THE ROLE OF NUTRITIONAL INDEPENDENCE IN THE PATHOGENICITY OF NEISSERIA MENINGITIDIS IN THE CHICK EMBRYO

by

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INTRODUCTION

A. Historical Advances in Nutrition and Pathogenicity of Neisseria

1. The Nutritional Requirements of Neisseria meningitidis

Weichselbaum (49) in 1887 isolated and accurately described a diplococcus present in the meningeal exudate from 6 cases of cerebrospinal meningitis. His observations concerning the morphology, staining reaction and viability of this microorganism became the subject of such controversy among investigators that it was not until 1900 that they were finally confirmed, firmly establishing this diplococcus as the etologic agent of epidemic cerebrospinal meningitis. Strains of this bacterium have since been classified as Neisseria meningitidis. The early confusion concerning N. meningitidis has been perpetuated to the present time. Controversy still exists concerning the morphological, cultural and biological characters of the meningococcus.

The nutritional requirements of N. meningitidis strains have been met primarily by complex media and an atmosphere containing carbon dioxide. In 1909 Elser and Huntoon (16) conducted extensive studies on the cultural characteristics of the meningococcus and employed ascitic fluid in solid and liquid media for its isolation and maintenance. Gordon and Hine (27) advocated a pea meal extract agar medium for carrier detection in 1916. This medium consisted essentially of a tryptic digest of meat, a 5% sodium chloride extract of pea flour, and agar. In 1941, Mueller and Hinton (41) developed a medium which was based on the essential component parts of the Gordon and Hine pea meal extract agar. They discovered that the active growth-promoting

fraction of the pea flour extract was due to starch and could be replaced by numerous varieties of starch. Their final medium consisted of starch, meat infusion and a complete acid hydrolyzate of casein. This relatively simplified medium was found superior to chocolate agar in a series of primary isolations of N. meningitidis strains. Other complex solid media which have been employed in the cultivation of the meningococcus include blood agar, chocolate agar and dextrose-starch agar. Recent studies by Jyssum (29) indicate that strains of N. meningitidis may be grown and repeatedly subcultured on solid media containing ammonium ion as the sole nitrogen source provided they are first cultured on blood agar plates containing glucose.

Liquid culture media advocated for the growth of the meningococcus have been as varied and as complex in their available nutrients as the solid media. It was not until 1942 that a serious attempt was made to precisely define the nutritional requirements of strains of N. meningitidis. In that year, Frantz (21) developed a simple, chemically defined medium in which he was able to obtain growth of 14 of the 15 strains of N. meningitidis tested. This medium consisted essentially of two amino acids, glucose and inorganic salts (Table 3). These compounds were found to represent the essential factors present in Mueller and Hinton medium necessary for growth of the meningococcus. Later, in 1945, Grossowicz (28) defined a simple liquid medium in which he was able to grow all 5 strains of N. meningitidis tested (Table 4). He emphasized the growth-stimulating effect of calcium ions and the inhibitory effect of certain amino acids. Subsequent use of Frantz medium by other investigators confirmed the original findings of Frantz (18,33,46,47).

2. Studies of the Pathogenicity of Neisseria meningitidis

Investigators have in general agreed that strains of N. meningitidis possess only a moderate grade of pathogenicity for laboratory animals. The role of endotoxin in the pathogenicity of these strains is of particular importance in view of the large numbers of bacteria generally employed in these studies. Mice have been used extensively and have been shown to be particularly susceptible to intraperitoneal inoculations (5,16,42) as have young guinea pigs (15, 16,19). Branham succeeded in producing meningitis in guinea pigs (6) and rabbits (5) using intracisternal injections of living cultures, heat-killed cultures, filtered suspensions of living cultures, and broth filtrates. Flexner (20) and McDonald (40) used intraspinal inoculations in producing a meningitis in monkeys simulating that occurring in man. Davis (15) inoculated strains of N. meningitidis in the nasal cavities and veins of monkeys in attempting to more closely approximate the portals of entry important in the initiation of this disease in man. He was unable to reproduce this disease using these routes.

Virulence studies of N. meningitidis strains in chick embryos followed the initial report of Goodpasture and Anderson (25) concerning the bacterial invasion of the chorio-allantoic membrane of these embryos. Buddingh and Polk (8,9,10) established the susceptibility of 12-day old chick embryos to a recently isolated virulent strain of N. meningitidis. Strains grown for long periods on artificial media were avirulent in chick embryos. In many instances histological changes substantiated the presence of meningitis following chorio-allantoic inoculations.

3. The Effect of Nutritional Requirements on Pathogenicity

The pathogenicity of N. meningitidis strains is dependent to a great extent on their ability to survive intracellularly following phagocytosis. This type of existence necessarily imposes nutritional restrictions within which the phagocytized bacteria must continue to meet essential metabolic requirements. This may in part explain the inability of the nutritionally dependent nonpathogenic strains of Neisseria to survive phagocytosis. Ordal and Busch (44) studied 14 strains of N. sicca and found that biotin was required for growth and that a mixture of 19 amino acids replaced the vitamin-free acid hydrolyzed casein also necessary for growth of these strains. Fitting and Scherp (17) succeeded in growing a strain of N. catarrhalis in Frantz medium only after adding a mixture of amino acids equivalent to those in casein. Nemes et al. (43) discovered that 47 of 57 strains of nonpathogenic Neisseria required biotin or niacin or both for growth in a glucose salts medium containing vitamin-free acid hydrolyzed casein. Martin et al. (39) showed that 4 strains of N. perflava required putrescine for growth in a medium containing 18 amino acids, biotin and salts. These requirements assume importance in that they determine the chemical environment within which these nonpathogenic strains must exist and are considerably more complex than those requirements of strains of N. meningitidis which are capable of growth in a simple chemical environment.

Mutation to nutritional dependency within strains of N. meningitidis might markedly alter the virulence of the affected strains. Reke (45) compared the growth in broth of recently isolated virulent strains of N. meningitidis with similar strains maintained

for long periods of time on artificial media. He found that recent isolates were readily grown in hormone broth medium in comparison to the stock strains which grew poorly if at all in this same medium. Scherp and Fitting (46) observed that some recent isolates from patients with meningitis grew well from minute inocula on first subculture in Frantz medium while some stock strains required heavy inocula. Recent isolates have been found to be more virulent in animal studies than have stock strains (9,19,42). However, these avirulent stock strains were found to retain their toxic properties.

No studies have been conducted to determine the effect of nutritional dependency on the pathogenicity of N. meningitidis strains. Studies have been conducted using other bacteria in establishing a relationship between nutritional requirements and pathogenicity.

Bacon et al. (1) studied the effects of biochemical mutation on the virulence of strains of Salmonella typhosa. They found that mutants with requirements for purines or for p-aminobenzoic acid possessed little virulence following intraperitoneal injection in mice in comparison with the virulent, nutritionally independent parent type. The loss of virulence was attributed to the limited availability of these specific growth factors in the peritoneal cavity of the host. Full virulence was restored by injection of these specific growth factors into the host or by reversion of the mutants to their former level of nutritional independence. Mutants requiring aspartic acid were also found to be relatively avirulent for mice. Garber et al. (23) studied biochemical mutants of Klebsiella pneumoniae. Five

different amino acid-requiring mutants[1] and one pyrimidine-requiring mutant were found to be as virulent as the parent strain when inoculated into the peritoneal or nasal cavities of mice. However, they observed that purine-requiring mutants were avirulent regardless of the route of inoculation. Reversion to purine independence or the injection of purine into the peritoneal cavity restored virulence. Furness and Rowley (22) successfully employed transduction by phage in establishing a high level of virulence in 2 strains of Salmonella typhimurium which were formerly avirulent for mice. These strains were adenine-dependent in their avirulent state but became adenine-independent following transduction by phage to the virulent state. A strain of Pseudomonas pseudomallei (34), and strains of Pasteurella pestis (11) have also been found to lose virulence for mice following mutation to purine dependency.

B. Objectives of this Study

The objectives of the present study are as follows:

1). To establish the minimal nutritional requirements of strains of N. meningitidis.

2). To determine the virulence of these strains using the chick embryo as the host.

3). To determine if a relationship exists between nutritional independence and virulence of N. meningitidis strains as has been shown for other strains of bacteria.

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1. The mutants possessed individual requirements for threonine, tyrosine, methionine, leucine, and histidine.

MATERIALS AND METHODS

A. Nutritional Requirements

1. Bacterial Cultures

Seven strains of Neisseria meningitidis were investigated with respect to their in vitro minimal nutritional requirements. Four were obtained from the American Type Culture Collection, two from the stock culture collection of the Department of Bacteriology of the University of Oregon Medical School, and one was freshly isolated from the nasopharynx of a symptomless medical student. The specific designation of each strain, and its history, are presented in Tables 1 and 2. All strains were reconfirmed as N. meningitidis according to the taxonomic criteria outlined in Bergey's Manual (7). Upon acquisition of cultures, each strain was lyophilized, and stock cultures were instituted and maintained by means of stab inoculations at approximately monthly intervals in dextrose-starch agar (38). This medium, hereafter referred to as DSA, consists of 1.5% Difco Proteose-Peptone No. 3, 2.0% gelatin, 0.2% dextrose, 1.0% soluble starch, 0.5% NaCl, 0.3% Na_2HPO_4 and 1.0% agar. All stock cultures were incubated and held at 37 C in stoppered test tubes.

2. Media

The basal, synthetic, liquid medium employed for evaluation of minimal requirements was modified slightly from that initially advocated by Frantz (21) in 1942, which consists of the two amino acids L-cystine and L-glutamic acid, dextrose and inorganic salts (Table 3). A single change in composition was made by reducing the concentration of magnesium sulfate by one-half to 0.03%. The procedure of preparation

varied only in that the final pH was brought to 7.6 prior to autoclaving instead of 7.3. The solutions were stored at room temperature in sealed 125 ml flasks.

The Grossowicz (28) medium, which was utilized in early studies, varied from Frantz medium in that sodium thiosulfate (25 mcg/ml) was substituted for L-cystine and both calcium chloride (100 mcg/ml) and thiamine (1 mcg/ml) were added. On addition of agar neither Grossowicz nor Frantz medium permitted growth of the meningococcus, thus necessitating the use of an entirely different solid medium.

The solid medium utilized was formulated by Mueller and Hinton (41) in 1941. Initially, it was prepared according to their original procedure, except for the substitution of Difco "Beef Heart for Infusion" for raw, chopped beef heart. However, the subsequent acquistion of Difco dehydrated Mueller and Hinton Medium greatly facilitated preparation. DSA was only occasionally used.

All media were sterilized by autoclaving at 121 C and 15 pounds pressure for 10 minutes. All solutions subsequently added to standard media were sterilized in the same way.

3. Determination of Essential Nutrients

a. Turbidimetric Assay of Growth and Survival

Growth of N. meningitidis strains in synthetic media was measured by a Bausch and Lomb "Spectronic 20" colorimeter-spectrophotometer. The resistors of this unit were replaced according to the procedure of Creamer (14) to increase the reliability of the instrument. The cuvettes employed were 13x100 mm Exax culture tubes which were etched with both reference reading mark and identifying number. The tubes were matched according to the density reading with 2.5% cupric

sulfate. The operations of cleaning, cotton plugging, autoclaving and incubation were carried out with care to prevent etching. The tubes generally contained 3.0 ml of culture medium, and never more than 4.0 ml, to prevent soaking the cotton plug upon thorough agitation of the contents prior to each density reading. All optical density measurements were made at 520 millimicra.

Frantz medium was employed as the basal solution to which additions or deletions were made in determining the individual minimal nutritional requirements. Double strength Frantz medium was used and diluted to final volume with solutions of additives or sterile distilled water. This method was followed to facilitate additions to this medium while maintaining the proper concentrations of solutes. If growth was obtained in the basal medium on continued subculture, deprivation studies were carried out in which the amino acids L-cystine and L-glutamic acid were eliminated completely, or sodium thiosulfate (12 mcg/ml) was substituted for L-cystine.

If growth was not obtained in the basal medium, it was subsequently enriched with Difco Yeast Extract. Growth in the enriched medium led to attempts at delineation of the essential growth-promoting substances present. In an attempt to sustain growth in a synthetic basal medium, yeast extract was replaced with solutions containing vitamin-free, acid and enzyme hydrolyzed casein, vitamins, purines, pyrimidines and amino acids.[2] These solutions were added individually or in combination, at concentrations reported in the results section.

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2. Vitamin-free casein was obtained in acid hydrolyzed form from Difco and Nutritional Biochemicals Corporation while the enzyme hydrolyzed preparation, vitamins, purines, pyrimidines, and amino acids were obtained from Nutritional Biochemicals Corporation.

The inocula consisted of either a wire loop transfer from stock DSA cultures or, more frequently, of 0.1 ml of a 24-hour liquid culture. All inoculated tubes were incubated in either a three or five gallon screw-cap candle jar at 37 C.

In many cases where liquid inocula were used, colony counts were employed in an effort to correlate density reading of the inoculum and number of viable cells. This procedure was used in order to standardize the number of living cells in each inoculum and to determine the survival of individual strains. The agar plates were poured, then incubated in a single layer at 37 C twenty-four hours prior to the time of inoculation. This was necessary to reduce the moisture content so that discrete colonies would develop. Plate inoculations consisted of spreading 0.05 or 0.1 ml of a cell suspension or dilution over the agar surface with triangular glass spreaders. If necessary, bacterial cultures were diluted with sterile saline or Frantz medium to provide a distribution of colonies that would allow accurate counts. Incubation was carried out at 37 C for 48 hours in one-gallon candle jars. A colony counter facilitated counts as all solid media used readily transmitted light.

b. Paper Chromatography

In some experiments, paper chromatography was resorted to in an attempt to determine the Rf ranges of active components in complex growth-promoting substances such as yeast extract and casein hydrolyzates on which some strains were dependent. The procedure of Bentley and Whitehead (2) consisting of ascending chromatography with pyridine-water (65:35) as solvent was followed. Materials to be evaluated were prepared in aqueous solution at pH 7.6. A sheet of

Whatman 3 MM chromatography paper (18x22 inches) was ruled with a horizontal line three inches from the dependent margin. Solutions were individually deposited in 10 lambda volumes at duplicate predetermined sites at least one inch apart on the ruled line. All spots were thoroughly dried before additional volumes were deposited and prior to solvent exposure. A cylinder was formed by stapling the opposed free margins and was subsequently placed in an air-tight cylindrical glass tank, containing freshly prepared pyridine-water solvent at a depth of one inch. Migration of the solvent front was allowed to proceed for approximately 24 hours at room temperature, after which the paper was removed from the tank and thoroughly dried in the hood. The presence of compounds containing free amino groups was determined by the color reaction on spraying with 0.1% ninhydrin in N-butanol and heating for one minute at 100 C.

Evaluation of Rf values of essential components was based on the visible growth of dependent strains in the presence of sections of chromatography strips. After evaporation of all solvent from the chromatography paper, one inch wide strips were cut comprising both the remnants of the initial spot and the pathway of migratory constituents. One strip of a pair was sprayed while the other strip was reduced to ten segments of equal size. Any adsorbed material on the ten segments was eluted by placing each segment in a culture tube containing 1.5 ml of distilled water and autoclaving for 10 minutes at 15 pounds pressure. Sterile double strength Franta medium was then added to a final volume of 3.0 ml, and finally, the contents were inoculated and incubated at 37 C in candle jars. Controls for each solution chromatographed were provided by depositing equivalent volumes

on small segments of chromatography paper. The dried spots were cut out with an adequate margin, eluted, then treated in the fashion just mentioned. Visual evaluation of turbidities and the relationship of the individual segments to the position of the solvent front were important in evaluation of the Rf values of the growth-stimulating components. Use of the "Spectronic 20" in optical density determinations was prohibited by the presence of the papers.

B. Pathogenesis in Chick Embryos

1. Chick Embryos

All embryos were obtained locally[3] and were of Cornish-Cross origin. In view of the results obtained by Buddingh and Polk (8,9), 12-day old embryos were used exclusively. The eggs were secured at least 24 hours before the day of inoculation and maintained at 37 C to provide a period of environmental adaptation. Maintenance of viability was by means of a small temperature-controlled incubator. A constant humidity was obtained by placing shallow pans of water at the bottom of the incubator. The uninoculated eggs were turned 45 to 90 degrees at least once daily. Both inoculated and uninoculated eggs were incubated with the longitudinal axis in the horizontal plane.

2. Method of Preparation for Inoculation

The chorio-allantoic route of infection was utilized and closely approximated the technique of Goodpasture and Buddingh (26). The eggs were candled prior to inoculation with particular attention paid to location of the air sac and large veins, and embryonic movements.

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3. Santa Clara Northwest Hatcheries, Portland, Oregon

Candling was somewhat difficult due to the variation in the coloration of the eggs and the relatively thick shells. A small triangle was penciled on the shell in a region relatively free of underlying chorio-allantoic veins. Seventy percent alcohol was used to cleanse the shell at the sites of entrance. A small flamed probe was passed through the shell and shell membrane into the air sac. An electric, rotary hand-tool fitted with a conical, abrasive tip was used in cutting the triangular shell window. Care was exercised in cutting through the shell only to the underlying shell membrane to avoid microhemorrhages in the chorio-allantoic membrane. After removal of the isolated shell fragments, the shell membrane was gently perforated with a blunt, flamed probe allowing the chorio-allantoic membrane to fall away from the shell membrane. In some cases, the air sac had to be bulb aspirated because of failure of the chorio-allantoic membrane to descend spontaneously upon perforation of the shell membrane.

3. Cultures and Inoculation

Inoculation of the chorio-allantoic membrane with strains of Neisseria followed immediately upon removal of the shell membrane to minimize air-borne contamination. The strains investigated included all those listed in Tables 1 and 2 and one nonpathogenic strain, Neisseria catarrhalis, which was obtained from the culture collection of the Department of Bacteriology, University of Oregon Medical School.

Two types of media, complex and minimal, were used in culturing strains prior to inoculation. DSA slants in 6x$\frac{3}{4}$ inch test tubes constituted the complex form of medium while Frantz solution formed the minimal medium in those strains in which it supported growth. In those strains in which minimal requirements were not defined, only DSA-grown

cultures were utilized as sources of bacteria. A DSA slant was streaked with bacteria from a stock DSA deep, stoppered and incubated at 37 C for 24 hours. Wire loop inocula were then taken from this 24 hour slant and used in initiating cultures in both DSA and Frantz media, each of which was subcultured after 24 hours. The 24 hour growth of these subcultures served as the source of cells for chorio-allantoic inoculation.

In an attempt to obtain inocula with equal numbers of living bacteria the following procedure was used. Cells from both DSA slants and Frantz medium were suspended in solutions of sterile saline or Frantz medium. In earlier experiments sterile saline was employed in all cell suspensions and dilutions whereas Frantz medium was used for this purpose in later studies. The DSA slant culture was suspended in a 4.0 ml volume and aseptically centrifuged at 17,000 G for ten minutes. The supernatant was decanted and the cells were resuspended in 3.0 ml volume. The Frantz cultures were similarly centrifuged, decanted and resuspended in 3.0 ml. Determination of viable cells present in the density adjusted cell suspensions was by means of colony counts of organisms grown on Mueller and Hinton plates according to the procedure previously described in the section concerning nutritional studies. Both the final adjusted cell suspensions and the dilutions of these suspensions made for counting purposes were used for inoculations.

Inocula of 0.1 or 0.05 ml were delivered through a 0.1 or 1.0 ml pipette onto the chorio-allantoic membrane without contacting the margins of the window. Following inoculation a sterile coverslip was applied over the window and sealed with a melted, 50-50 mixture of vaseline and paraffin. Three minutes were required to complete the procedure with each egg before it was returned to the incubator. Embryos

were inoculated in groups of five for each individual cell suspension or dilution. Five control eggs were inoculated with equivalent volumes of the fluid in which the bacteria were suspended.

4. Observations of Infection

The embryos were observed through the coverslips for signs of death to the day of hatching (21st day). Death of the embryo was based upon failure to observe movements and absence of arterial pulsations in the chorio-allantoic membrane. Candling was employed as a means of reaching a final judgment on expiration of the embryo.

Specimens for culture were obtained as follows from all embryos which died and from some embryos which survived to the day of hatching. The coverslip was removed and a flamed nichrome loop was passed through the chorio-allantoic membrane into the underlying spaces and structures. The loop was then streaked over the surface of a BSA slant which was subsequently stoppered and incubated at 37 C until colonies appeared or for one week in the absence of growth. Smooth, gray, translucent, moist-appearing colonies, which were oxidase positive as indicated by their color reaction to p-dimethyl-phenylene-diamine, were recorded as Neisseria. Suspected contaminants were examined microscopically using the Gram stain.

Occasionally slides were prepared of the embryonic fluids, which were obtained in a fashion similar to specimens for culture. They were Gram stained and examined microscopically for bacteria, inflammatory cells and phagocytosis.

RESULTS

A. Nutritional Studies

1. Choice of Basal Medium and Growth Response Groupings

Both Frantz (Table 3) and Grossowicz (Table 4) media were initially considered in choosing a basal medium. However, the Grossowicz medium, as shown in Table 5, promoted a granular type of turbidity and a brief inquiry was made into the causative factors responsible for this phenomenon. The components of Grossowicz medium foreign to Frantz medium were investigated using strain M-1. Solutions of differing composition were inoculated in duplicate with this strain and subsequent turbidities were examined visually. Turbidity measurements and plate counts at 24 and 48 hours indicated that the reaction of M-1 to this medium was one of survival rather than actual growth. The results are shown in Table 5. It is apparent that calcium chloride plays an important role in this phenomenon as it is capable of promoting a granular type of growth when added alone or in combination with other inorganic salts. Since growth in Grossowicz medium with or without calcium chloride was no better than in Frantz medium, its use was discontinued and Frantz medium subsequently formed the basis of all nutritional studies.

On the basis of the results to be described, the seven strains could be divided into three groups according to their nutritional requirements. Group I represents those strains of *N. meningitidis* which would grow in Frantz medium on initial transfer from the stock complex DSA. Group II, however, required supplementation of Frantz medium initially but did grow on subsequent transfer into the simple

synthetic medium. Group III could not be adapted to growth in Frantz medium without supplementation.

2. Group I

This group consists of strains M-1027, M-1628 and M-2092 which were obtained from the American Type Culture Collection. These strains grew on initial wire-loop transfer into Frantz medium from stock DSA. All nutritional studies involving this group employed inocula which were taken from a 24-hour culture of these strains in Frantz medium. The liquid test media were inoculated with 0.1 ml of a 24-hour culture. In the event that a volume greater than 3.0 ml was necessary for inoculation of test solutions, culture tubes were matched according to their 24 hour turbidity measurements in an attempt to approximate an equal number of viable cells in all inocula. The growth response of strain M-1027 in Frantz medium (Table 3) is representative of this group (solid line in Figure 1).

From Figure 1 it is evident that growth was relatively slow in the synthetic media; but, with time, the density in complete Frantz medium (shown by the solid line) reached that in medium supplemented with 0.1% yeast extract. It is interesting to note that growth was obtained when the medium was simplified by the deletion of both glutamic acid and cystine, that is, it contained only glucose and inorganic salts (labeled: No Addition). The addition of cystine to this simple glucose-salts medium completely inhibited growth (lowest curve), in contrast to the increment obtained when it was combined with glutamic acid in complete Frantz medium (labeled: + Glutamic + Cystine). Despite the fact that growth was obtained in the simple glucose-salts medium without addition, difficulty was experienced in maintaining serial

subcultures in this medium.

However, the addition of 0.0012% sodium thiosulfate not only improved the growth rate but also permitted serial subcultures in the absence of glutamic acid and cystine (Figure 2). These subcultures indicate that these Group I strains possess remarkable synthetic ability in their response to a chemical environment consisting only of glucose and inorganic salts. The exact time of subculture was not fixed but varied from 144 hours to 192 hours. The delay in transfer was probably a major factor in the failure of strain M-1027 to grow in the third subculture. The second subculture of strain M-1027 had been in the stationary phase of growth for at least 72 hours before the third subculture was instituted whereas the other strains were in the log phase of the growth curve. In subsequent experiments strain M-1027 was grown and subcultured repeatedly in the simple glucose-salts medium when the organisms were transferred during the log phase of growth.

The minimal medium for maintenance of growth and metabolic processes essential to Group I strains of N. meningitidis is thus met by a simple chemically defined solution (Table 6).

3. Group II

This group consists of strains M-2 and Bell which required a single passage through Frantz medium with yeast extract supplementation before growth could be obtained in Frantz medium without supplementation. This initial passage consisted of incubating a wire loop transfer from DSA at 37 C in Frantz medium supplemented with 0.1% yeast extract for a period of 24 hours. Inocula of equal volumes (0.1 ml) were subsequently transferred into Frantz medium with or without supplements.

The growth response of strains M-2 and Bell in Frantz medium

supplemented with 0.2% yeast extract is rapid and sustained (Figure 3). Comparison of turbidities in the initial 48 hour period reveals comparable growth of the two strains. However, following this initial period, the Bell strain exhibits a response in growth which is approximately 1.3 fold greater than that of strain M-2 and is maintained at this level to 168 hours of incubation. Regardless of the slight individual differences, both strains presented marked turbidity increments in yeast extract supplemented Frantz medium, in contrast to the absence of growth in unsupplemented Frantz medium on primary transfer from complex medium.

Following an initial 24 hour culture period in yeast extract supplemented Frantz medium, these strains are capable of continuous growth in unsupplemented Frantz medium (Figure 4). This is well demonstrated in spite of the irregular times of subculture varying from a minimum of 96 hours to a maximum of 240 hours. This latter time of subculture no doubt played an important role in the subsequent loss of viability of strain M-2 in the fifth subculture, as the stationary phase of growth had been reached at least three days previously in the fourth subculture. Subculturing at a specified time in the log phase of growth would have produced a more uniform viable cell population in each inoculum and aided in the reproduction of comparable growth curves. The 144-hour turbidity measurements shown are small in comparison to the response to yeast extract supplemented Frantz medium at this same interval (Figure 3) but are apparently not due to yeast extract carried over in the inocula employed in the initial subculture. If this mechanism were operating, one would expect a decline in the growth response with each consecutive subculture. However, the results present

no evidence for the importance of such a carry-over mechanism.

An experiment was conducted to evaluate the effect of complex growth-promoting substances on strain M-2. This was performed in conjunction with an experiment involving a Group III strain. Strain M-2 was incubated for 24 hours at 37 C in 0.1% yeast extract supplemented Frantz medium and was then inoculated in equal volumes into the solutions shown in Figure 5. Comparison of densities at 72, 144 and 216 hours of incubation reveals several interesting differences among the complex media. A concentration of 0.1% yeast extract added to Frantz medium enhances the early growth response without significantly stimulating the late response beyond that occurring in unsupplemented Frantz medium. Acid and enyzme hydrolyzed casein in equal concentrations did not markedly enhance the growth rate of strain M-2 in comparison to the Frantz medium control and, in fact, there appeared to be a tendency toward growth inhibition in cultures containing these substances. The presence of the vitamins, biotin (5 mcg/ml) and niacin (5 mcg/ml) did not improve the growth response of strain M-2 to 0.01% casamino acids (Difco). The failure of these vitamins to appreciably affect the growth of strain M-2 was of interest as Pelczar and Doetsch (43) found these vitamins to be required in the nutrition of the majority of the nonpathogenic strains of Neisseria studied.

The inhibitory effect of amino acid mixtures on the growth response of strain M-2 to yeast extract is presented in Figure 6. This strain was employed as a control in an experiment involving a Group III strain subjected to the same amino acid mixtures to assess possible inhibitory characteristics of the test solutions. The early inhibitory effect of the complete solution containing 18 amino acids is evident

in comparison of the 48 and 96 hour turbidity measurements of strain M-2 in the presence of 0.2% yeast extract and 0.2% yeast extract plus 18 amino acids. A pronounced inhibitory effect was present at 48 hours and remained in effect through the 96 hour period. However, 168 hours of incubation rendered this inhibitory mechanism inoperative and coincided with the appearance of relatively good growth in the same medium lacking yeast extract. The growth in the presence of 12 of the 18 amino acids was less than the growth in Frantz medium with or without the 18 amino acids.

4. Group III

This group comprises strains M-1 and M-158 of N. meningitidis, which required supplementation of Frantz medium for growth in primary culture and in subsequent subculture. Strain M-1 has been maintained for many years on DSA with yearly subculture in the Department of Bacteriology, while M-158 is an American Type Culture Collection strain. The bulk of investigation in this group has been centered around strain M-1 as it was the first strain encountered and presented an interesting problem in nutrition, since it would not grow in Frantz medium in contrast to the strains of the other two groups.

Strain M-1 was repeatedly wire-loop transferred from DSA deep cultures into Frantz medium and on all occasions the density measurements could be attributed solely to the size of the inoculum transferred into the synthetic medium. Plate counts at 48 hours of incubation revealed very few viable cells remaining in spite of the fact that the density measurements had decreased very little since the time of inoculation. Yeast extract was employed as a supplement to Frantz medium to stimulate growth and to provide a means of measuring the relative

potency of other nutrients in promoting growth of strain M-1. The effect of graded increments of yeast extract in improving growth of strain M-1 is shown in Figure 7. A concentration of 0.1% yeast extract seemed to be the optimal concentration above which further increments did not markedly improve the rate of growth.

The lack of growth in Frantz medium may be explained by the absence of an essential nutrient and/or the presence of toxic factors. Growth in Frantz medium containing added yeast extract would imply reversal of one or both of these growth-inhibiting situations. In the present study, experiments to define a minimal solid medium for each group resulted in failure on every occasion in which 1.5% agar was added to the minimal liquid medium. However, the addition of 0.1% yeast extract reversed the apparent inhibitory effect of agar. Frantz (21) noted this identical phenomenon in 1942 when attempting to employ his minimal medium in agar form. Mueller and Hinton (41) described a similar growth-promoting effect of starch in solid media and suggested that it acts as a "protective colloid" against the inhibitory effects of amino acids. Dialysis was employed to determine whether compounds of high molecular weight in yeast played a similar role. A yeast extract solution was dialyzed against one liter of distilled water for a period of 18 hours at 7 C. The dialyzate was distilled under vacuum to a volume equal to that remaining inside the bag. Activity in the dialyzate would be attributed to compounds of low molecular weight, probably acting as nutrients, while activity in the residue would probably be due to the protective action of high molecular weight compounds. The results are shown in Figure 7. The activity was found to be present in the dialyzate with almost complete recovery while the

residue added to Frantz medium failed to support the growth of strain M-1. This would suggest that yeast extract provides a dialyzable nutrient essential to the growth of strain M-1 in Frantz medium.

In attempting to determine the nature of this dialyzable nutrient, solutions of Frantz medium with added vitamins, purines, pyrimidines and casein hydrolyzates were evaluated for their ability to promote growth in comparison to growth in Frantz medium with added yeast extract. Vitamins, purines and pyrimidines were found to be without effect either alone or in combination in enabling strain M-1 to grow in Frantz medium. Vitamin-free casamino acids (Difco acid hydrolyzed casein) revealed an ability to sustain growth of this strain in Frantz medium as shown in Figure 8. Although the early response is less than seen with yeast extract (Figure 7), later turbidity measurements are in general much higher than with equivalent concentrations of yeast extract. This is particularly true of the lower concentrations of casamino acids (0.05% and 0.1%) which show prolonged log phases. The 192 hour growth response of strain M-1 upon continued subculture in this medium is presented in Figure 9. The initial culture was transferred at 96 hours of incubation whereas all following subcultures were initiated after 48 hours of incubation. A concentration of 0.05% casamino acids appears optimal in continued subculture as well as in initial cultures (Figure 8).

Vitamin-free, acid and enzyme hydrolyzed casein (Nutritional Biochemicals Corporation) were also evaluated to determine if any significant difference existed in their growth-stimulating potentials. A better growth response in the presence of enzyme hydrolyzed casein would suggest that strain M-1 might be dependent upon small peptides.

The results of graded increments of both enzyme and acid hydrolyzed casein on the growth of strain M-1 in Frantz medium are shown in Figure 10. As with casamino acids the optimal concentration of both types of hydrolyzates is 0.05%. The graded growth response to the increments of acid hydrolyzed casein is in sharp contrast to the growth response in the presence of increasing concentrations of the enzyme hydrolyzed casein preparation. The enzyme hydrolyzed preparation in concentrations up to 0.1% promoted growth which was comparable to that with acid hydrolyzed casein in the first 72 hours of incubation. However, concentrations of enzyme hydrolyzed casein greater than 0.1% showed partial inhibition of growth; and, at 0.2%, complete inhibition occurred (Figure 10B). The presence of small concentrations of yeast extract (0.003%) tended to reverse this inhibitory effect.

The 72 hour growth response of strain M-1 to increments of the various types of supplementation mentioned above is shown in Figure 11. The inhibitory effect of enzyme hydrolyzed casein in concentrations above 0.1% is quite evident (curve labelled: Enzyme Hydrolyzed Casein). The growth response to the acid hydrolyzed preparation was practically identical to that obtained with casamino acids in similar experiments. The turbidity measurements of strain M-1 in the presence of increasing amounts of yeast extract are approximately twice as great as in the casein hydrolyzates at equivalent concentrations with the exception of the enzyme hydrolyzate above 0.1%.

The experiments testing the effect of casein hydrolyzates on the growth of strain M-1 suggested that either an amino acid or combination of amino acids was essential for the maintenance of growth and metabolic processes of this strain. A mixture of 18 amino acids

was employed in the same concentrations that Martin and Hansen (39) used in obtaining growth of nonpathogenic strains. The complete mixture, and eighteen mixtures with a single different amino acid deleted, failed to support the growth of strain M-1 in Frantz medium lacking L-glutamic acid and L-cystine (L-glutamic acid and L-cystine were among the 18 amino acids tested). Subsequent use of Group II strain M-2 in evaluating possible inhibitory influences of the mixtures indicated that the individual absence of 6 amino acids resulted in an enhanced growth response. These amino acids, L-threonine, DL-tryptophan, DL-valine, L-cystine, L-tyrosine and glycine, were eliminated from the total mixture and the 12 remaining were again evaluated with strain M-1. The results are presented in Figure 12. The inhibitory effect of the mixture of 12 amino acids on the early growth response of strain M-1 to yeast extract is apparent on comparison of the 48 hour density measurements with 0.1% yeast extract, and 0.1% yeast extract plus the mixture of 12 amino acids, in modified Frantz medium. A marked lag in the response to yeast extract was seen at 48 hours in the presence of the amino acids and had disappeared at 96 hours at which time growth was apparent in the amino acid mixture without added yeast extract. The absence of L-glutamic acid and L-cystine in modified Frantz medium containing yeast extract resulted in a diminished growth response at 96 and 168 hours while not influencing growth at 48 hours. The individual deletions of DL-serine, DL-isoleucine, L-lysine and L-leucine tended to increase the 48 hour growth response without appreciably altering the later growth in comparison to that seen with the mixture of 12 amino acids. However, when DL-aspartic acid and L-glutamic acid were deleted a lag was observed at the 96 and 168 hour stages of incubation without

a marked disturbance at the 48 hour interval. The relative lag in growth was more apparent in the absence of DL-aspartic acid. The results suggest that strain M-1 may possess a requirement for DL-aspartic and L-glutamic acids.

The results with growth of strain M-1 in amino acid mixtures indicated a complex interaction between the bacteria and the amino acids present. Although the growth response in the presence of 12 amino acids was comparable to that obtained in acid hydrolyzed casein (Figure 10A), the absolute amino acid requirements were not defined; paper chromatography was utilized to more accurately determine the nature of these requirements. Solutions of the complex nutrients[4] previously employed were subjected to chromatography, and chromatographed fractions were incubated with strain M-1 to determine the Rf location of the growth promoting components. This method of determining the active Rf ranges is described in the Materials and Methods section. The results are shown in Table 7. The Rf locations of the active components of yeast extract extended from 0.3 to 0.5 and were identical with those obtained with the dialyzate of yeast extract. The growth response of strain M-1 indicated that the active components were located within much larger Rf boundaries in the cases of casamino acids, acid hydrolyzed casein and enzyme hydrolyzed casein than was the case with the yeast extract preparations. In contrast, the color reactions of the separated components of equivalent quantities of yeast extract, the dialyzate of yeast extract, casamino acids, and acid hydrolyzed casein were confined to practically identical Rf boundaries. This apparent discrepancy in

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4. Yeast extract, casamino acids and casein hydrolyzates.

the growth response may have been due to concentration differences, absence of inhibitory substances within these ranges or to totally different active substances in the nutrients investigated. All of the nutrients tested exhibited a color reaction within the Rf range 0.7 to 0.8; however, only in the case of enzyme hydrolyzed casein was growth obtained in this range.

On the basis of these chromatography results individual amino acids were investigated in respect to their Rf ranges. The Rf range extending from 0.4 to 0.6 was arbitrarily chosen as a means of selecting amino acids which would most likely promote growth of strain M-1 in Frantz medium. Eight amino acids were found to possess Rf values within this range and included DL-aspartic acid, L-glutamic acid, DL-alanine, L-threonine, DL-serine, L-histidine, L-cystine, and glycine. A mixture of these amino acids was evaluated for its ability to promote growth of strain M-1 and was found to lack any stimulus in this respect.

B. Virulence Studies

1. Neisseria catarrhalis

This organism, considered nonpathogenic for man, appeared relatively benign in its effect on chick embryos following inoculation of the chorio-allantoic membrane. The results are presented in Figure 13. Whether the observed deaths were actually due to an established infection may be questioned, as on no occasion was a positive culture of N. catarrhalis obtained from a dead embryo (Table 8). This strain was employed as a control representative of the nonpathogenic strains of Neisseria. It was grown solely on DSA medium as were other strains for which a minimal medium was not defined. No attempt was made to

explore the minimal requirements of this organism.

2. Strains of Group I

Strain M-1027 appeared to be as benign in its effect on chick embryos as was N. catarrhalis. The results of in vivo experiments with this organism are presented in Figure 14 A. Growth in Frantz medium did not enhance the virulence of this strain. It is apparent that a certain amount of variability existed in that lesser numbers of bacteria appeared more lethal than larger numbers grown on the same medium. Among the contributing factors may be included individual differences in susceptibility of the embryos to infection, stresses experienced by the bacteria throughout the extended procedure of chick embryo inoculation, and experimental error.

The lethal effect of strain M-2092 on 12-day old chick embryos was more pronounced than with strain M-1027 and here again growth in Frantz medium did not enhance virulence (Figure 14B).

Strain M-1628 proved to be the most virulent for the chick embryo of all strains tested. This strain was also one of the most consistent in its manifestation of virulence (Figure 15). Bacteria grown in Frantz medium were more virulent than were bacteria grown on DSA medium. Very few organisms grown in Frantz medium were necessary to establish an infection while larger numbers of bacteria resulted in early death of all embryos. An experiment was conducted to decide if endotoxin may have played an important role in these latter deaths. Bacteria were heat-killed by boiling dilutions for 10 minutes at 100 C and inoculating equivalent numbers of living and dead bacteria. Inocula of the heat-killed suspension included heat-stable endotoxin liberated upon death of the bacteria. Inocula containing 2.2×10^6

DBA-grown or 6.2×10^4 Frantz-grown, heat-killed bacteria and liberated endotoxin failed to have any lethal effect in chick embryos.

3. Strains of Group II

Strains M-2 and Bell are equally virulent in chick embryos (Figure 16) and are surpassed in this respect only by strain M-1628. Frantz medium again failed to enhance the virulence of these strains.

4. Strains of Group III

In general, strains M-1 and M-158 (Figure 17) exhibited no greater virulence in the chick embryo than that resulting from inoculation with N. catarrhalis (Figure 13).

These Group III strains were responsible for many changes in the inoculating procedure of all strains used in subsequent experiments. Their decline in viability was so great in the sterile saline initially used for suspensions and dilutions that it became necessary to routinely use Frantz medium for these purposes and to perform more frequent counts of viable cells present. All strains employed did poorly in sterile saline and the one hour losses ranged from 40 to 70% of the viable cells present initially. In contrast, the Frantz medium supported growth of some strains and reduced the loss of viable cells very little in other strains. Differences here were due in part to the ability of strains to survive in Frantz medium and in part to individual strain requirements for carbon dioxide. The use of dilutions necessitated thorough mixing with resultant aeration and marked reduction in carbon dioxide concentration. The deleterious effects of aeration on the viability of N. meningitidis strains in Frantz medium were demonstrated by Tuttle and Scherp in 1952 (47). For this reason and because of the length of the procedures involved, plate counts were routinely used to a greater

extent than previously in determinations of the number of viable bacteria in the primary suspensions from which the inocula were derived.

5. A Recent Isolate from an Active Case of Meningitis

This N. meningitidis strain, designated Heagle (Table 1), was the only strain which was acquired from an acute case of meningitis during the course of this study. Its virulence in chick embryos was of particular interest in this respect. The lethal effect of this strain grown in DSA (Figure 18) compared favorably with that of strain M-1628 grown on this same medium (Figure 15A). Growth in 0.1% yeast extract supplemented Frantz medium did not alter the virulent manifestations of this strain. Although a thorough nutritional study was not conducted, strain Heagle was found to grow in Frantz medium. Buddingh and Polk (9) found that strains of meningococci grown on artificial culture media for long periods of time were avirulent for chick embryos, while a strain which they studied shortly after it was isolated from an active case of meningitis was virulent for the chick embryo.

6. Observations of Chick Embryos Infected with Neisseria

Microscopic examinations of allantoic fluid containing exudate from the chorio-allantoic membrane were conducted to ascertain presence of phagocytosis. Material from embryos which died from infection with M-1027 was examined in this fashion using the Gram stain. Slides prepared from embryos which died 48 hours following inoculation revealed both intracellular and extracellular Gram negative diplococci. Cells of the monocytic series seemingly predominated and contained from one pair to as many as 10 to 15 pairs of bacteria. Relatively few disintegrated monocytes were found although the granulocytes were present in varying states of cellular disintegration. Very few intact granulocytes

were found to contain more than one or two pairs of bacteria. Presence of phagocytosis, extracellular bacteria, and a cellular inflammatory response were thereby established.

Attempts at recovery of inoculated N. catarrhalis within 16 hours after death of the embryo failed (Table 8). Group III strains were also relatively difficult to recover in comparison to recovery rates of strain Heagle and Groups I and II. A negative culture was not interpreted as an indication of a non-specific death or the absence of infection according to the results obtained by Buddingh and Polk (9).

DISCUSSION

A. Nutritional Requirements of N. meningitidis

Five of the 7 strains studied were grown in the simple, synthetic medium advocated by Frantz (21). The remaining 2 strains possessed nutritional requirements which were not satisfied by this medium. The results obtained in these experiments agree in general with observations made by earlier investigators studying the nutritional requirements of strains of N. meningitidis in that the majority of these strains demonstrate a marked degree of nutritional independence.

The growth of the American Type Culture Collection strains M-1027, M-1628, and M-2092 in Frantz medium with carbon dioxide available indicated that these strains possessed remarkable biosynthetic abilities. This became even more apparent when growth was obtained after further simplification of Frantz medium and upon continued subculture in this resultant, simple, glucose-salts medium. Frantz (21) stated that glutamic acid was essential to growth or that its absence greatly delayed growth. The observations in the present experiments indicate that it is not an absolute growth requirement and did not markedly stimulate the growth of these strains in the simple, glucose-salts medium. The role of cystine in the nutrition of N. meningitidis strains was studied by Grossowicz (28). He found that cystine exhibited an effect on the growth of 2 strains of N. meningitidis which was dependent upon the concentration of this substrate and the size of the inoculum. Concentrations of cystine of 0.005% and higher inhibited growth while "minimal" concentrations stimulated growth. The "minimal" concentrations which stimulated growth of the strains he tested were not defined. He observed the inhibitory action

of cystine when it was added to a basal medium containing both glutamic acid and sodium thiosulfate in addition to glucose and other inorganic salts. The results of the present experiments indicate that glutamic acid tends to reverse the inhibitory effect of cystine; the reversal is evident at a concentration of cystine as low as 0.0012%. The mechanism of this inhibitory action of cystine on growth of N. meningitidis strains has not been elucidated.

Scherp and Fitting (46) succeeded in obtaining good growth with 4 strains of N. meningitidis tested in Frantz medium. These strains were subsequently transferred to Frantz medium lacking glucose and were found to grow as well in the absence as in the presence of glucose providing carbon dioxide was supplied. No studies have thus far been reported in which strains of N. meningitidis have been successfully grown in a liquid medium as simple as the glucose-salts medium employed in the present experiments.

Jyssum (29) succeeded in growing 3 strains of N. meningitidis on a solid medium containing only glucose and inorganic salts. A 24-hour period of growth on blood agar supplemented with glucose was found to be necessary before growth could be obtained on the simple agar medium. This initial step was found to consist of an adaptation of the meningococci to metabolize glucose. The 3 strains cultivated on blood agar directly after isolation did not produce measurable quantities of acid from glucose. However, suspensions of adapted meningococci rapidly produced appreciable quantities of acid from glucose. The presence of casein hydrolyzate or glutamate facilitated the adaptation to utilization of glucose. This was thought to be an indication that glucose was not metabolized in any appreciable quantity by the wild strains of meningococci.

Similar results were reported by Fitting and Scherp on strains of N. meningitidis (18) and on a strain of N. catarrhalis (17). The results of the present study indicate that a similar phenomenon may be operative with respect to the growth of strains M-2 and Bell (Group II strains) in Frantz medium. No studies were conducted to determine whether the necessary period of growth of these 2 strains in 0.1% yeast extract supplemented Frantz medium represented an adaptive process or a selection of mutants.

The effect of complex nutrients on the growth of a relatively fastidious strain of N. meningitidis is exemplified in results obtained with M-2. The primary effect of yeast extract and casein hydrolyzates is limited to the early period of growth and represents a stimulation of the rate of growth. These nutritive substances did not result in any marked increases in the late growth response when compared to the response obtained with simple synthetic media. The lack of growth stimulation by biotin and niacin is in sharp contrast to the importance of these vitamins in the growth of nonpathogenic species of Neisseria (39,43,47). Grossowicz (28) found that a mixture of vitamins was without effect in altering the growth response of N. meningitidis strains in a simple synthetic medium.

Strains M-1 and M-158 represented the 2 strains out of the total of 7 studied which required supplementation of Frantz medium. These strains were of particular interest in that investigators have been in general successful in culturing N. meningitidis strains in simple synthetic media. Studies on the nutritional requirements of strains which failed to grow in simple synthetic media have not been reported. The experiments performed with strain M-1 indicated that this strain

possesses a requirement for one or more amino acids. The studies involved revealed many interesting nutritional problems with respect to the presence of amino acids in the culture medium. A marked difference in bacterial growth was obtained with equal concentrations of vitamin-free preparations of enzyme and acid hydrolyzed casein. The enzyme hydrolyzed casein preparation tended to inhibit growth in concentrations greater than 0.1% and completely inhibited growth at 0.2%. It was not determined whether this difference was due to the presence of small peptides or of tryptophan in the enzyme hydrolyzed preparation. Small concentrations of yeast extract tended to reverse this inhibitory effect. Acid hydrolyzed casein did not inhibit growth within these concentrations. Vitamin-free acid hydrolyzed casein (Difco) increased the growth rate of strain M-1 over a concentration range of 0.01% to 0.2%. The growth rate of strain M-1 was almost independent of concentration increments of this casein preparation above 0.05% (Figure 8B). One or more rate-limiting biochemical reactions appeared to play an important role in this phenomenon. Among these possible reactions may be included the fixation of carbon dioxide, active membrane transport and utilization of amino acids.

Mixtures of amino acids were studied with respect to their effect on the growth of strain M-1 and their ability to replace casein hydrolyzates. A mixture of 12 amino acids produced a pronounced lag in the growth response of strain M-1 to yeast extract which largely disappeared after 96 hours of incubation. Experiments employing deletion of single amino acids from the mixture of 12 indicated that no one amino acid was absolutely essential for growth. However, the absence of aspartic or glutamic acid resulted in a diminished growth response. The studies

of Gladstone (24) demonstrated that diminished or absent growth following the deletion of an amino acid from a mixture of amino acids does not necessarily imply that that amino acid is indispensable. Some amino acids were found to be "indispensable" only in the presence of other amino acids. A mixture of 12 amino acids was found to replace the growth-promoting compounds present in acid hydrolyzed casein preparations.

The results with paper chromatography indicate that the growth-promoting substances within the different complex nutrients tested may not be of the same nature. The presence of potential growth-inhibiting compounds may have influenced the results obtained employing this method. A mixture of 8 amino acids selected on the basis of these chromatography experiments failed to support growth of strain M-1.

The complex interactions of amino acids in the nutrition of bacteria are evident in the studies reported by numerous investigators. This is quite apparent in view of the reports on the nutritional requirements of nonpathogenic species of Neisseria. The synthetic media employed for growth of these species has generally contained all the amino acids present in casein hydrolyzates (17,39,44). Reports of the absolute amino acid requirements of these nonpathogens have not appeared. Gladstone (24) reported the first extensive studies concerning the inter-relationship of amino acids in the growth of bacteria. He discovered that isoleucine, valine or leucine inhibited the growth of B. anthracis when added singly to a synthetic medium capable of supporting the growth of this strain in their absence. However, when added together these 3 amino acids improved growth. He observed that the toxic effect of valine was antagonized by leucine and that the reverse was also true.

However, the presence of both valine and leucine was required for growth with isoleucine. Similar inter-relationships were noted between other amino acids of similar chemical structure. Two possible mechanisms were advanced to explain this phenomenon. The first mechanism involved inhibition of the incorporation of amino acids into the cell while the second implied inhibition of bacterial synthesis of amino acids. Subsequent studies by Umbarger and Brown (48) indicated that isoleucine in excess prevented the uptake of valine by an isoleucine and valine-requiring mutant of E. coli, while not affecting the utilization of valine formed within the cell. Using another strain of E. coli their results indicated that valine may inhibit the formation of isoleucine within the cell. Other examples of growth-inhibiting phenomena, due to an interference with the penetration of essential nutrients into bacterial cells, have been reported by Mandelstam (36,37). These inter-relationships complicate determinations of absolute amino acid requirements and seemingly played an important role in the failure to discover the minimal amino acid requirements of strain M-1 employing the deletion technique in the present study.

Detailed studies of the biochemical reactions essential in the metabolism of strains of N. meningitidis will facilitate the further definition of minimal nutritional requirements. Jyssum (30) has demonstrated that 3 strains of N. meningitidis possessed enzymatic activities representative of the intermediate reactions of the tricarboxylic acid cycle with but one exception. The malic dehydrogenase characteristic of this cycle was not demonstrated in these 3 strains. However, a TPN dependent malic enzyme was present which oxidatively decarboxylated L-malate to pyruvate. No aspartase activity was

discovered. Previously, Jyssum (29) had established the presence of transaminase in these same strains. He also discovered that strains adapted to growth on a minimal, glucose-salts-agar medium possessed enhanced activity of a DPN dependent glutamic dehydrogenase while both adapted and wild strains exhibited high activity of a TPN dependent glutamic dehydrogenase (31). Jyssum et al. (32) have recently reported the presence of enzymatic activities in cell-free extracts from meningococci characteristic of the conventional Embden-Meyerhof route of glycolysis. A significant increase in glucokinase activity occurred in strains adapted to growth on a simple agar medium containing glucose and inorganic salts. Aside from these studies no other information is available concerning the specific enzymatic reactions present in strains of N. meningitidis.

B. Pathogenicity of Neisseria

The results presented indicate that the chick embryo provides an ideal experimental animal for evaluation of host-parasite relationships. Six of the 8 strains tested were far more virulent in chick embryos than the control strain of N. catarrhalis. Two strains did not markedly differ from the relatively avirulent manifestations of the control strain in chick embryos. The virulence demonstrated by the majority of strains which were maintained on artificial media is in sharp contrast to the avirulent manifestations of similar strains studied by Buddingh and Polk (10). Three of the strains studied were as virulent as a freshly isolated strain taken from an active case of cerebrospinal meningitis.

The results obtained indicate that the chick embryo is more susceptible to infection with strains of N. meningitidis than formerly believed.

The extensive studies of Buddingh and Polk (8,9,10) were not quantitative and were conducted using only 2 strains of N. meningitidis. Both strains had been recently isolated from active cases of cerebro-spinal meningitis. The large numbers of bacteria which they inoculated on the chorio-allantoic membrane resulted in death of the majority of embryos within 48 hours. Cultures were obtained from living embryos following inoculation of the amniotic fluid (10). Specimens of blood, amniotic fluid and brain were cultured from as few as 3 hours to as many as 96 hours following inoculation. They failed to obtain positive cultures of N. meningitidis later than 72 hours following inoculation. In the present experiments bacteria were recovered from chick embryos which died as late as 168 hours following inoculation with virulent strains. The avirulent strains M-1 and M-150 were relatively difficult to recover from chick embryos which died subsequent to inoculation with these strains. No organisms were ever recovered from chick embryos which died following inoculation with N. catarrhalis. The data suggest that the virulent strains multiplied more readily in the chick embryo than did the avirulent strains. This was even more evident when embryos died following inoculation of exceedingly small numbers of the most virulent strain, M-1628, followed by recovery of bacteria upon death of these chick embryos. The results indicate that endotoxin did not play an important role in the early death of embryos inoculated with large numbers of bacteria of strain M-1628.

The virulence of the majority of strains was not significantly altered by growth in Frantz medium. Only in the case of strain M-1628 was a difference noted. The virulence of these bacteria grown on Frantz medium was greater than that exhibited by cells grown on a

complex solid medium.

A considerable amount of variability existed in the virulent manifestations of individual strains. This was noted to a certain degree throughout all experiments and was apparently due to the numerous variables existing in the experimental technique and the relationship between host and parasite.

C. The Relationship Between Nutrition and Pathogenicity in Neisseria

The results of this study indicate that virulence of N. meningitidis strains in chick embryos does not depend on nutritional independence alone. All virulent strains tested were capable of continued growth in Frantz medium or a simple, glucose-salts medium with added carbon dioxide. Two of the 4 relatively avirulent strains were also found to possess minimal nutritional requirements and could be maintained in the glucose-salts medium with added carbon dioxide. However, the other 2 avirulent strains were found to be nutritionally dependent as they could not be grown in Frantz medium without supplementation. All strains which possessed minimal nutritional requirements were more easily recovered from dead embryos than were the nutritionally dependent strains.

Other factors which may play an important role in the virulence of N. meningitidis include antigenic structure, presence of capsules, endotoxin production, and others as yet unknown. Determinants of virulence other than nutritional independence have been observed in other bacteria. Burrows (11) reported that the determinants associated with virulence of P. pestis in mice included the presence of a surface envelope, production of "V and W antigens", pigment production on a

medium containing hemin, and purine independence. His results indicated the existence of other determinants of virulence in strains of P. pestis although their exact nature is not known as yet. Furness and Rowley (22) were successful in simultaneously conferring virulence and purine independency on 2 strains of S. typhimurium by means of transduction. Another avirulent, purine independent strain possessing the typical antigenic structure of S. typhimurium was not made virulent by transduction. The avirulence of this strain could not be attributed to its antigenic structure or to a biosynthetic deficiency.

The minimal nutritional requirements of strains M-1027, M-2092 and M-1628 suggest that serological groups A, B and C may be largely composed of nutritionally independent strains. Other strains belonging to these groups have been studied by other investigators and have been found to be nutritionally independent (18,28,29,35,46,47). The nutritional requirements of Group D strains have not been studied previously. The present data obtained with strain M-158, the American Type Culture Collection neotype strain for Group D, suggests that strains of this group may not be as independent in their nutritional requirements as strains of the other serological groups. Strains of Group D have rarely been isolated from cases of cerebrospinal meningitis in man (3). If nutritional dependency proved to be characteristic of this group, such a state might constitute a major factor in their infrequent association with cases of meningitis in man.

The data presented in this study indicate that a relationship exists between virulence and nutritional independence of strains of N. meningitidis in chick embryos. Nutritional independence apparently represents one of the determinants of virulence of N. meningitidis

strains in chick embryos and may well contribute to the virulence of these same strains in man. Many interesting experiments remain to be conducted in order to clarify this relationship. Catlin (12,13) has been successful in transformation experiments in which crude extracellular DNA was used in altering the streptomycin sensitivity of strains of N. meningitidis. She used crude extracellular DNA from streptomycin resistant strains of N. sicca and N. meningitidis and discovered that streptomycin sensitive strains of N. meningitidis were transformed by these crude DNA preparations such that they were resistant to streptomycin. Similar transformation experiments could be employed in studies relating to nutritional requirements and virulence. Intracellular survival in polymorphonuclear leukocytes would provide an in vivo measurement of virulence under conditions more closely approximating the disease process in man. The final definition of the determinants of virulence of N. meningitidis strains will aid in the resolution of many questions concerning the pathogenesis and treatment of cerebrospinal meningitis in man.

SUMMARY AND CONCLUSIONS

Frantz medium was selected and utilized as a simple, chemically defined medium for evaluation of the minimal nutritional requirements of strains of *N. meningitidis*.

Seven strains of *N. meningitidis* were studied in their ability to grow in Frantz medium. The strains were divided into 3 groups according to their minimal nutritional requirements. Group I consisted of 3 strains which grew in a simple, glucose-salts medium. Group II consisted of 2 strains which were capable of growth in Frantz medium following a 24 hour period of growth in Frantz medium supplemented with yeast extract. Group III included 2 strains which required supplementation of Frantz medium for growth.

The absolute requirements of Group III strains were not defined although experiments indicated that one or several amino acids were necessary for the growth of one of these strains. The problems encountered in these experiments with respect to delineation of absolute amino acid requirements were noted and discussed.

Eight strains of *N. meningitidis* and one strain of *N. catarrhalis* were evaluated with respect to their virulence in 12-day old chick embryos. Four strains of *N. meningitidis* were found to be as avirulent in chick embryos as was the strain of *N. catarrhalis*. Three strains of *N. meningitidis* were as virulent in chick embryos as was a strain freshly isolated during the course of this study from an active case of cerebrospinal meningitis. Only in the case of one strain was virulence affected by the type of medium upon which it was grown.

Nutritional independence seemingly represented one of the determinants

of virulence of *N. meningitidis* strains in the chick embryo. Nutritional dependence was associated with avirulence. A similar relationship may well exist with respect to the nutritional independence and pathogenicity of *N. meningitidis* in man.

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TABLE 1

A History of Neisseria meningitidis Strains Used in this Study
Obtained from the University of Oregon Medical School

	Departmental		Recent Isolates	
Strain Number	M-1	Bell	M-2	Heagle
Serological Group	Unknown	Unknown	Unknown	Unknown
Date Isolated	1-'42	12-'42	11-'60	12-'61
Place Isolated	Portland, Oregon	Portland, Oregon	Portland, Oregon	Portland, Oregon
Received From	Clinical Pathology	Clinical Pathology	Medical Student	Clinical Pathology
Source	Spinal Fluid	Spinal Fluid	Nasopharynx	Spinal Fluid
Comment	Department of Bacteriology Laboratory Strain	Isolated during an Epidemic	Isolated during a Bacteriology Laboratory Experiment	Acute Case with Subsequent Recovery in a 5 year old Girl

TABLE 2

A History of Neisseria meningitidis Strains Used in this Study
Obtained from the American Type Culture Collection (4)

Strain Number	M-1027	M-2092	M-1628	M-158
Serological Group	A	B	C	D
Date Isolated	4-'37	1-'48	11-'49	8-'28
Place Isolated	Chicago, Ill.	Boston, Mass.	Hartford, Conn.	Chicago, Ill.
Received From	C.P. Miller, M.D.	Jamaica Plain Lab.	F.L. Mickle, M.D.	Chicago Dept. Health
Source	Spinal Fluid	Spinal Fluid	Spinal Fluid	Spinal Fluid
Comment	Neotype Strain for Group A and for Species	Neotype Strain for Group B	Neotype Strain for Group C	Neotype Strain for Group D

TABLE 3

Composition of Frantz Medium (21)

Compound	Gm/L
L-Glutamic Acid	1.3
L-Cystine	0.012
$NaH_2PO_4 \cdot H_2O$	2.5
KCl	0.09
NaCl	6.0
NH_4Cl	1.25
$MgSO_4 \cdot 7H_2O$	0.6
Glucose	5.0

TABLE 4

Composition of Grossowicz Medium (28)

Compound	Gm/L
Sodium Glutamate	1.0
$Na_2S_2O_3$	0.025
$Na_2HPO_4 \cdot 12H_2O$	2.5
KH_2PO_4	0.35
NaCl	5.0
NH_4Cl	0.3
$MgSO_4 \cdot 7H_2O$	0.3
Glucose	2.0
$CaCl_2 \cdot 4H_2O$	0.1
$FeSO_4$	0.001
$MnSO_4$	0.001
Thiamine	0.001

TABLE 5

The Effect of Constituents of Grossowicz Medium on Growth of Strain M-1

Compounds Deleted from Grossowicz Medium	Compounds Replaced	Type of Growth
None	None	Granular
Glutamate, $Na_2S_2O_3$, Thiamine	Glutamate	Granular
	Glutamate, $Na_2S_2O_3$	Granular
	Glutamate, $Na_2S_2O_3$, Thiamine	Granular
Na_2HPO_4, KH_2PO_4, $CaCl_2$, $FeSO_4$, $MnSO_4$	Na_2HPO_4	Non-granular
	Na_2HPO_4, KH_2PO_4	Non-granular
	Na_2HPO_4, KH_2PO_4, $CaCl_2$	Granular
	Na_2HPO_4, KH_2PO_4, $CaCl_2$, $FeSO_4$	Granular
	Na_2HPO_4, KH_2PO_4, $CaCl_2$, $FeSO_4$, $MnSO_4$	Granular

TABLE 6

Minimal Medium for Group I Strains of *N. meningitidis*

Compound	Gm/L
$Na_2S_2O_3$	0.012
$NaH_2PO_4 \cdot H_2O$	2.5
KCl	0.09
NaCl	6.0
NH_4Cl	1.25
$MgSO_4 \cdot 7H_2O$	0.3
Glucose	5.0

TABLE 7

The Relative Growth Response of Strain M-1 to the Migratory Components of Complex Nutrients Separated by Paper Chromatography

Rf	Yeast Extract		Difco Casamino Acids		Acid Hydrolyzed Casein		Enzyme Hydrolyzed Casein	
	Hours Incubated with Strain M-1		Hours Incubated with Strain M-1		Hours Incubated with Strain M-1		Hours Incubated with Strain M-1	
	72	144	72	144	72	144	72	144
0.0-0.1	0[b]	0	0	0	0	0	0	0
0.1-0.2	0	0	0	0	0	0	0	0
0.2-0.3	0	0	0	0	0	0	0	0
0.3-0.4	tr	tr	tr	tr	tr	tr	tr	tr
0.4-0.5	+	++	tr	++	tr	+++	tr	tr
0.5-0.6	0	0	tr	++	+	+	tr	tr
0.6-0.7	0	0	0	0	++	+++	tr	tr
0.7-0.8	0	0	0	0	0	0	++	+++
0.8-0.9	0	0	0	0	0	0	0	0
0.9-1.0	0	0	0	0	0	0	0	0
Control[a]	++++	++++	++++	++++	++++	++++	++++	++++

a. Growth response to an equivalent amount of each nutrient which was not subjected to chromatography.

b. Symbols: 0 represents absence of growth;
+ represents presence of growth, graded from trace (tr) to four plus.

TABLE 8

Cultures from Chick Embryos Which Died Subsequent to Inoculation with *Neisseria*[a]

Strains Inoculated	Embryos Cultured	Hours Following Inoculation 24	48	72	96	120	144	168	Totals
Group I[b]	No. Positive	1	23	14	1	2	1	1	43
	No. Negative	0	0	2	0	3	0	2	7
	Total	1	23	16	1	5	1	3	50
	% Positive	100	100	88	100	40	100	33	86
Group II[c]	No. Positive	0	17	6	3	2	1	3	32
	No. Negative	0	0	0	1	5	2	1	9
	Total	0	17	6	4	7	3	4	41
	% Positive	0	100	100	75	29	33	75	80
Group III[d]	No. Positive	0	2	0	1	0	0	0	3
	No. Negative	0	0	0	1	4	3	2	10
	Total	0	2	0	2	4	3	2	13
	% Positive	0	100	0	50	0	0	0	23
Heagle	No. Positive	0	7	3	0	2	1	0	13
	No. Negative	0	0	0	1	0	0	1	2
	Total	0	7	3	1	2	1	1	15
	% Positive	0	100	100	0	100	100	0	87
N. catarrhalis	No. Positive	0	0	0	0	0	0	0	0
	No. Negative	0	0	0	2	2	0	1	5
	Total	0	0	0	2	2	0	1	5
	% Positive	0	0	0	0	0	0	0	0

a. All cultures were initiated within 16 hours after death of the embryos.

b. Group I consists of strains M-1027, M-2092 and M-1628.

c. Group II consists of strains M-2 and Bell.

d. Group III consists of strains M-1 and M-158.

FIGURE 1

The Effect of Additions to Frantz Glucose-Salts Solution on the Growth Response of *N. meningitidis* (M-1027)

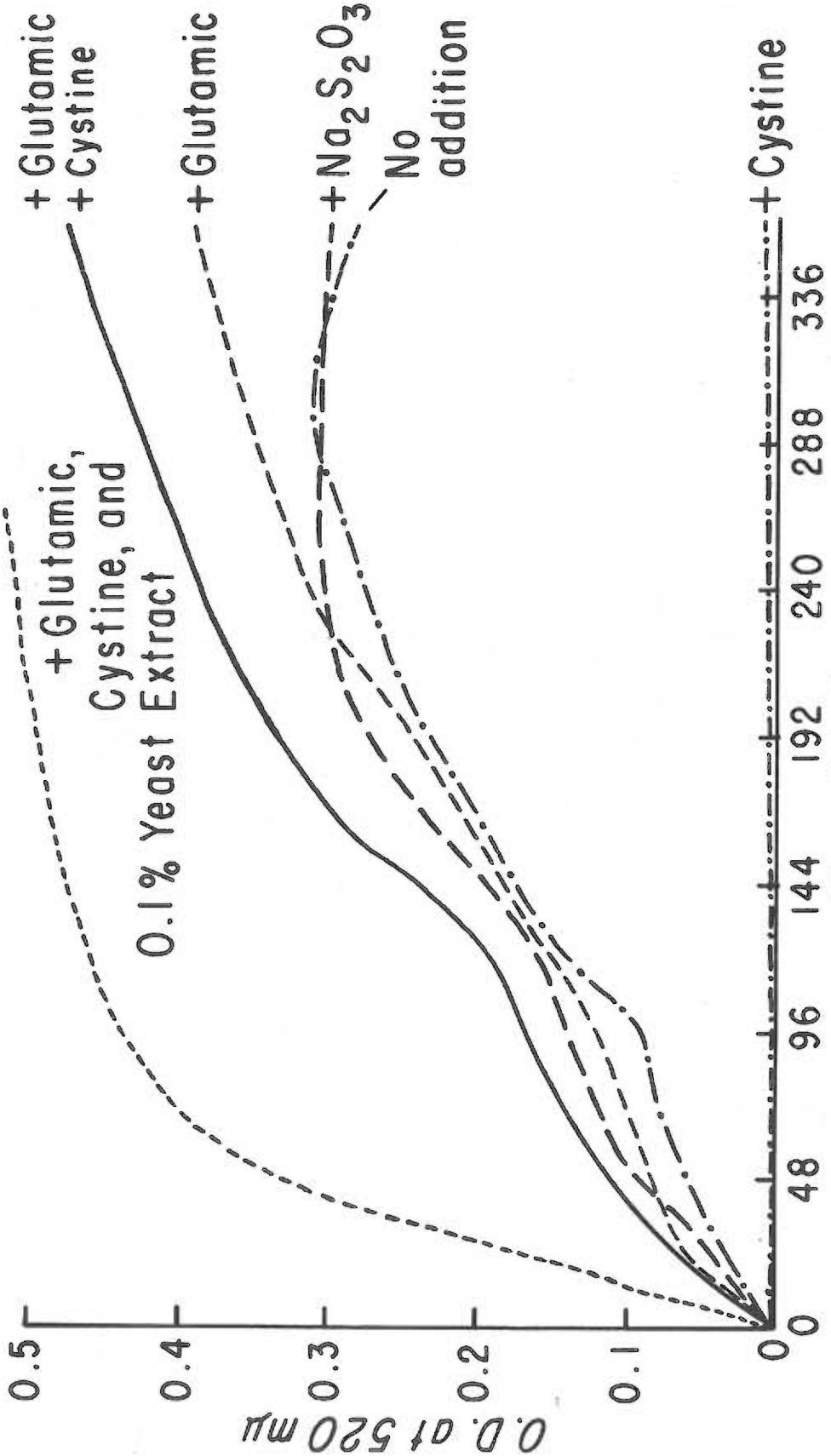

+ Glutamic
+ Cystine
+ Glutamic, Cystine, and 0.1% Yeast Extract
+ Glutamic
+ $Na_2S_2O_3$
No addition
+ Cystine
0.5
0.4
0.3
0.2
0.1
0
48
96
144
192
240
288
336
Time (Hrs)
O.D. at 520 mμ

FIGURE 2

Growth Response at 144 Hours of Group I Strains

Upon Continued Subculture in Simple Glucose-Salts Medium

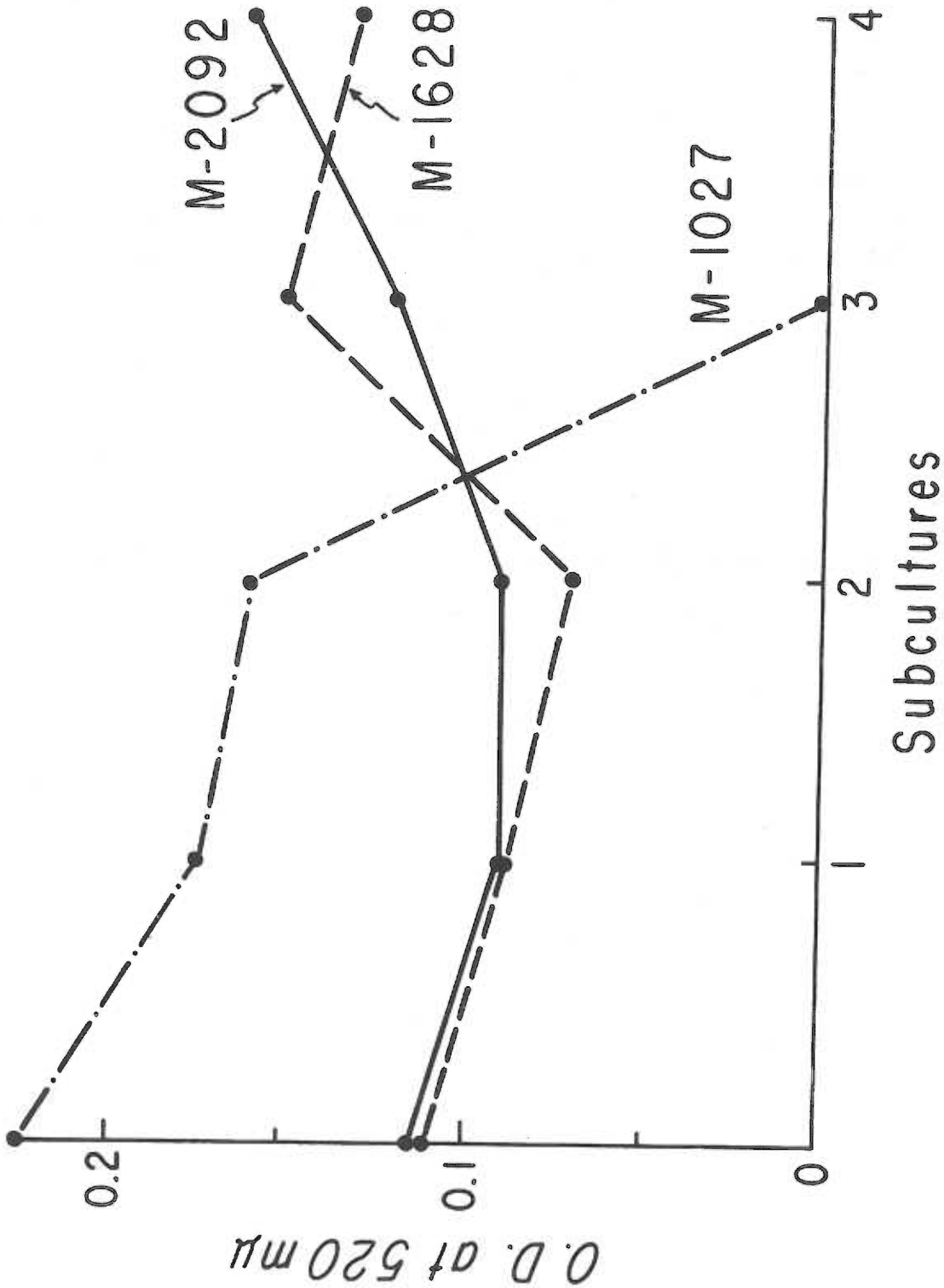

M-2092
M-1628
M-1027
0.2
0.1
0
1
2
3
4
Subcultures
O. D. at 520 mμ

FIGURE 3

Growth Response of Group II Strains

in Frantz Medium Supplemented with 0.2% Yeast Extract

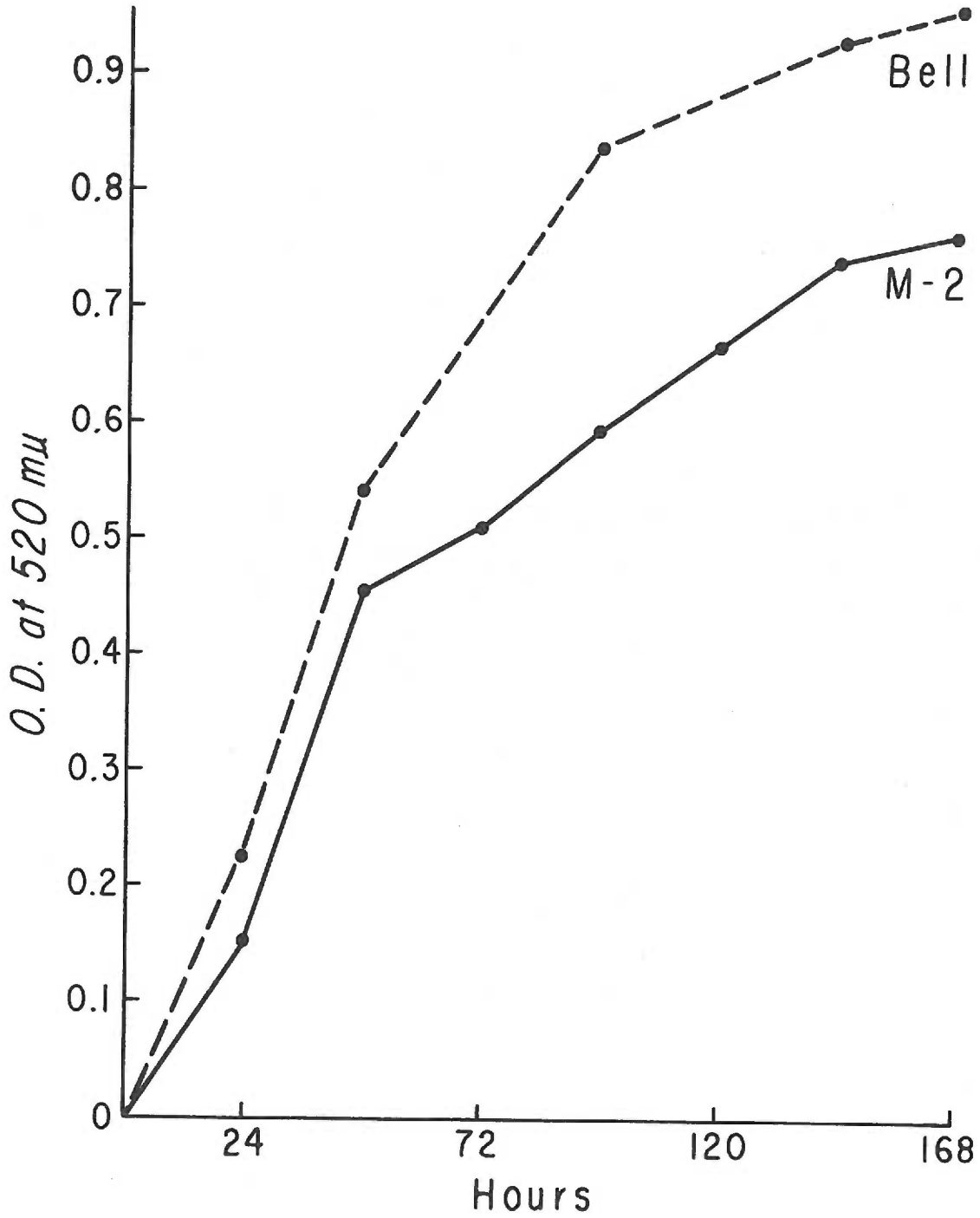

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 520 mμ
Bell
M-2
24
72
120
168
Hours

FIGURE 4

Growth Response at 144 Hours of Group II Strains Upon Continued Subculture in Frantz Medium Following Initial Culture in Frantz Medium Supplemented with Yeast Extract

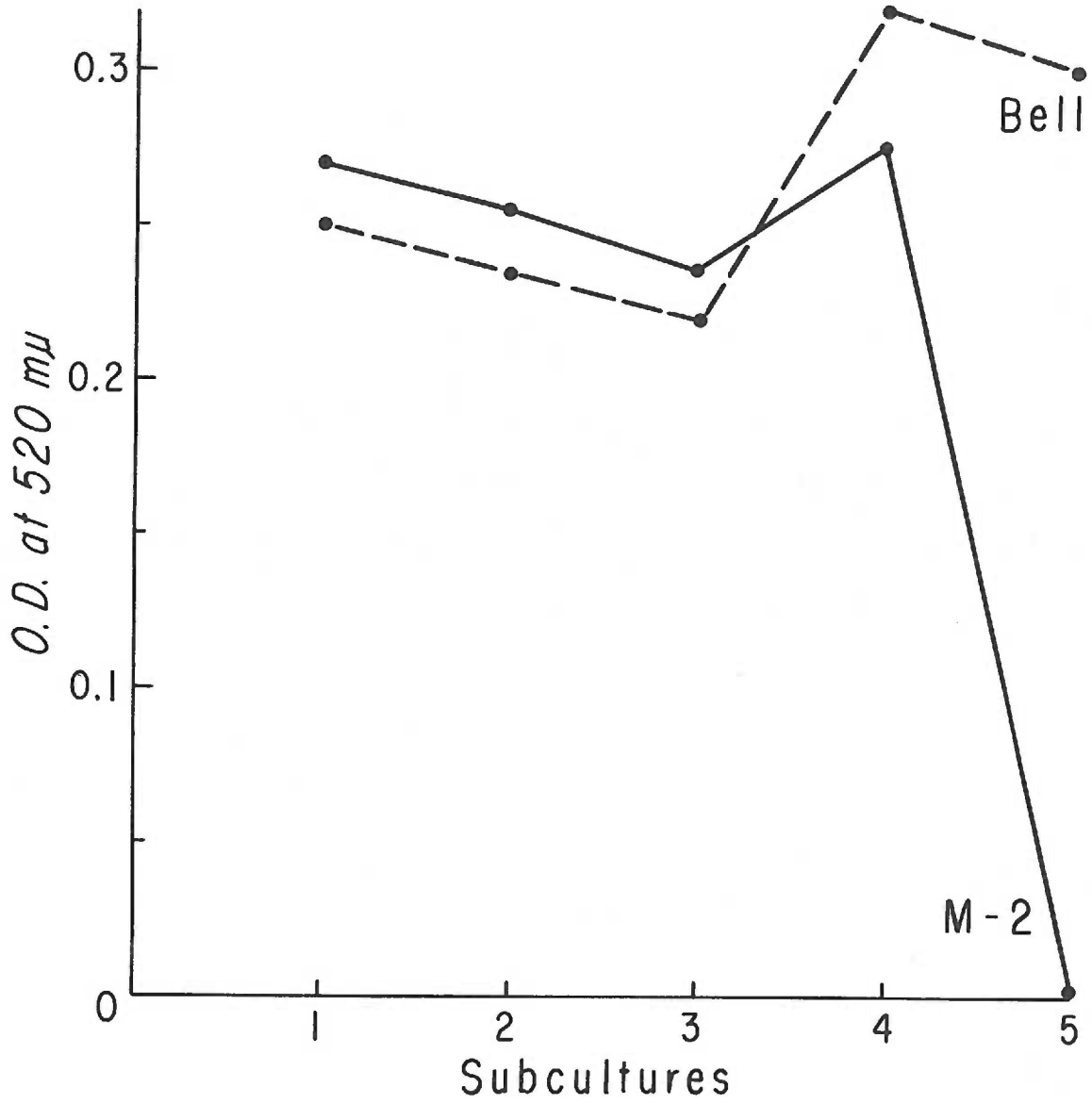

0.3
0.2
0.1
0
O.D. at 520 mμ
Bell
M-2
1
2
3
4
5
Subcultures

FIGURE 5

Growth of Strain M-2 in Supplemented Frantz Medium

Key:

1. YE - yeast extract (Difco)
2. EHC - enzyme hydrolyzate of casein (Nutritional Biochemicals Corporation)
3. AHC - acid hydrolyzate of casein (Nutritional Biochemicals Corporation)
4. CA - casamino acids (Difco acid hydrolyzed casein)
5. CA+Biotin+Niacin - casamino acids (Difco) plus biotin (5 mcg/ml) and niacin (5 mcg/ml)

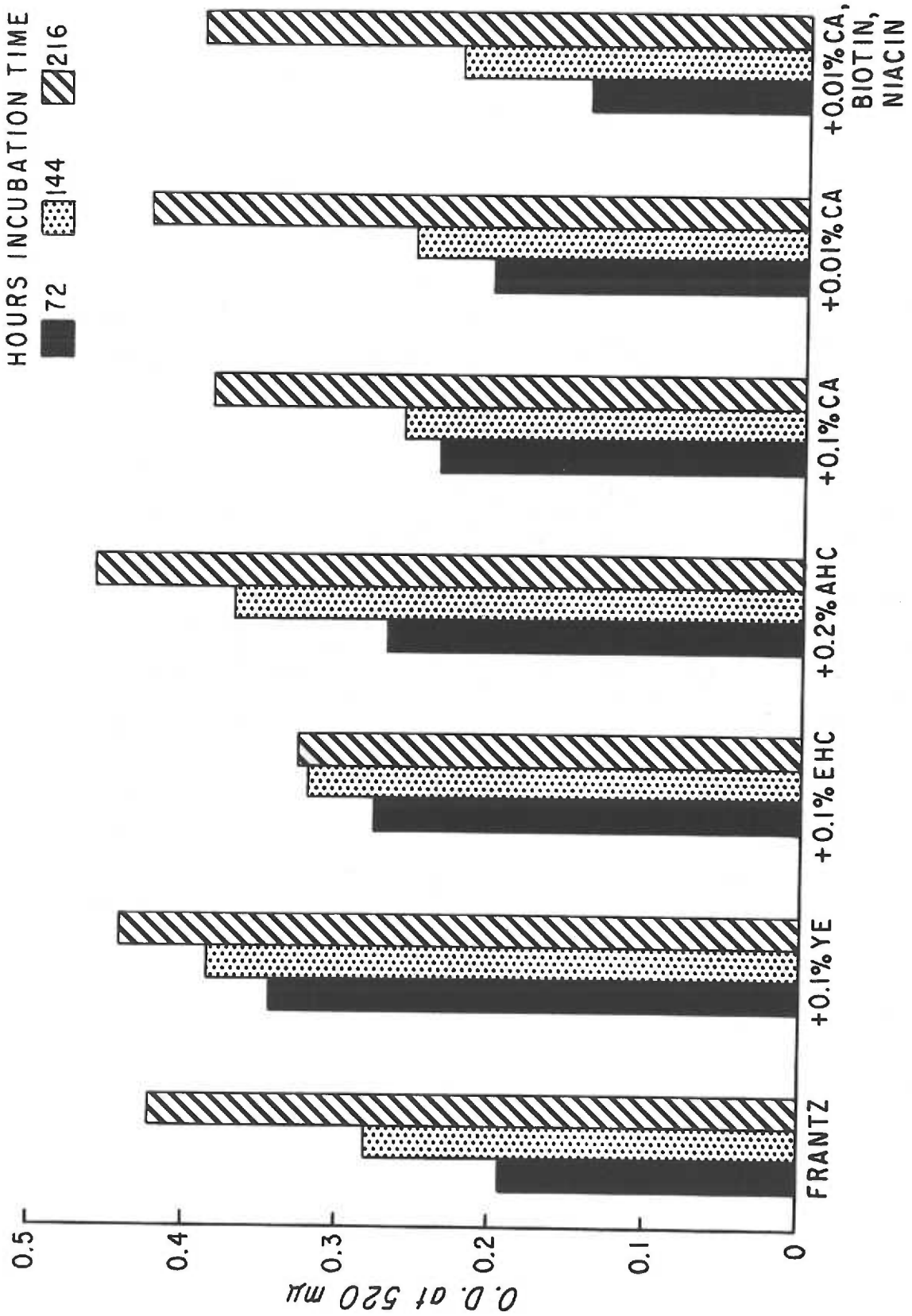

HOURS INCUBATION TIME
72
144
216
O.D. at 520 mμ
0.5
0.4
0.3
0.2
0.1
0
FRANTZ
+0.1% YE
+0.1% EHC
+0.2% AHC
+0.1% CA
+0.01% CA
+0.01% CA, BIOTIN, NIACIN

FIGURE 6

Effect of Amino Acid Mixtures on the Growth Response of Strain M-2 in Unsupplemented and Yeast Extract Supplemented Frantz Medium

Key:

1. Frantz - unmodified Frantz medium
2. 0.2% YE - 0.2% yeast extract (Difco) in Frantz Medium lacking L-glutamic acid and L-cystine (modified Frantz medium)
3. 0.1% YE - 0.1% yeast extract (Difco) in modified Frantz medium
4. 18 AA - solution of 18 amino acids in modified Frantz medium:

Amino Acid	Gm/L
1). DL-aspartic acid	1.0
2). L-glutamic acid	1.0
3). DL-alanine	1.0
4). L-arginine	0.2
5). DL-methionine	0.2
6). DL-serine	0.2
7). DL-phenylalanine	0.2
8). DL-isoleucine	0.2
9). L-lysine.	0.2
10). L-leucine	0.1
11). L-proline	0.1
12). L-histidine.	0.1
13). L-threonine.	0.2
14). DL-tryptophan	0.2
15). DL-valine	0.2
16). L-cystine	0.1
17). L-tyrosine	0.1
18). Glycine	0.1

5. 0.2% YE+18 AA - 0.2% yeast extract (Difco) and mixture of 18 amino acids listed above in modified Frantz medium
6. 12AA - first 12 of the 18 amino acids listed above in modified Frantz medium

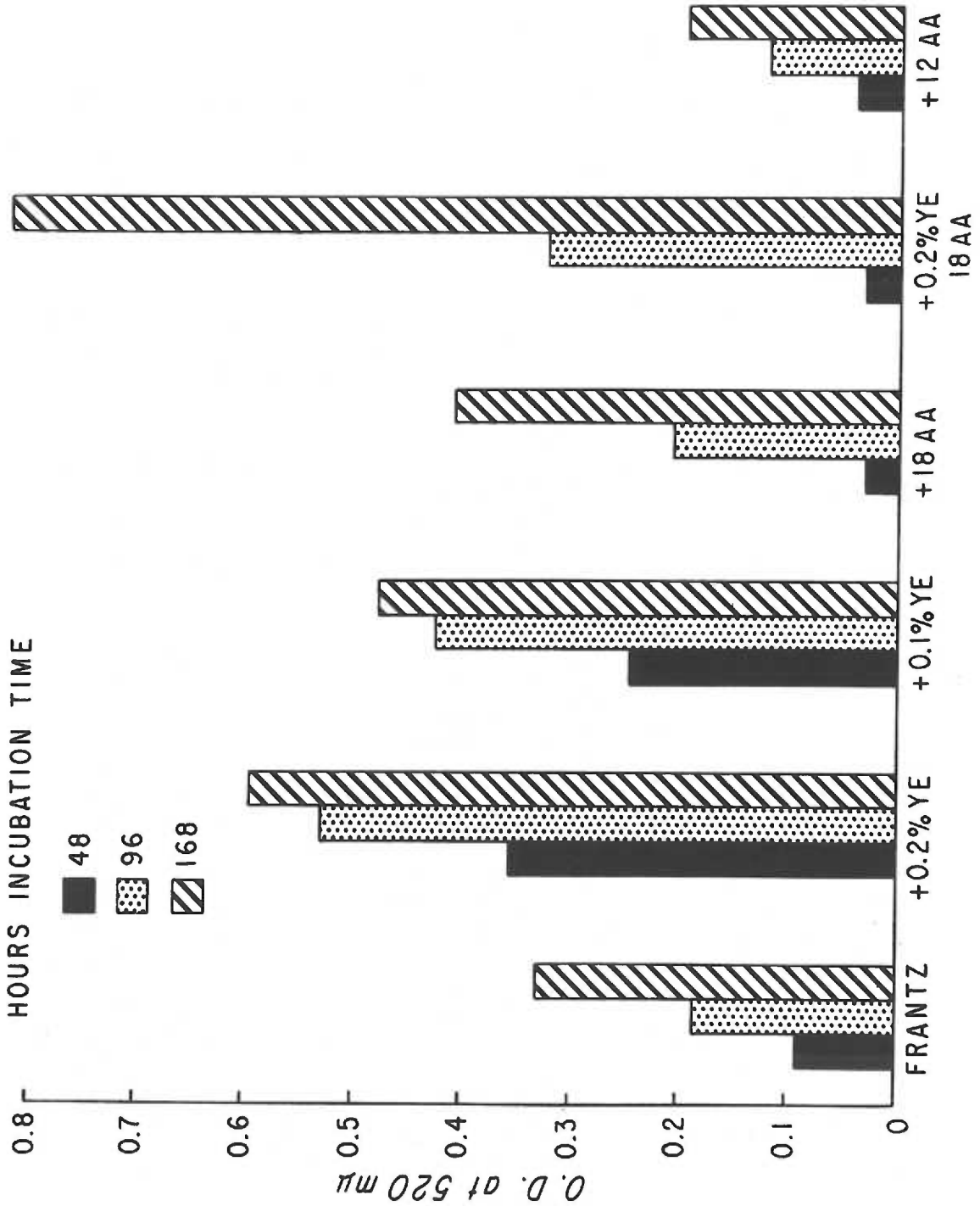

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O. D. at 520 mμ
HOURS INCUBATION TIME
48
96
168
FRANTZ
+0.2% YE
+0.1% YE
+18 AA
+0.2% YE
18 AA
+12 AA

FIGURE 7

Growth Response of Strain M-1 in Frantz Medium Containing Yeast Extract and the Components of Dialysis of Yeast Extract

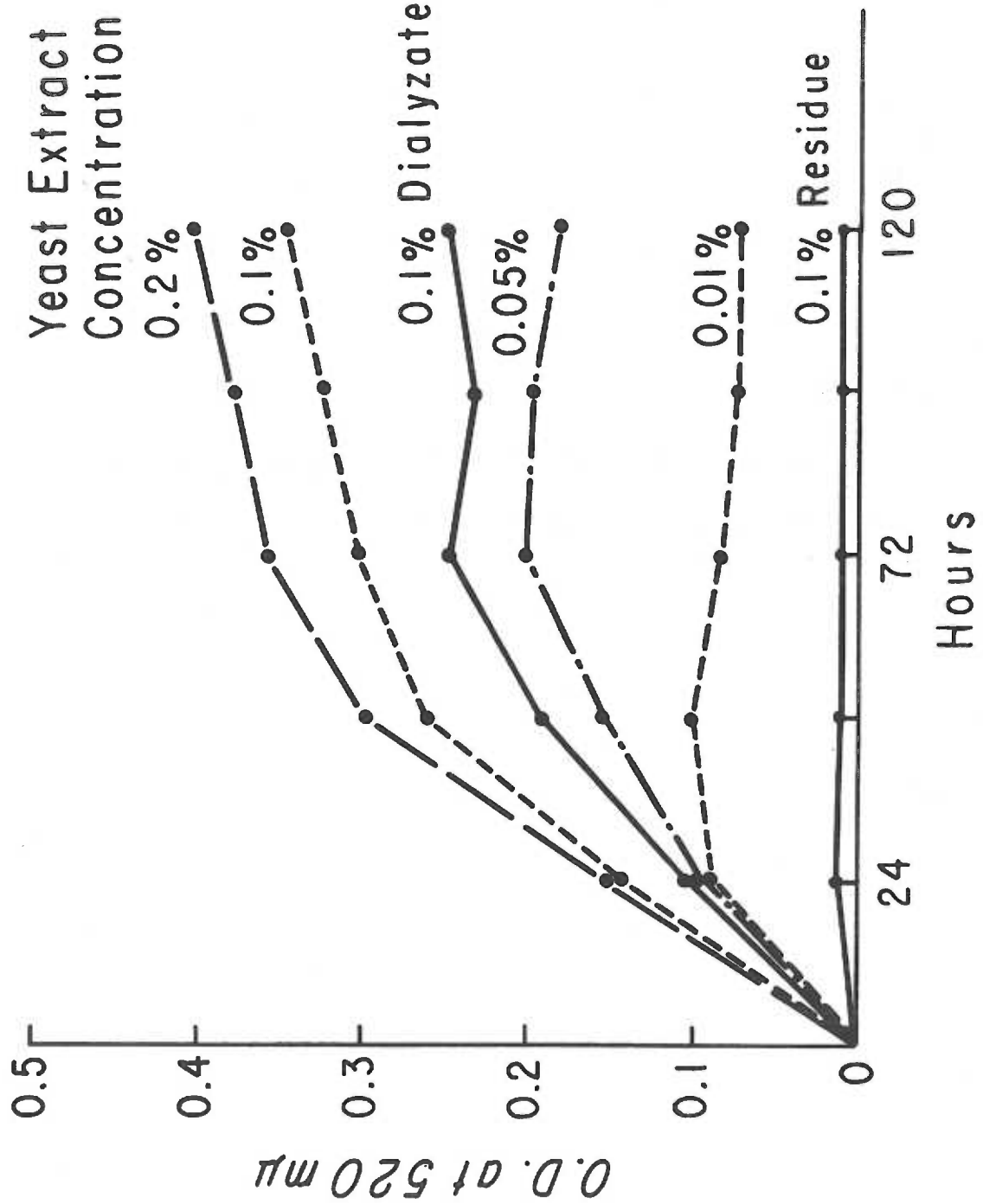

Yeast Extract Concentration
0.2%
0.1%
0.1% Dialyzate
0.05%
0.01%
0.1% Residue
0.5
0.4
0.3
0.2
0.1
0
24
72
120
Hours
O.D. at 520 mμ

FIGURE 8

A. Growth Response of Strain M-1 in Frantz Medium with Added Difco Vitamin-Free Casamino Acids

B. Semilogarithmic Plot of the 96 Hour Growth Response of Strain M-1 in Frantz Medium Containing Graded Increments of Difco Vitamin-Free Casamino Acids

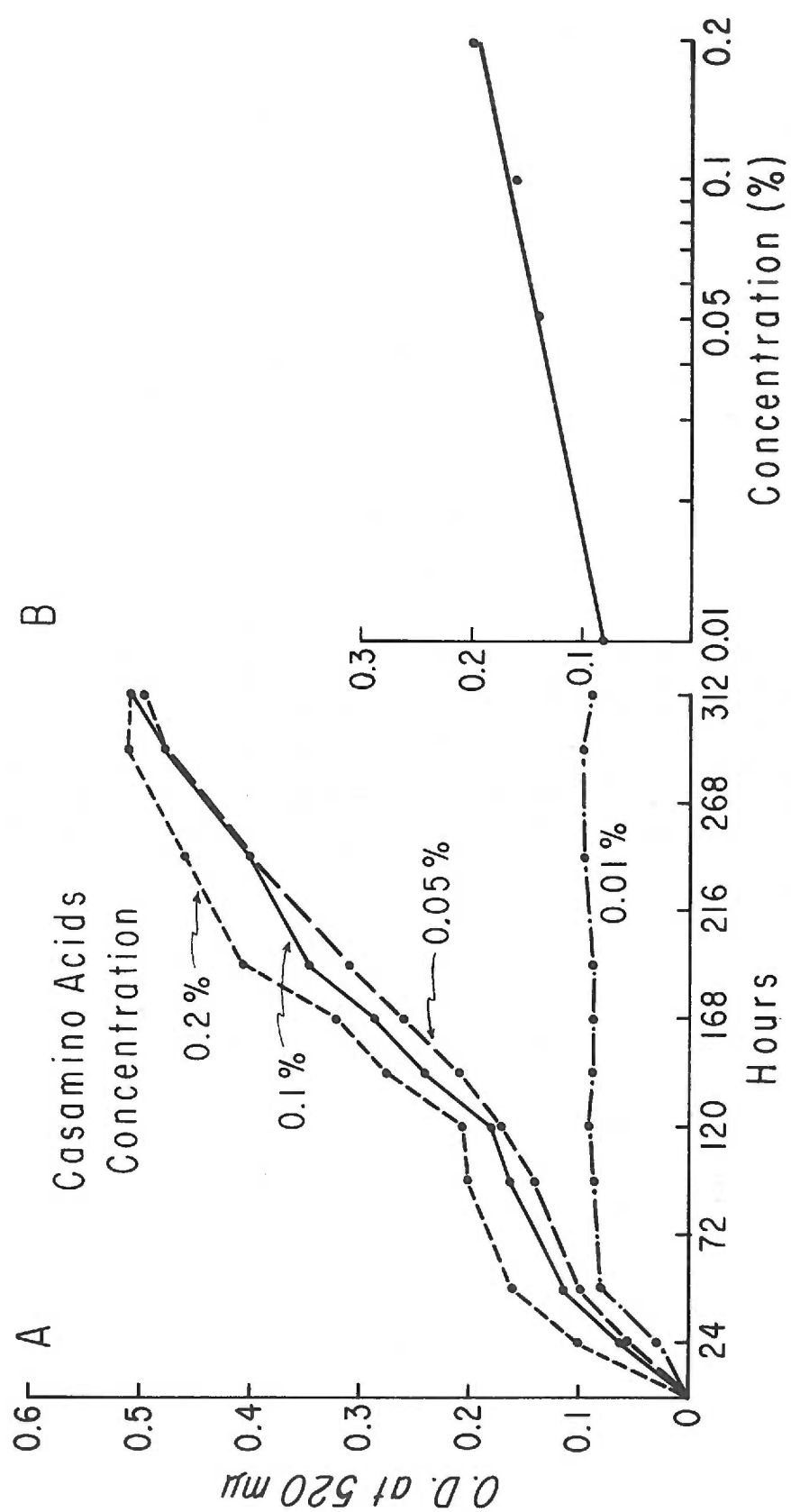

A
B
Casamino Acids
Concentration
0.2%
0.1%
0.05%
0.01%
O.D. at 520 mμ
Hours
Concentration (%)
0
24
72
120
168
216
268
312
0.01
0.05
0.1
0.2
0.3
0.4
0.5
0.6

FIGURE 9

Growth Response at 192 Hours of Strain M-1 Upon Continued Subculture in Frantz Medium with Added Difco Vitamin-Free Casamino Acids

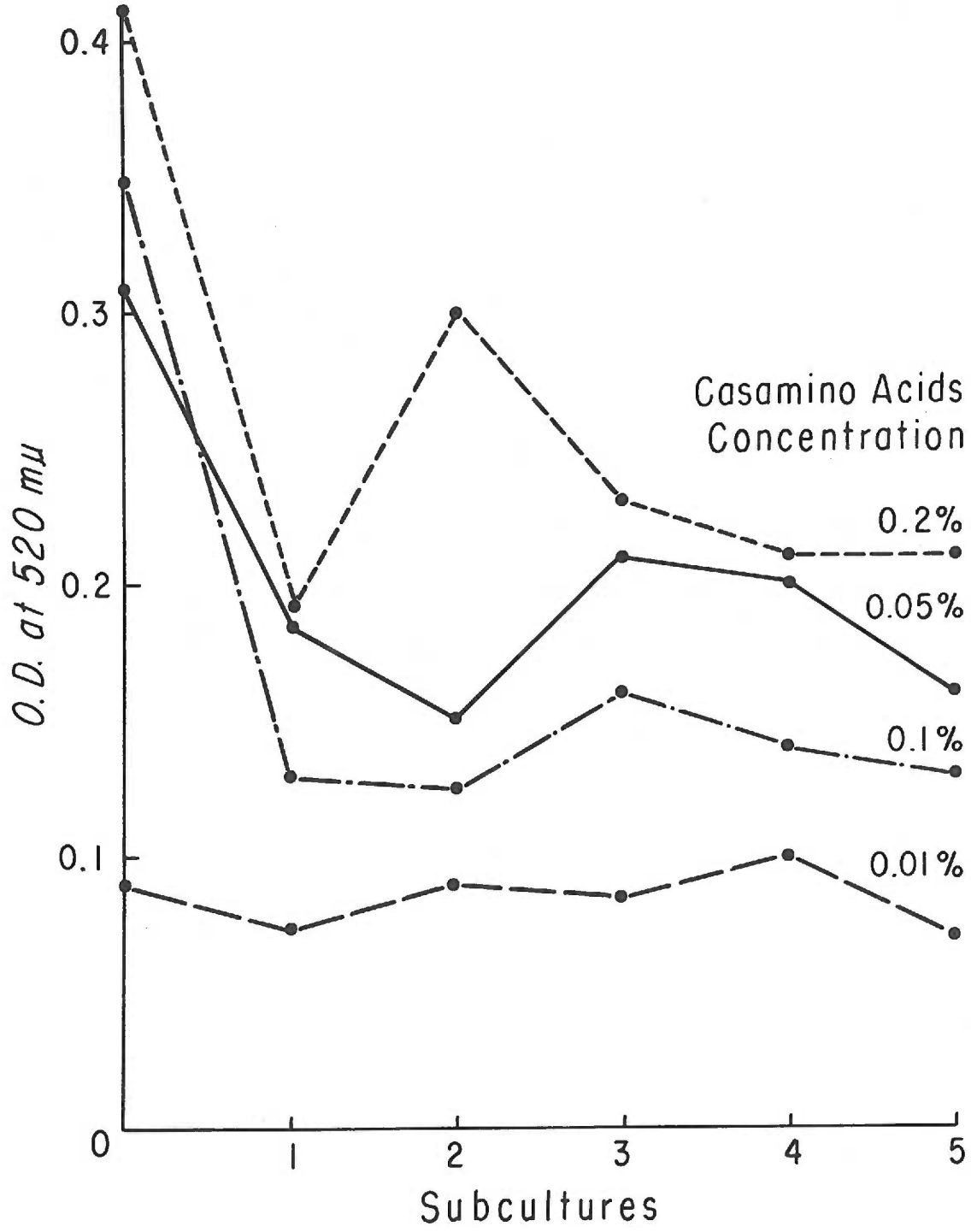

0.4
0.3
0.2
0.1
0
O.D. at 520 mμ
Casamino Acids
Concentration
0.2%
0.05%
0.1%
0.01%
1
2
3
4
5
Subcultures

FIGURE 10

Growth Response of Strain M-1 in Frantz Medium with Concentration Increments of Vitamin-Free Casein Hydrolyzates

A. Acid Hydrolyzed Casein (Nutritional Biochemicals Corporation)

B. Enzyme Hydrolyzed Casein (Nutritional Biochemicals Corporation)

Key:
0.003% YE - 0.003% yeast extract (This represents a 1:30 dilution of that amount of yeast extract carried over in each 0.1 ml inoculum from a culture containing 0.1% yeast extract)

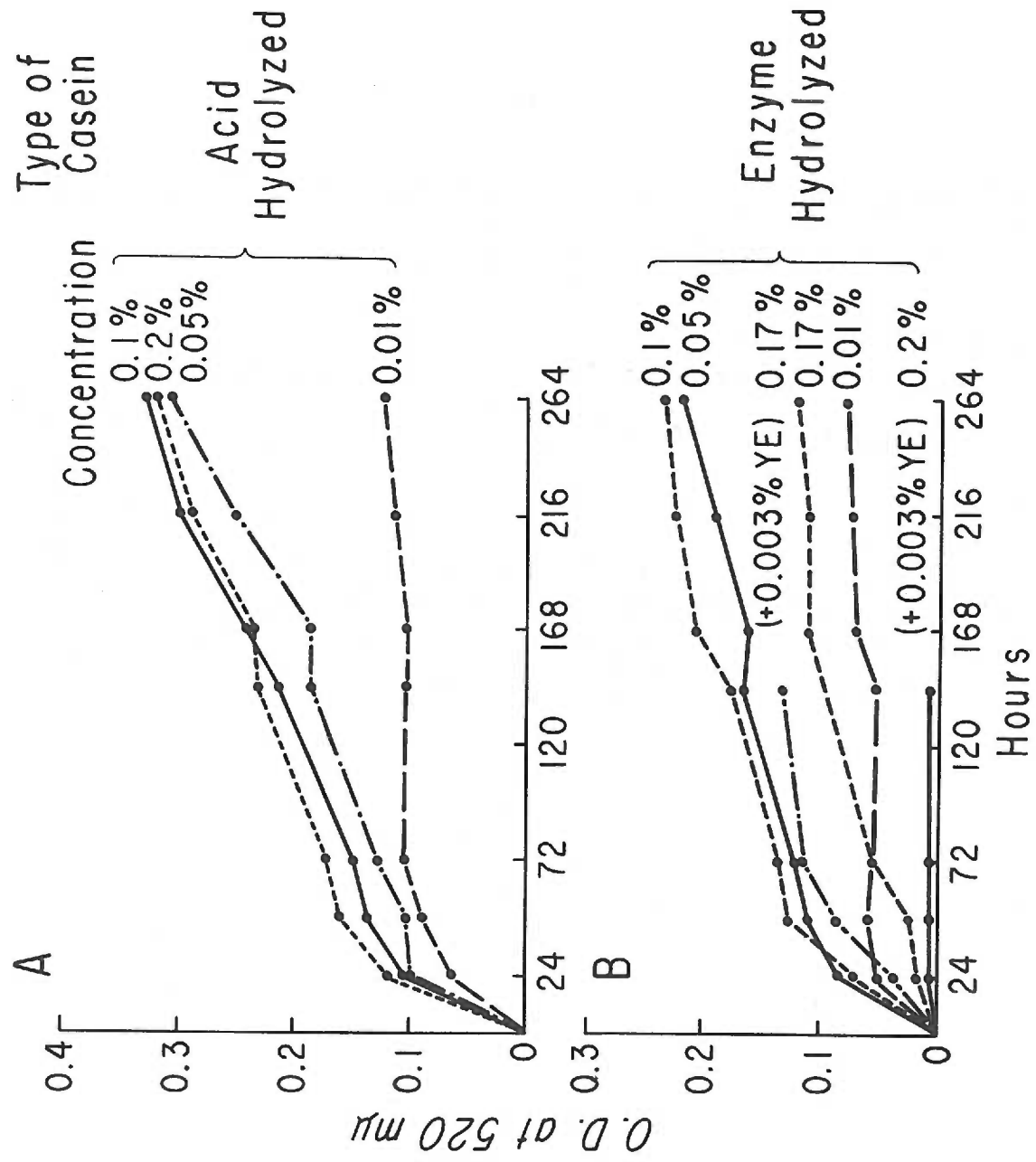

Type of Casein
Acid Hydrolyzed
Concentration
0.1%
0.2%
0.05%
0.01%
A
B
Enzyme Hydrolyzed
0.1%
0.05%
(+0.003% YE) 0.17%
0.17%
0.01%
(+0.003% YE) 0.2%
O.D. at 520 mμ
0.4
0.3
0.2
0.1
0
24
72
120
168
216
264
Hours

FIGURE 11

Growth Response of *N. meningitidis* (M-1) at 72 Hours in Supplemented Frantz Medium

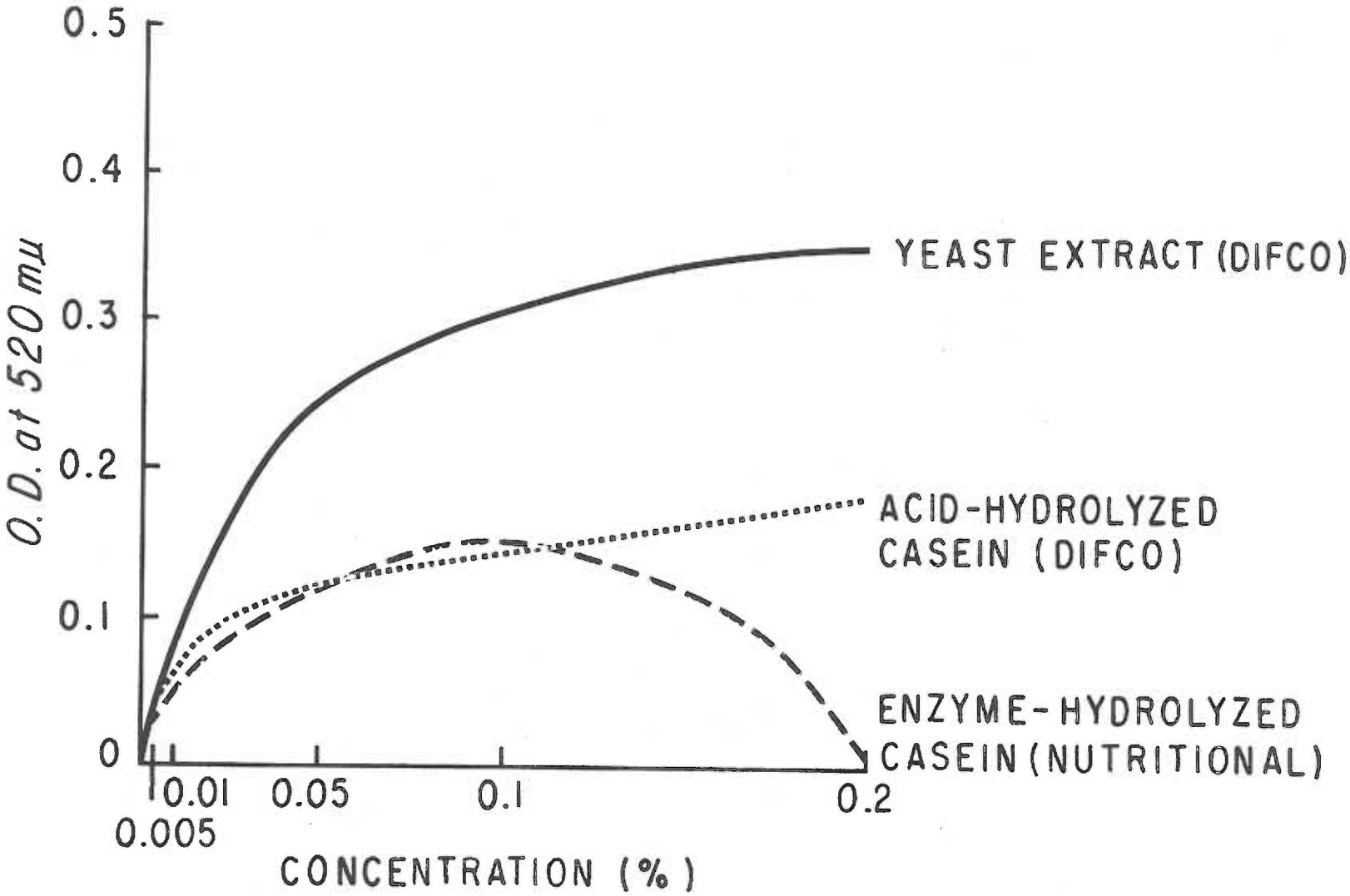

0.5
0.4
0.3
0.2
0.1
0
O.D. at 520 mμ
YEAST EXTRACT (DIFCO)
ACID-HYDROLYZED CASEIN (DIFCO)
ENZYME-HYDROLYZED CASEIN (NUTRITIONAL)
0.005
0.01
0.05
0.1
0.2
CONCENTRATION (%)

FIGURE 12

The Effect of Amino Acids on the Growth of Strain M-1

Key:

1. Frantz - unmodified Frantz medium
2. Mod.Frantz- modified Frantz medium (L-glutamic acid and L-cystine were deleted)
3. 0.1% YE - 0.1% yeast extract (Difco)
4. TM - total mixture of 12 amino acids in the following concentrations:

Amino Acid	Gm/L
1). DL-aspartic acid	1.0
2). L-glutamic acid	1.0
3). DL-alanine	1.0
4). L-arginine	0.2
5). DL-methionine	0.2
6). DL-serine	0.2
7). DL-phenylalanine	0.2
8). DL-isoleucine	0.2
9). L-lysine	0.2
10). L-leucine	0.1
11). L-proline	0.1
12). L-histidine	0.1

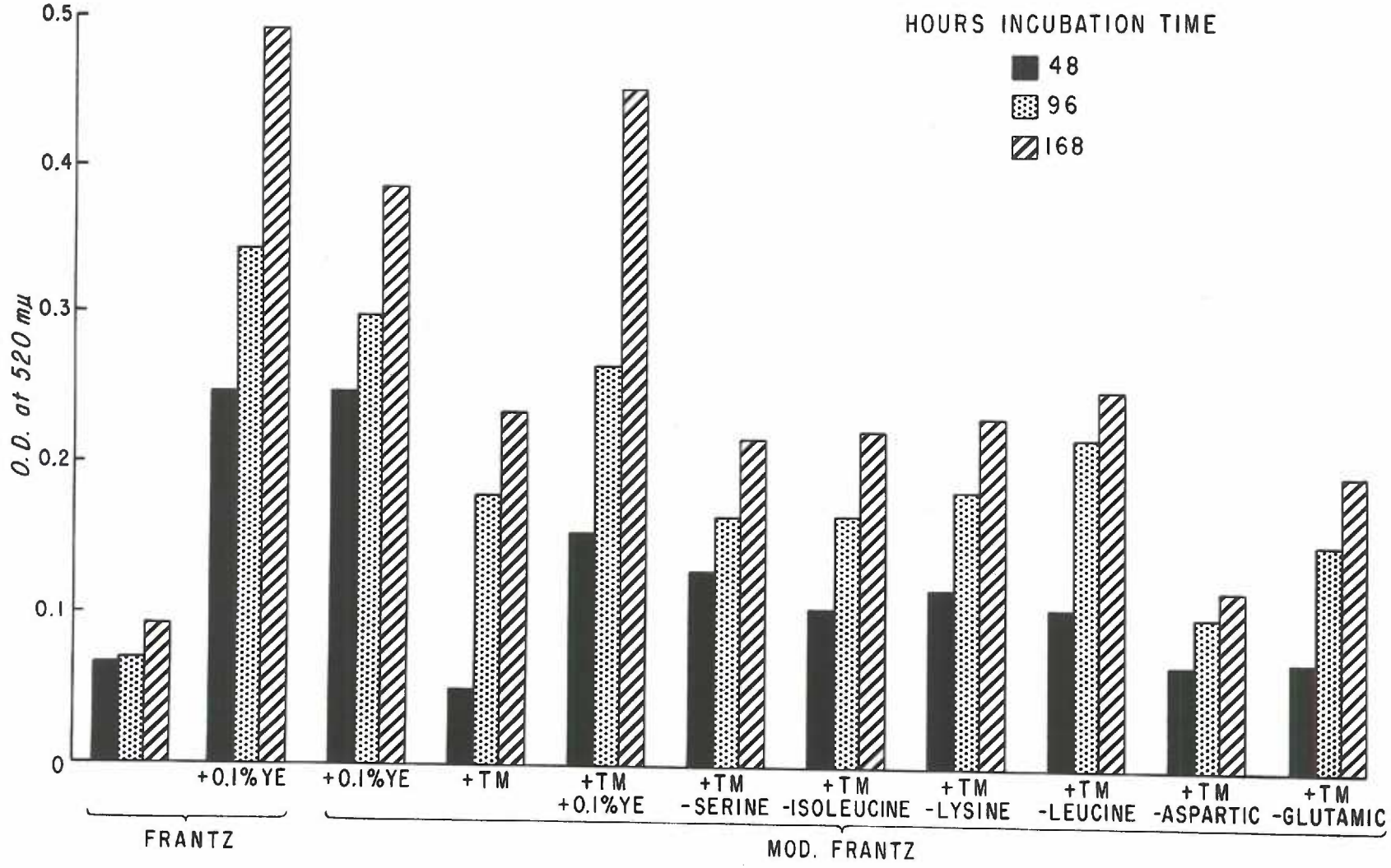

HOURS INCUBATION TIME
48
96
168
O.D. at 520 mμ
0.5
0.4
0.3
0.2
0.1
0
+0.1%YE
+0.1%YE
+TM
+TM +0.1%YE
+TM -SERINE
+TM -ISOLEUCINE
+TM -LYSINE
+TM -LEUCINE
+TM -ASPARTIC
+TM -GLUTAMIC
FRANTZ
MOD. FRANTZ

FIGURE 13

Mortality in Chick Embryos Inoculated with a Strain of *Neisseria catarrhalis* Cultured on DSA Medium

Experiment	No. Bacteria Inoculated
# 1	2.2×10^5, 2.2×10^4
# 2	6×10^5, 6×10^4

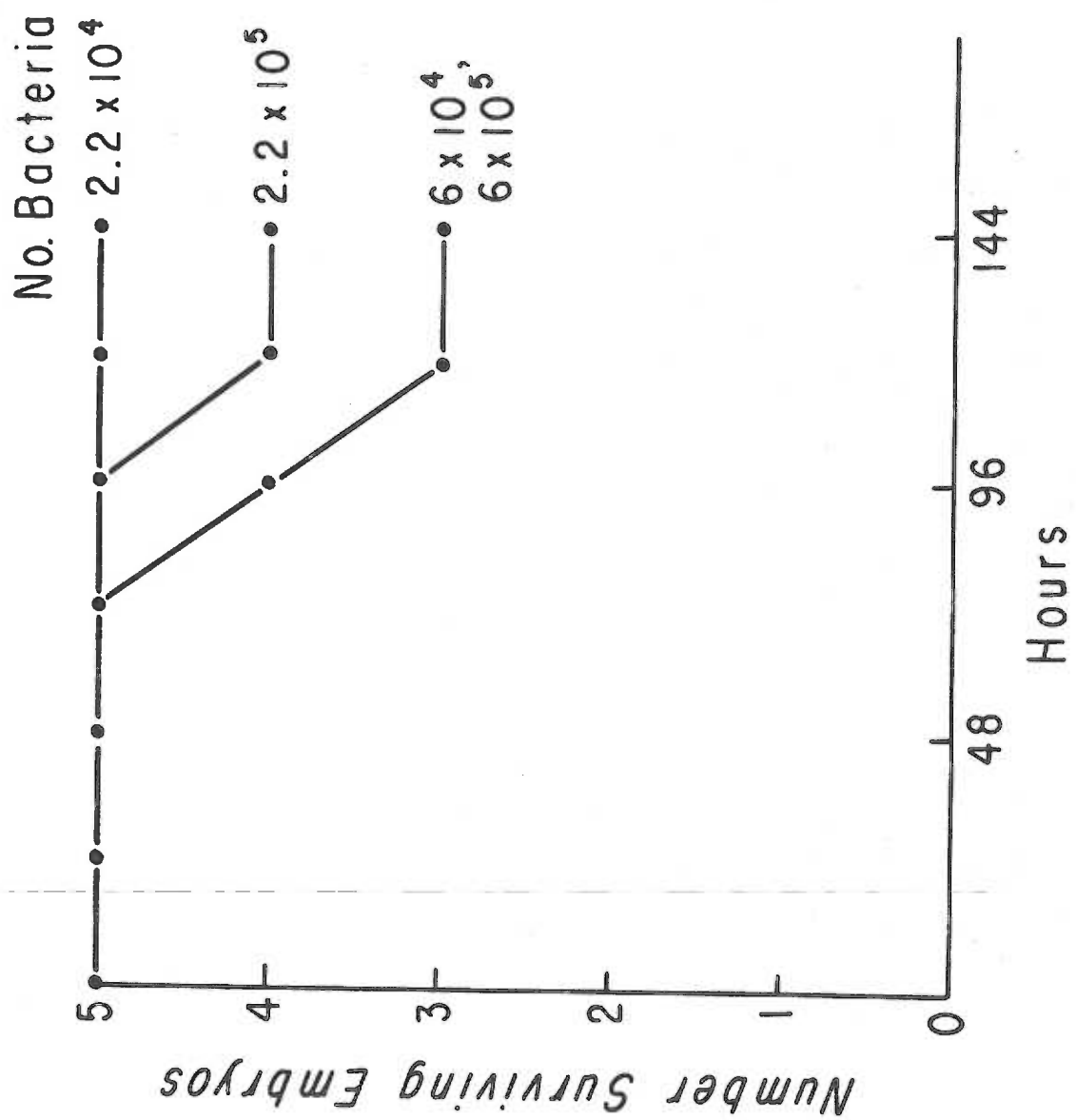

No. Bacteria
2.2 x 10^4
2.2 x 10^5
6 x 10^4
6 x 10^5
Number Surviving Embryos
0
1
2
3
4
5
48
96
144
Hours

FIGURE 14

Mortality in Chick Embryos Inoculated with Group I Strains, M-1027 and M-2092, Cultured on Frantz and DSA Media

A. Strain M-1027

1. Grown on Frantz Medium (-o-)
2. Grown on DSA Medium (-•-)

B. Strain M-2092

1. Grown on Frantz Medium (-o-)
2. Grown on DSA Medium (-•-)

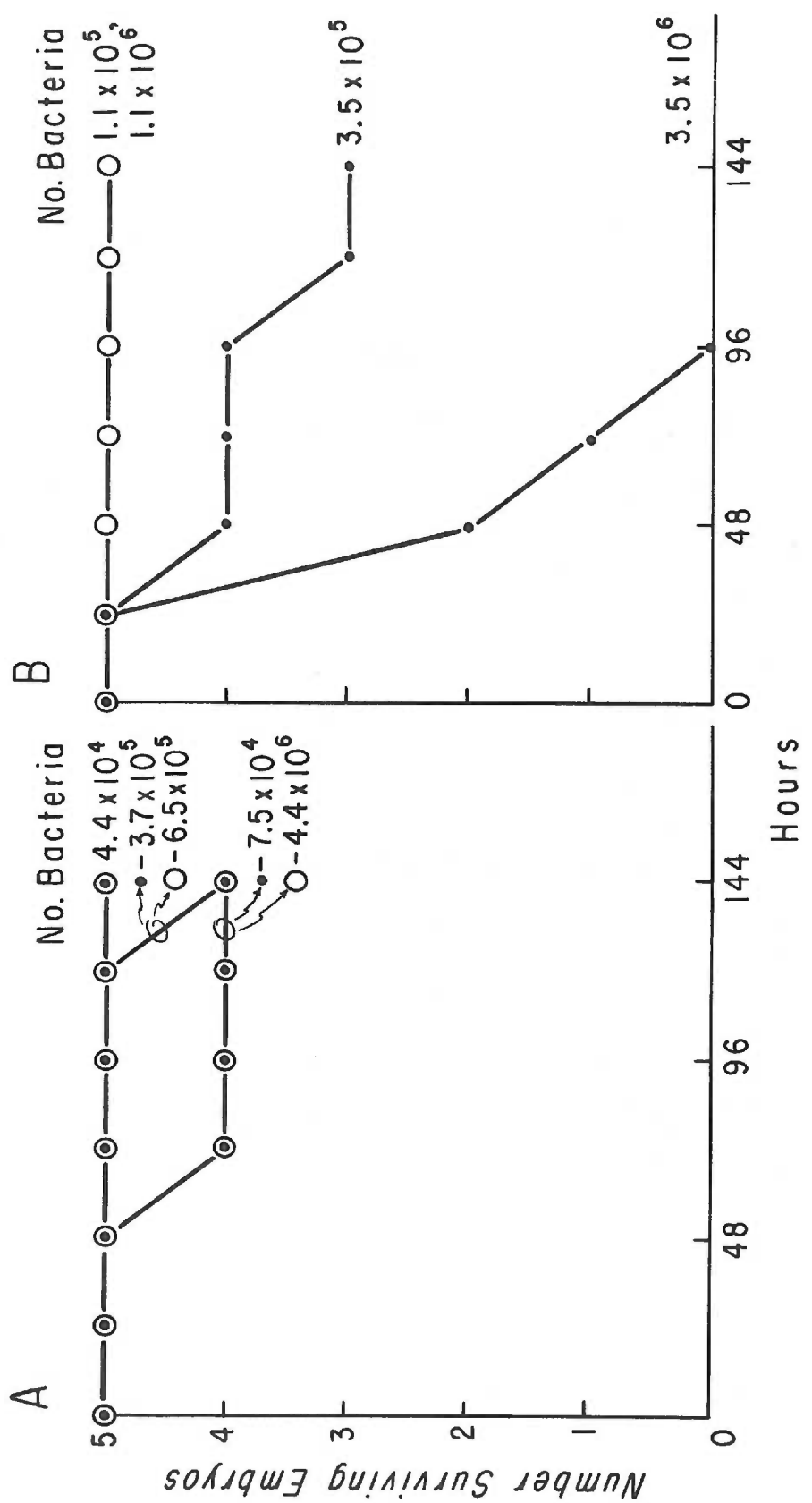

A
B
No. Bacteria
4.4 x 10^4
3.7 x 10^5
6.5 x 10^5
7.5 x 10^4
4.4 x 10^6
No. Bacteria
1.1 x 10^5, 1.1 x 10^6
3.5 x 10^5
3.5 x 10^6
Number Surviving Embryos
0
1
2
3
4
5
48
96
144
Hours

FIGURE 15

Mortality in Chick Embryos Inoculated with Group I Strain M-1628

A. Cultured on DSA Medium

Experiment	No. Bacteria Inoculated
# 1	2.2×10^6, 2.2×10^5
# 2	2.7×10^5, 2.2×10^4, 2.2×10^3

B. Cultured on Frantz Medium

Experiment	No. Bacteria Inoculated
# 1	5.4×10^5, 5.4×10^4
# 2	5.4×10^4, 3.1×10^2, 6.6×10^1

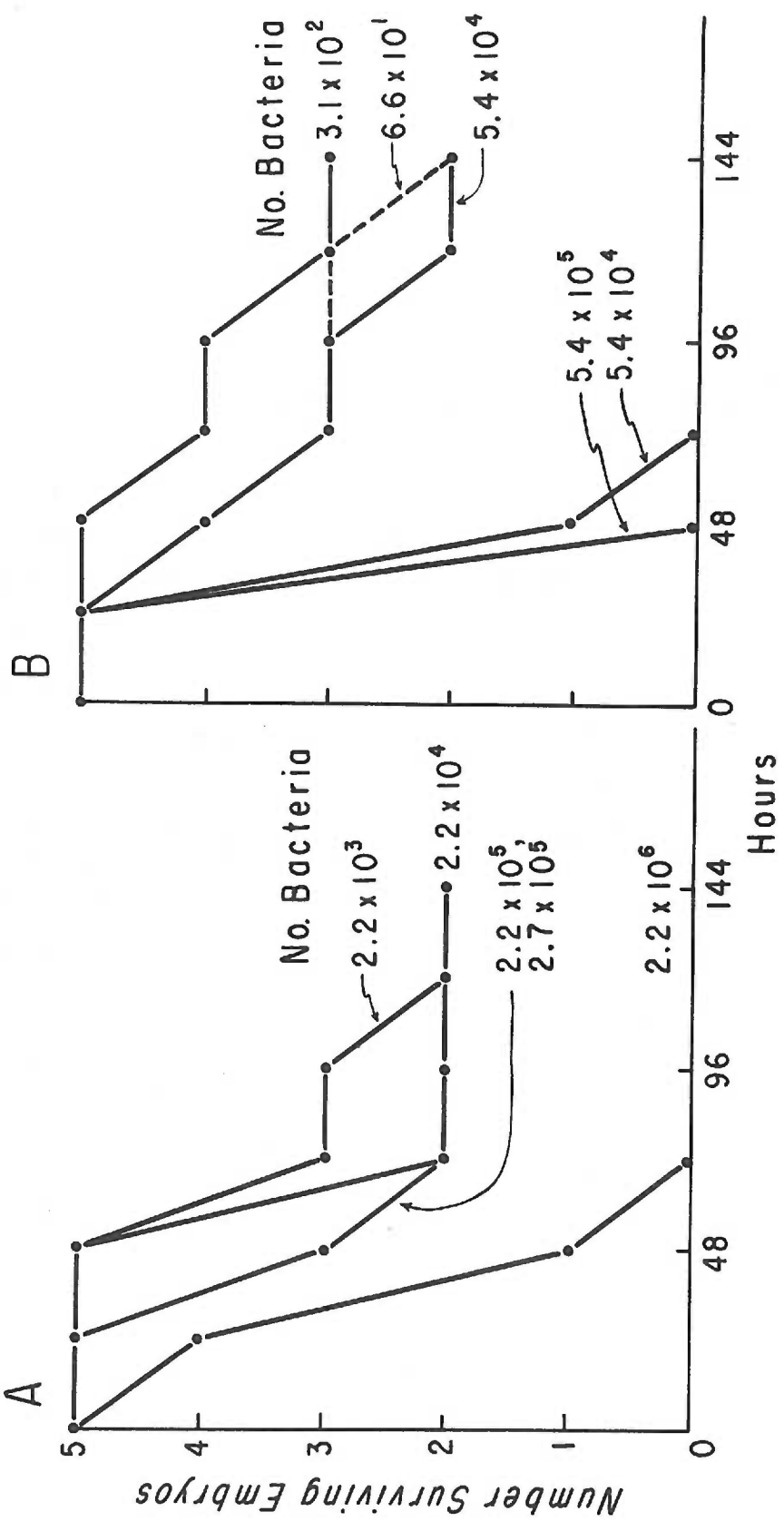

A
B
No. Bacteria
2.2 x 10^3
2.2 x 10^4
2.2 x 10^5, 2.7 x 10^5
2.2 x 10^6
No. Bacteria
3.1 x 10^2
6.6 x 10^1
5.4 x 10^4
5.4 x 10^5
5.4 x 10^4
Number Surviving Embryos
0
1
2
3
4
5
48
96
144
Hours

FIGURE 16

Mortality in Chick Embryos Inoculated with Group II Strains Grown on Frantz and DSA Medium

A. Strain M-2

1. Grown on Frantz Medium (-o-)

Experiment	No. Bacteria Inoculated
# 1	4.2×10^5, 4.2×10^4
# 2	1.5×10^6

2. Grown on DSA Medium (--·-)

Experiment	No. Bacteria Inoculated
# 1	5.5×10^5
# 2	4.1×10^5, 4.1×10^4

B. Strain Bell

1. Grown on Frantz Medium (-o-)

Experiment	No. Bacteria Inoculated
# 1	1.2×10^6, 1.2×10^5

2. Grown on DSA Medium (--·-)

Experiment	No. Bacteria Inoculated
# 1	6.9×10^5, 6.9×10^4

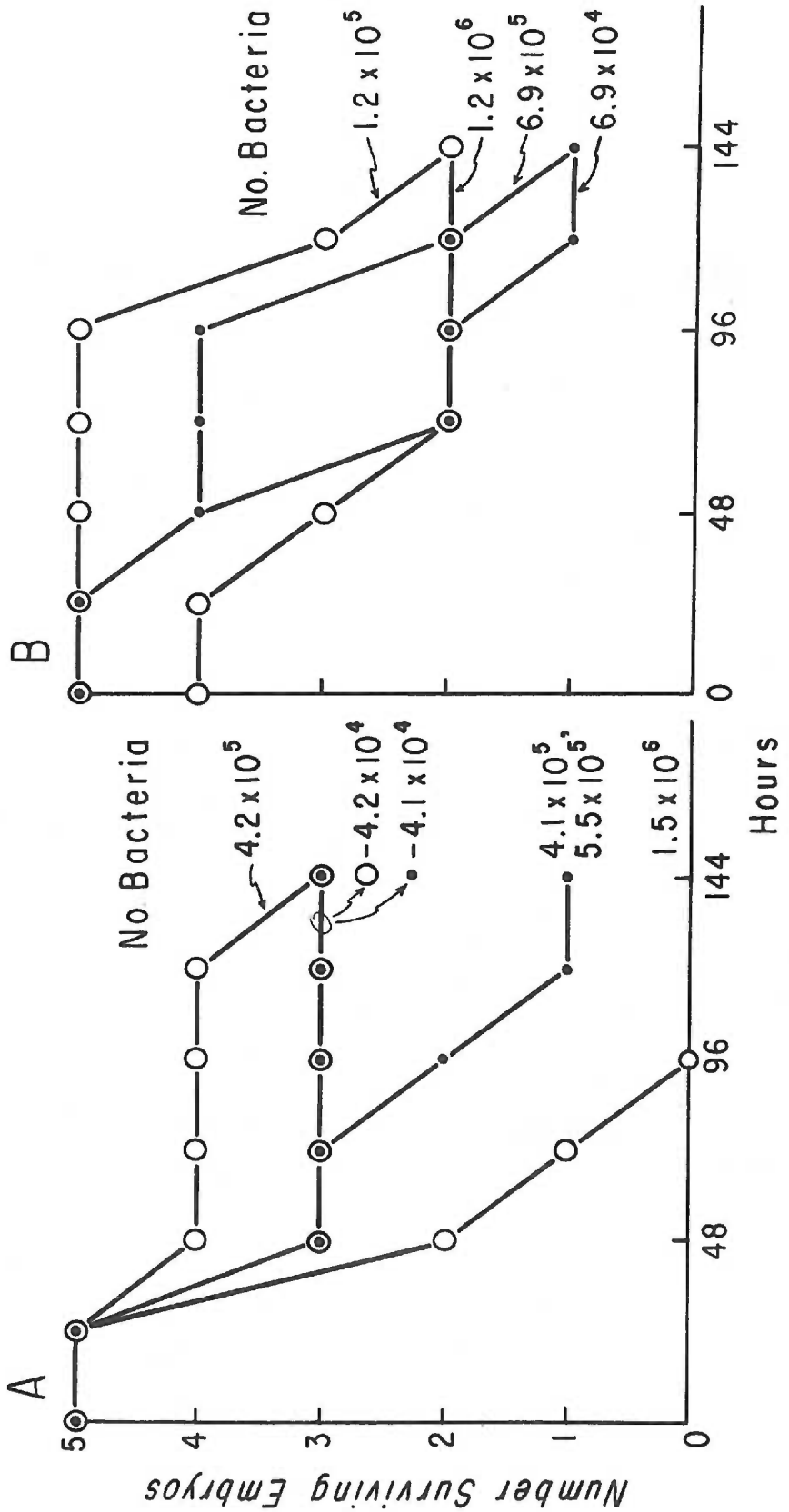

A
B
No Bacteria
No. Bacteria
4.2 x 10^5
4.2 x 10^4
4.1 x 10^4
4.1 x 10^5
5.5 x 10^5
1.5 x 10^6
1.2 x 10^5
1.2 x 10^6
6.9 x 10^5
6.9 x 10^4
Number Surviving Embryos
Hours
0
1
2
3
4
5
48
96
144

FIGURE 17

Mortality in Chick Embryos Inoculated with Group III Strains of *N. meningitidis* Cultured on BSA Medium

A. Strain M-1

Experiment	No. Bacteria Inoculated	
# 1	8.7×10^5,	2.6×10^5
# 2	4×10^6,	2.6×10^5

B. Strain M-158

Experiment	No. Bacteria Inoculated	
# 1	2.1×10^6,	9×10^4
# 2	6.6×10^5,	5.5×10^4

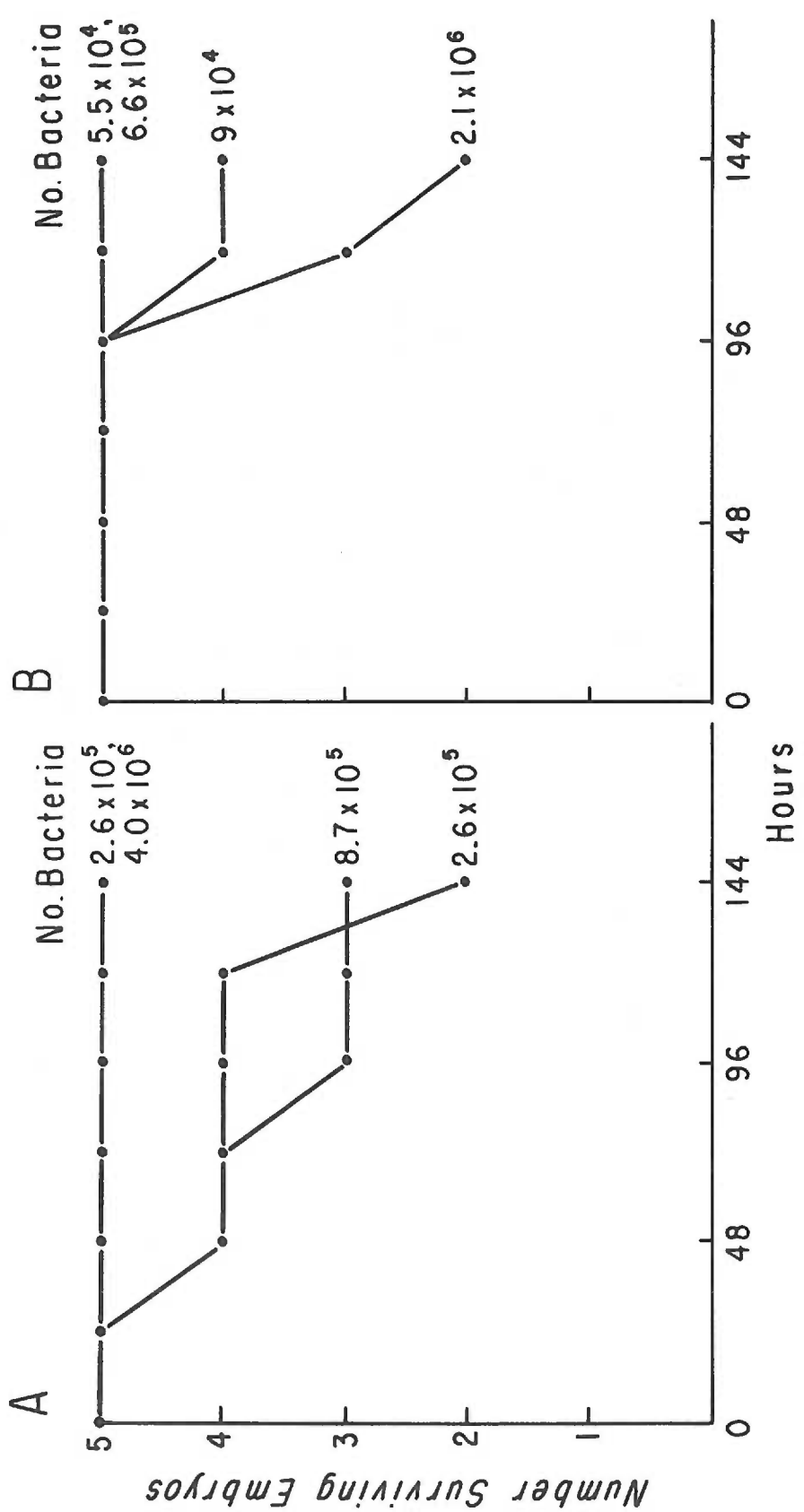

A
B
No. Bacteria
2.6 x 10^5; 4.0 x 10^6
8.7 x 10^5
2.6 x 10^5
No. Bacteria
5.5 x 10^4; 6.6 x 10^5
9 x 10^4
2.1 x 10^6
Number Surviving Embryos
5
4
3
2
1
0
48
96
144
Hours

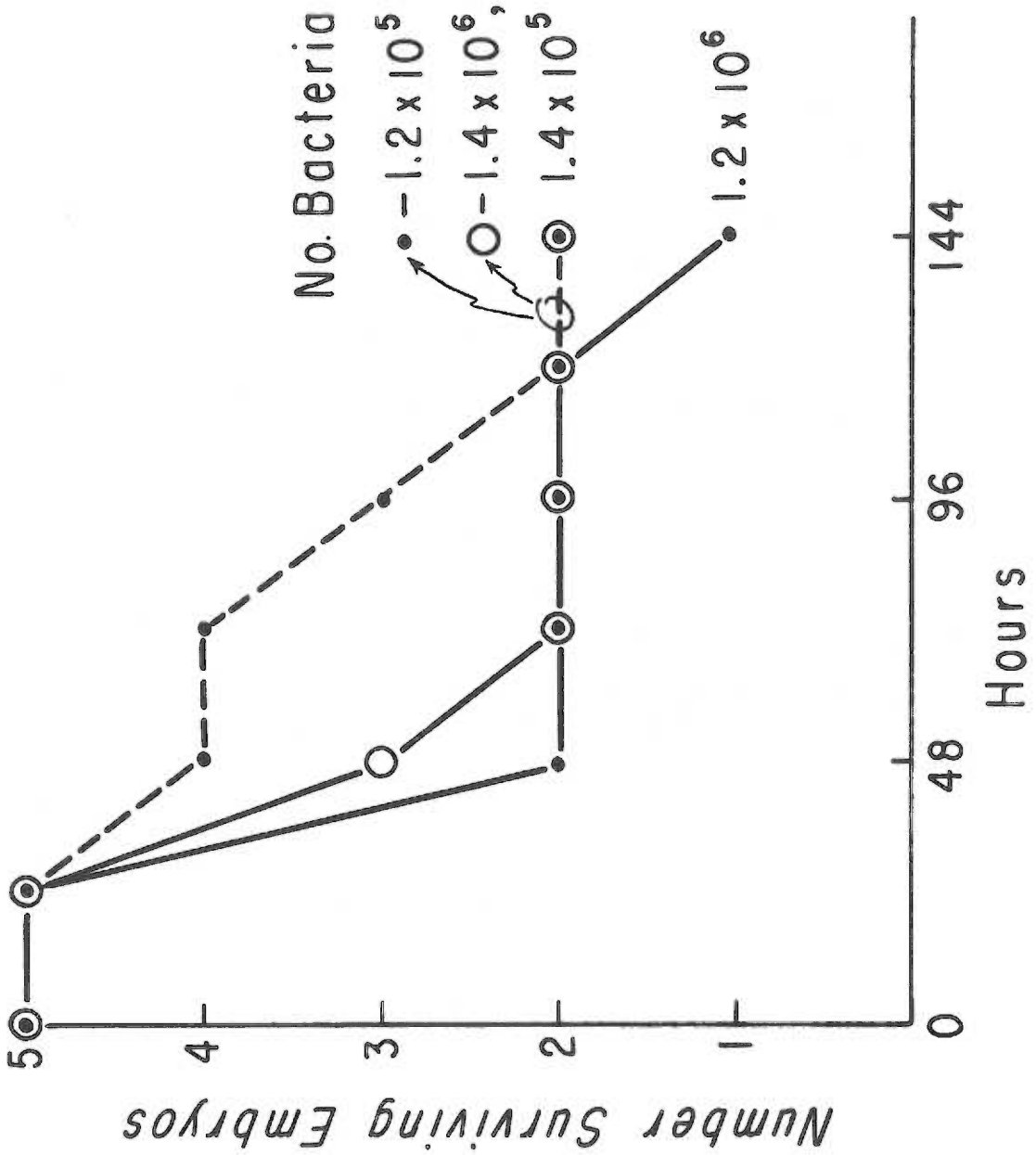
FIGURE 18

Deaths in Chick Embryos Inoculated with *N. meningitidis*

Strain Heagle

Key:

1. Grown on DSA Medium (-•-)
2. Grown in Frantz Medium Supplemented with 0.1% yeast extract (-o-)


No. Bacteria
1.2 x 10^5
1.4 x 10^6,
1.4 x 10^5
1.2 x 10^6
Number Surviving Embryos
5
4
3
2
1
0
48
96
144
Hours