 hours) was increased although maximum counts decreased. The "Phoenix phenomenon" was reproduced in nearly all the age of inocula studies and thus, the age of inoculum did not appear to be appear to be the answer to the "Phoenix phenomenon".

The effect of oxygen and oxidized media was then investigated. Using the anaerobic techniques outlined by Holdeman and Moore (1972) and pre-reduced media and diluents, all exposure of the organism to oxygen and oxidized media was essentially eliminated. This technique allowed C. perfringens HT_9 and HT_{10} to grow at a higher temperature than with oxygen present. Furthermore, the Phoenix effect was observed 0.5 C higher than in media which was not pre-reduced which was a significant increase at this temperature level. It was found that minimum and maximum counts describing the "Phoenix phenomenon" were attained after a longer period of time as compared to studies using media which was not pre-reduced. It was concluded from this study that strict anaerobic conditions did not eliminate the "Phoenix phenomenon".

The effect of the assay medium was examined to determine if the sulfite-iron agar (SIA) medium was a stress medium since viable cells may still be present and the assay medium used was possibly inhibitory. SIA was compared to peptone-yeast extract agar (PYA), tryptose-soytone-yeast extract agar (TSY) and veal agar (VA). No difference in counts was observed and the "Phoenix phenomenon" was reproduced. Similarly, using strict anaerobic conditions throughout, PYA was compared to PVA, and again, there was no difference in counts and the "Phoenix phenomenon" was reproduced. Direct microscopic counts (DMC's) were attempted but the number of cells were too low to be detected by this method.

A study was performed to determine whether the increase in counts from a minimum level decribed by the "Phoenix phenomenon" to the initial level (Phase II) was a result of growth or a result of recovery of injured cells. By the addition of the minimal inhibitory concentration (MIC) of the metabolic inhibitor, nalidixic acid (NA), at the minimum point described by the "Phoenix phenomenon" under both aerobic and anaerobic conditions, it was found for C. perfringens HT_9 and HT_{10} that recovery of injured cells was taking place and not growth.

A final investigation was made on the effect of diluent. The temperature of the diluent was found to be independent of the final counts obtained, but the veal diluent did affect the counts obtained on veal agar. Using both aerobic and anaerobic conditions the veal diluent afforded a less pronounced Phoenix effect and appeared to relieve some of the stress to the organism.

It is concluded that the "Phoenix phenomenon" consists of three phases: injury, recovery, and growth. The cells of C. perfringens HT_9 and HT_{10} were injured, recovered, and later grown in the same "injury vessel" at a constant temperature. The injured and recovered cells were detected on all the assay media studied, and either all these media exhibited stress to the organism and a non-stress medium might still be found, or there is no non-stress medium. However, viable cells may still be present in the heating menstruum during Phase I and II and these cells can repair this injury and finally grow. By using a veal diluent some of the stress on the organism was relieved. The growth phase (Phase III) was found to occur only part of the time and appeared to be extremely sensitive to all factors involved in the experiment.

One study remaining to further elucidate the "Phoenix phenomenon" is an in depth study on the mechanism of the recovery process. One method that has successfully been used is a radiotracer technique of Tomlins and Ordal (1971). They followed incorporation of uracil-6-^{3}H and L-leucine-U-^{14}C into recovering cells of Salmonella typhimurium to observe the extent of RNA and protein synthesis, and followed the incorporation of thymidine-methyl-^{3}H and L-leucine-U-^{14}C to observe the extent of DNA synthesis. An adaptation of this technique to the present study would give much information on the mechanism of recovery in the "Phoenix phenomenon".

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A STUDY OF THE "PHOENIX PHENOMENON" IN CLOSTRIDIUM PERFRINGENS

by

SHARON L. PAYNE

(ABSTRACT)

The "Phoenix phenomenon" as first noted by Collee et al. (1961) was investigated for Clostridium perfringens HT_9 and HT_{10}. The "Phoenix phenomenon" is described by an initial decrease in counts (Phase I), followed by an increase to the initial level of counts (Phase II), and continued increase above the initial counts to give maximum counts (Phase III). Under similar conditions as employed by Collee et al. (1961), the "Phoenix phenomenon" was reproduced for C. perfringens HT_9 and HT_{10} at 51.7 C and 51.5 C, respectively. The effect of sporulation, age of inoculum, anaerobiosis, assay medium, growth inhibitors, and diluent were studied. The "Phoenix phenomenon" was reproduced in experiments using sporulation negative mutants derived from HT_9, various ages of inocula, and under strict anaerobic conditions. Similarly, all of the assay media tested: sulfite-iron agar, tryptose-soytone-yeast extract agar, peptone-yeast extract agar, veal agar, and pre-reduced veal agar, gave the "Phoenix phenomenon". The presence of a DNA inhibitor at the minimum point of the growth curve (end of Phase I) had no effect on the appearance of the "Phoenix phenomenon" (Phase II), however Phase III was completely inhibited. This indicated that Phase I and II was an injury-recovery process. The type and temperature of diluent was varied and the results indicate that although temperature of diluent had no effect on enumeration, a veal diluent relieved some of the stress to the organism in giving a less pronounced Phoenix effect.