THE ISOLATION AND CHARACTERIZATION OF A SLOW GROWTH RATE AND STARVATION RESISTANT MUTANT OF *ESCHERICHIA COLI*

By

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CHAPTER I

LITERATURE SURVEY

Many studies have been made upon bacterial cells exposed to harmful conditions such as radiation, desiccation, freezing, heat, adverse pH and drugs. However one is able to find in the literature very few investigations of the response of bacterial cells merely held at growth temperature in the absence of nutrients, i.e., under starvation conditions. Certainly in nature cells are faced with this simple predicament as often as with the more strikingly harmful circumstances mentioned above. It is desirable to know how cells respond to a simple absence of nutrients. Such knowledge may give insight into some of the factors controlling declining bacterial populations regardless of the particular harmful condition imposed upon them.

The survival of vegetative bacteria in a starvation condition has been studied by some workers. It was shown by Cohen (1922) that "Bacterium coli" and "Bacterium typhosum" in distilled water and dilute buffer died most rapidly at elevated temperature and high pH values. Winslow and Falk (1923a, 1923b) observed that "B. coli" survived longer in physiological saline than in distilled water and that the pH optima for survival were different in different media. Cook and Willis (1958) showed that a washed, buffered population of E. coli maintained high viability at room temperature compared with an unwashed and unbuffered aqueous suspension.

Ryan (1959) recorded exponential death of histidineless E. coli in spent medium, though during this period he showed the organisms had an appreciable mutation rate. According to Ryan, nondividing stationary phase cells of E. coli can still mutate. They represent not only an aging system but also one resembling human adult somatic cells, in as much as cell division is at a minimum.

Harrison (1960) observed that suspensions of Bacterium lactis aerogenes at various cell densities kept at growth temperature in the absence of added nutrients show survival which is maximum at a particular cell density for logarithmic phase cells. This critical density was observed to be approximately 10^8 cells/ml. At densities above and below this critical value survival is proportionally less. This observation was explained as a result of logarithmic phase cells dying in the absence of nutrient due to loss of cell substance. Concentration of this lost cell matter increases with time. At low densities released cell matter never becomes sufficiently concentrated to be recoverable so none of the cells survive. However, at higher densities the released cell matter as it is accumulated from larger numbers of cells, eventually becomes sufficiently concentrated to be able to sustain those cells that are still viable at that time. It was also observed that the maximum stationary phase cells survived better during starving conditions. This was accounted for by assuming that the stationary phase cells are less permeable and thus lose cell substance less rapidly. It was concluded that cells can maintain themselves or survive in adverse conditions by means of cannibalism.

Strange et al. (1961) published a study of the survival of stationary phase populations of the same strain of A. aerogenes used by

Harrison (1960) in nonnutrient buffer. They showed that the composition of growth medium, the phase of growth and the period during which the organisms had been in the stationary phase influenced the survival characteristics of the population. Survival curves for bacteria harvested from defined medium during the late exponential growth phase showed that extension of the stationary phase under growth conditions favored survival. Death of the population was preceded and accompanied by degradation of polymeric cell constitutents (protein, RNA, DNA, and polysaccharide) and excretion of fragments of these polymers. The mean contents of these polymers within the organism were influenced by the composition of the media from which the populations were harvested, as were the rates at which dying populations degraded those polymers, the order in which they were degraded and the death rates. Strange (1961) reported that these reactions decreased the ability of the organisms to form adaptive enzymes without affecting their viability or ATP content. Ryan (1959) showed that glucose accelerated the death of histidineless mutants. Strange et al. (1961) observed that addition of glucose to moribund populations accelerated their death rate. Harrison (1960) in contrast reported that traces of glucose prolonged survival.

Studies on survival at ordinary temperature in non-nutrient aqueous solutions showed that as soon as a portion of the population dies, nutrient may be released into the medium enabling the survivors to multiply. This phenomenon is well known in aging of bacterial cultures, as shown in the work of Winslow and Falk (1923a, 1923b). Ryan (1955) termed it cryptic growth and later concluded that it did not occur to any significant extent in non-multiplying populations of histidineless E. coli (Ryan, 1959). Strange et al. (1961) called it regrowth and

showed that it could be prevented by dialysing or filtering the bacterial suspension. Postgate and Hunter (1963) reported that a population of A. aerogenes taken from continuous culture required that about 50 organisms die to support the doubling of one survivor. Harrison (1960) invoked the phenomenon (termed cannibalism) to account for the increased survival time of dense bacterial suspensions as compared with more dilute ones, but he included within the term maintenance of viability. This refers to maintaining the cell viability as a result of the use of released cell material without cell division.

Postgate and Hunter (1963) observed, for a population cultured in a chemostat using a defined medium, a variety of survival curves ranging from logarithmic through linear (carbon-limited) to sigmoid (nitrogen and phosphorus-limited). For most nutritional conditions the growth rate was inversely related to death rate. The faster the population grew the slower death would occur. Magnesium-limited populations have provided an apparent exception. Harrison and Lawrence (1963) found in their experiments with A. aerogenes that the slower the growth rate the slower the death rate. Harrison and Lawrence (1963) showed that starvation-resistant mutants could be obtained from batch culture populations and that these were different in several ways from the "wild type". In particular their maximum exponential growth rate was slower. According to their view, starvation mutants owe their resistance to a more rapid conversion from log to postlog phase physiology. Thus they do not remain long enough in the more susceptible log phase state to succumb.

Monod (1942) introduced, in the form of an equation, a relationship between specific growth rate (μ) and the concentration of limiting

nutrient (S). The specific growth rate is defined as the rate of increase per unit of organism concentration $(dx/dt)(1/x)$. It was shown to be proportional to substrate concentration to a limiting saturation value as described by the equation

$$\mu = \frac{\mu_m S}{K_s + S}$$

μ_m is that value at which a further increase in initial substrate concentration causes no further increase in μ. K_s is numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\mu_m$. Both μ_m and K_s are constant under a given set of experimental conditions for a particular strain of bacteria. The equation has been shown to fit large amounts of experimental data for pure cultures and the results of a series of experiments designed to test the validity of the Monod equation for heterogeneous populations have been presented by Gaudy et al. (1967).

The continuous culture technique developed by Monod (1950), Novick and Szilard (1950) and Herbert et al. (1956) has increased the numbers of mathematical treatments describing growth kinetics. Applied to continuous culture, the Monod equation has again proven to be extremely useful. However, two faults become evident when applying the equation to this type of system. The first of these is the insensitivity of μ to small changes in S during the exponential growth phase where S is much higher than K_s. This has been called "slippage" by Gaudy et al. (1971). They have proposed that the symbol S_o be used in the Monod equation instead of S to represent initial substrate. Secondly, Mateles et al. (1965) showed that during the transient state in a continuous culture, Monod's equation could not be used to relate specific growth rates and

concentration of limiting nutrient. Continuous culture technique has been utilized in many studies involving bacterial physiology and genetics. All of these uses take advantage of the inherent capability of the system to allow balanced growth of microorganisms over a wide range of specific growth rates. The only change in the organisms' environment occurs when the dilution rate is changed. This results in a change in the steady state of the culture. Theoretical treatment as well as experimental studies by Monod (1950), Herbert et al. (1956) and Novick and Szilard (1950) have laid the foundation for use of the system. Probably the most obvious advantage of a continuous flow system other than the balanced environment is the operator's ability to hydraulically control the specific growth rate by simply controlling the dilution rate. This results in a system wherein responses to specific growth rate may be measured under the same set of conditions.

The yield (Y) for any particular strain of bacteria is a constant for a specific set of conditions. Monod (1942) defined the expression for substrate-based yield as

$$Y = \frac{\text{dry wt. of bacteria formed}}{\text{weight of substrate used}}$$

It was observed by Mennett and Nakayama (1971) that the specific growth rate and the yield increase with an increase in incubation temperature. This indicates a relationship between yield and specific growth rate. Monod presented early negative evidence, when he demonstrated with E. coli that the yield did not seem to be affected by specific growth rate. He obtained a yield of 0.23 mg/mg for E. coli by limiting aeration. According to Schulze and Lipe (1964), Monod used incomplete

nutrient media. There may have been other limiting factors controlling the rate of growth of the bacteria. It has been observed by many workers that as the specific growth rate increases cell yield also increases. The reverse situation is also true. Postgate and Hunter (1962) showed this effect with A. aerogenes. Schulze and Lipe (1964) showed the same effect using E. coli. The same findings were reported by Gaudy and Gaudy (1971) and by Sherrard et al. (1973) using mixed microbial populations in continuous culture. Explanation for this phenomenon has been formulated around the concept of endogenous metabolism and maintenance energy. The concept of maintenance energy was first postulated by Monod (1942) and later studied and defined by Dawes and Ribbons (1964) and McGrew and Mallette (1961) as that metabolic energy required to meet the demand of chemical and physical wear and tear of cells. To explain the higher yield values obtained when cells were grown at higher growth rates, they suggested that the relative proportion of energy needed for maintenance is less at higher growth rates compared to that at lower growth rates.

It was observed by Gaudy et al. (1967) and Ramanathan and Gaudy (1969) in continuous culture that, as dilution rate was increased above the critical value in mixed microbial populations, complete dilute-out of cells did not occur, but the system did apparently select for organisms with inherently faster specific growth rates. It was also observed by Gaudy and Srinivasaraghavan (1974) that in continuous flow systems with mixed microbial populations there is a decrease in yield with a decrease in specific growth rate; similarly, there is an increase in yield with an increase in specific growth rate. Further, they observed that yield in batch experiments using an inoculation from the

continuous flow reactor operated at various specific growth rates was the same as the yield in the continuous flow experiment from which the inoculum was taken, even though the specific growth rates were much higher in the batch flasks. It was explained that one possible reason for the change in yield at lower growth rates may be selection of species with a lower "true yield" from the heterogeneous population.

Jones (1974) showed that growth of cells in a chemostat at different dilution rates programmed yield characteristics into the cells. Cells from two different dilution rates were shown to have two different yield values which remained the same when inoculated into batch flasks with excess substrate. Under these conditions μ_m should be obtained and the true yield should be observed. One of the hypotheses presented to explain the effect was that yield mutants were being selected for at two different growth rates.

The present work was done in an attempt to isolate mutants with lower growth rates (and lower yield) and to determine the various growth characteristics of these mutants. These include yield, growth rate and starvation resistance.

Experiments were also designed to determine whether, when mixed populations of a slow growth rate, low yield mutant and the wild type, in a continuous flow system, were subjected to different dilution rates, a slow dilution rate would select for the mutant.

CHAPTER II

MATERIALS AND METHODS

The Organisms

The strain of *E. coli* used in this study as wild type was isolated in this lab from sewage. Wild type *E. coli* is designated *E. coli* IV. *E. coli* mutants were produced by subjecting wild type *E. coli* IV to starvation conditions. The procedure used for mutant isolation will be described in the Results section.

Biochemical Tests

To determine whether the mutants isolated were strains of *E. coli* and not contaminants, various biochemical tests were made. Glucose, lactose, mannitol, sucrose, nitrate, Koser's citrate and tryptone broths were used. As pH indicator bromcresol purple was used for fermentation tests. All the media except tryptone and citrate included Durham tubes to detect gas formation. Additional media used were methyl red-Voges Proskauer, Kligler's iron agar and litmus milk. All of the media were incubated at 37°C and readings were taken at 24 and 48 hours, except for methyl red, litmus milk and nitrate broth. Litmus milk was read at 1, 2 and 7 days, methyl red and nitrate broth were read at 5 days. These results were supplemented with Gram stains and wet mounts.

Isolation of Carbenicillin-Resistant *E. coli*

One ml of a 24-hr old *E. coli* IV cell suspension in glucose minimal medium was inoculated on glucose minimal medium plates with 10 μg/ml concentration of carbenicillin and incubated at 37°C until isolated colonies were observed. To make sure that these colonies were pure and carbenicillin-resistant, they were restreaked on the same medium. Slants were made of these isolated colonies. The carbenicillin-resistant mutant was designated as *E. coli* IVc.

Media

Glucose minimal medium was used to determine μ, μ_m and K_s and other characteristics of the organisms throughout this study, both in batch culture and in the continuous flow system. The composition of medium (per liter of solution) was: glucose, 1.0 gm; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4.7H_2O$, 0.1 g; $FeCl_3.6H_2O$, 0.5 mg; $CaCl_2.2H_2O$, 7.5 mg; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; yeast extract, 1 mg; and tap water, 100 ml. All ingredients of the medium were autoclaved separately, cooled and mixed aseptically.

Glucose minimal media containing glucose at concentrations of 100, 200, 400, 600, and 800 mg/l were also prepared to determine K_s. Plates of glucose minimal medium contained 2% agar (Difco).

Glucose minimal medium plates containing 10 μg/ml carbenicillin were prepared, by preparing as described above, glucose-minimal medium with 2% agar. The ingredients were cooled and mixed and the temperature of the medium was brought to 45°C. Carbenicillin was dissolved in 1 ml of distilled water, filter-sterilized and mixed with the medium to provide a final concentration of 10 μg/ml.

Nutrient broth with different combinations of glucose and/or yeast extract was prepared by adding to sterile nutrient broth the required volume of a sterile concentrated solution of glucose or yeast extract.

Tryptic soy agar (Difco) (TSA) plates were prepared by rehydrating the powdered medium with distilled water and autoclaving.

Cultivation of the Test Organisms

Each organism was streaked on TSA plates from stock cultures every month. An isolated colony from each plate was transferred to two new TSA slants. Inoculated slants were incubated at 37°C for 24 hours. One slant was stored at 4°C and one was kept at room temperature.

Batch Studies

Batch experiments were performed to determine specific growth rates of the organisms (wild type and isolated mutants) in different kinds of media. Values of μ_m and K_s for the organisms were determined in glucose minimal medium by batch study experiments. These constants were calculated from various μ values obtained at various substrate concentrations. A straight line form of the Monod equation $1/\mu = 1/\mu_m + K_s/\mu_m S$ (Monod, 1942) was used to determine μ_m and K_s. To measure a growth rate, 20 ml of medium was inoculated from a slant and incubated at 37°C until the density of cells was approximately 10^6 - 10^8 cell/ml. Normally this required 24 hours for the wild type and 48-72 hours for mutants. Then 0.5 ml of the cell suspension was inoculated into 20 ml of fresh medium of the same kind. Growth was followed on the Coleman Junior Spectrophotometer at 540 nm. Specific growth rate was determined first by

plotting absorbance versus time on semi-logarithmic paper. The specific growth rate μ, was calculated from this graph using the equation $\mu = 0.693/t_d$ where t_d is the doubling time. This equation is derived from $dx/dt = \mu X$, which is in the form of a first order differential equation. X represents the concentration of cells. The doubling time t_d is determined from the semilog plot and substituted into this equation. μ is then defined as $\mu = \ln 2/t_d = 0.0693/t_d$.

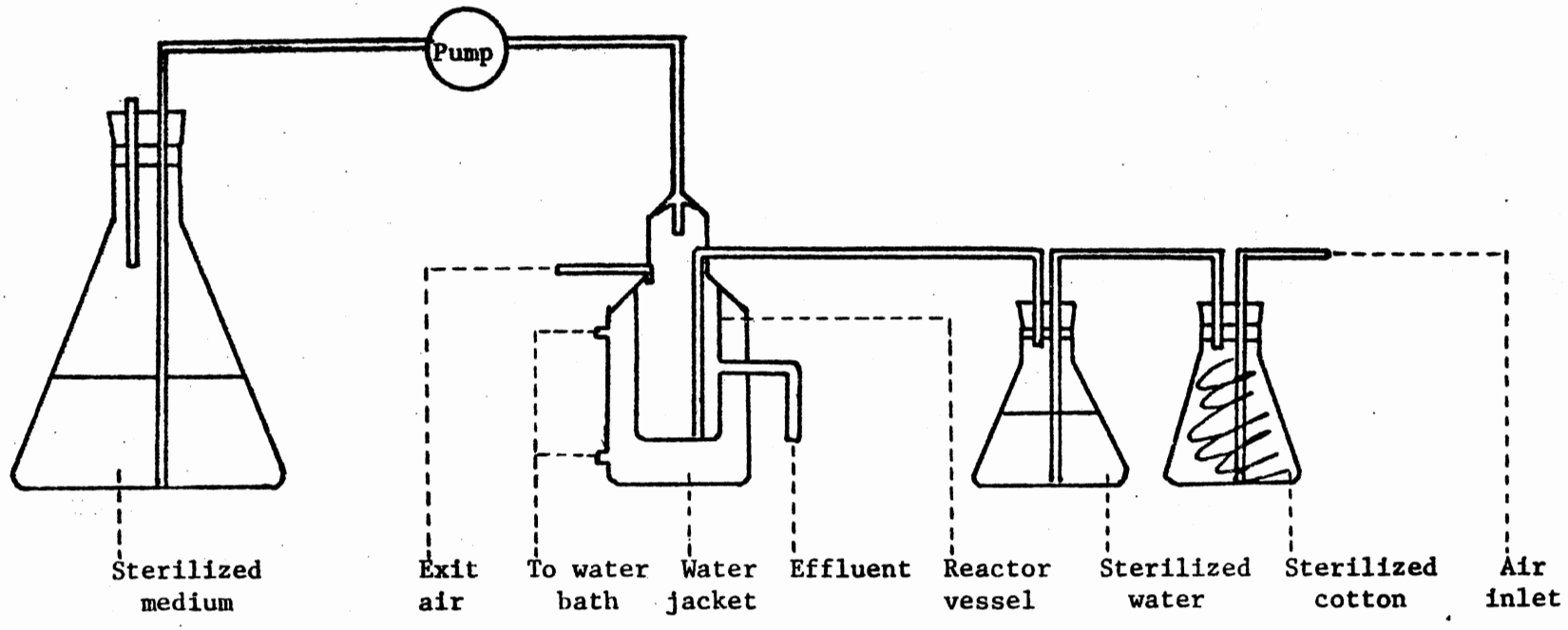
The batch experiments were performed in 250 ml sidearm flasks using 20 ml of medium (25 ml of medium for yield studies). These were incubated at 37°C in a Warner Chilcott shaking water bath at 105 cycles/min. Absorbance was measured at short time intervals.

Continuous Flow System

A chemostat was used to determine whether selection of organisms with different growth rates would occur at different dilution rates, and to test the hypothesis that the yield of organisms directly depends on dilution rate, i.e., on growth rate (dilution rate is the same as growth rate at steady state conditions in a once-through continuous flow system).

The apparatus used was fabricated wholly in glass. The design is shown in Figure 1. Figure 1 was obtained from the thesis of Tsay (1968) since she used the same apparatus. A four liter flask was used for storing glucose minimal medium containing 1000 mg/l glucose as the carbon and energy source to be fed into the chemostat. The feed line to the system was regulated by a liquid flow meter pump (Sigmamotor, Inc., Middleport, N. Y.) for which the discharge volume could be set at a

Figure 1. Schematic Drawing of the Continuous Flow System (from Tsay, 1968)


Pump
Sterilized medium
Exit air
To water bath
Water jacket
Effluent
Reactor vessel
Sterilized water
Sterilized cotton
Air inlet

definite rate. The aeration and mixing were effected by an air line passed through a flask filled with sterile cotton to filter the air and through a flask filled with sterilized water to saturate the air with water. The temperature of the system was maintained at room temperature. To initiate the operation, cells were grown in 20 ml of glucose minimal medium on the shaker until cell density was 10^9 cells/ml. Wild type E. coli and mutant E. coli were grown as mentioned above in separate flasks. One to 1.5 ml of each of the cell suspensions was introduced into the chemostat which was then filled to the overflow line with medium and aerated. The feed pump was started at a setting predetermined to achieve the desired dilution rate. The calibration data for the pump are shown in Figure 2 and Table I.

For calibration, the pump was fitted with tubing and prepared just as in the actual experiment. A flask was placed at the same level as the chemostat lid and pump delivery was collected in it. The pump was adjusted to a certain speed which was recorded as percentage of full speed and after a certain time period delivery was measured in a graduated cylinder. This was converted into the delivery expressed as ml/hr. A series of pump adjustments were made to prepare a calibration curve for each pump.

Yield

Yield measurements were obtained from batch systems and continuous flow systems. Samples of 20 ml were taken for yield analysis at the end of each run (at a particular growth rate) from the continuous flow system or at the end of the substrate removal phase in batch systems. The batch reactor contained 25 ml of growth medium. Growth in batch

Figure 2. Calibration of Sigmamotor Pump

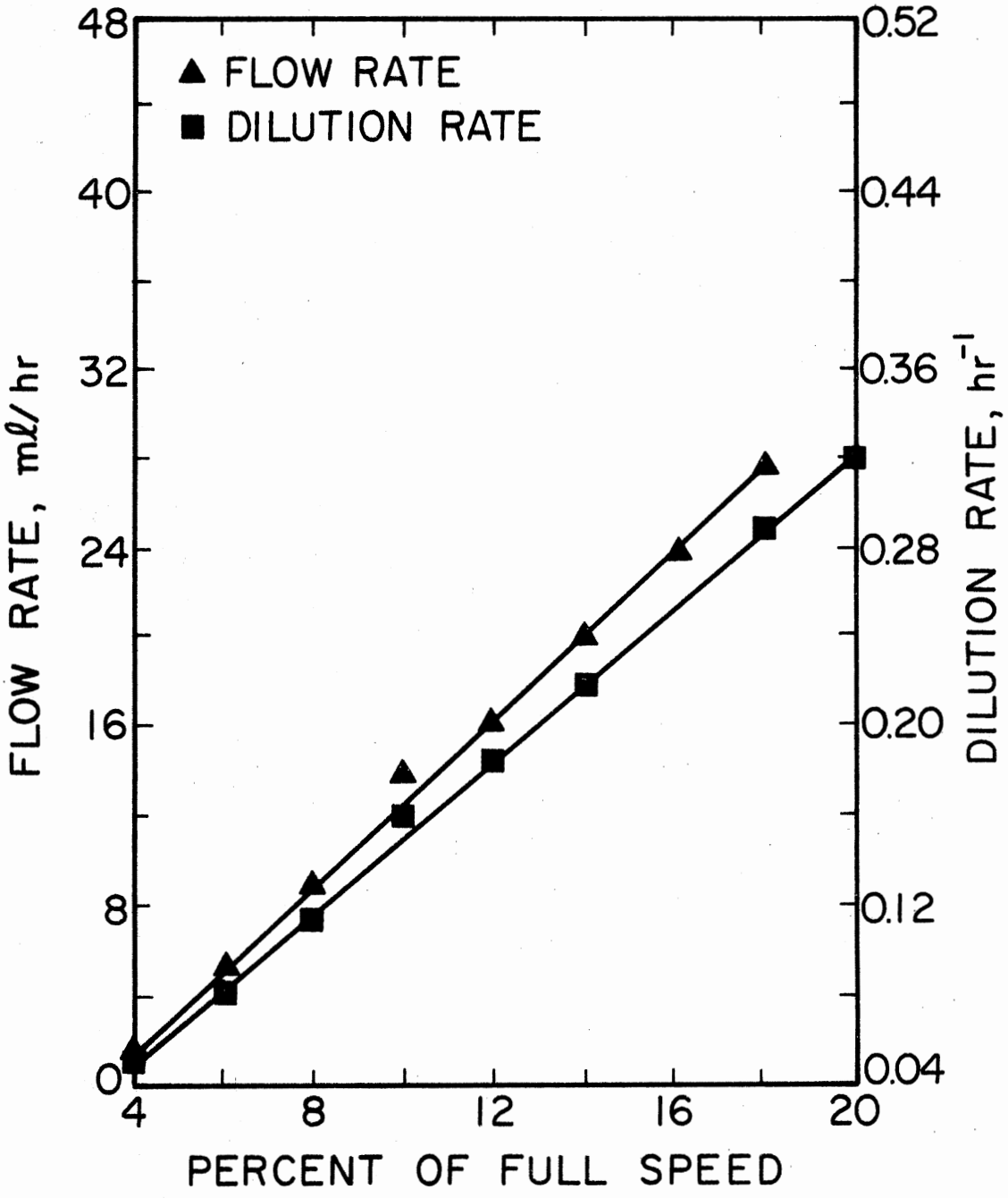

FLOW RATE
DILUTION RATE
48
40
32
24
16
8
0
0.52
0.44
0.36
0.28
0.20
0.12
0.04
4
8
12
16
20
FLOW RATE, mℓ/hr
DILUTION RATE, hr⁻¹
PERCENT OF FULL SPEED

TABLE I

DETERMINATION OF DILUTION RATE OF CHEMOSTAT

Pumping Rate (% of Capacity)	Flow Rate (ml/hr)	Dilution Rate (hr^{-1})
4	5.4	0.05
6	9.2	0.08
8	13.8	0.112
10	18.0	0.16
12	20.4	0.185
14	24.2	0.22
18	32.0	0.29
20	36.0	0.32

Volume of the chemostat = 110 ml.

Air flow was 2 liters/min.

systems was followed by spectrophotometry using a Coleman Junior Spectrophotometer Model D at 540 nm. The cells were filtered through tared membrane filters (pore size 0.45 nm) for determination of cell concentration (mg/l dry weight). The filtrate was removed to a prepared chemical oxygen demand (COD) flask and analyzed for total organic matter by oxidation with sulfuric acid--potassium dichromate solution (COD test, APHA, 1971). The cells were washed twice on the filter with distilled water and filters were dried to a constant weight in an oven overnight at 150°C. The dry weight of the sample was determined by weighing the dried filter paper and sample on an analytical balance. Yield was determined by dividing the dry weight of the sample by the change in the chemical oxygen demand (ΔCOD). The ΔCOD represents the difference between the initial feed COD and the sample filtrate COD.

CHAPTER III

EXPERIMENTAL RESULTS

Isolation of a Starvation-Resistant Strain of *E. coli*

Wild type *E. coli* IV from a slant was inoculated into 20 ml of glucose minimal medium. It was incubated at 37°C under aerobic conditions for 24 hours. Next day, 0.5 ml of the 24-hour old *E. coli* IV cell suspension was introduced into 20 ml of fresh glucose minimal medium in a sidearm flask. It was incubated at 37°C on the shaker. *E. coli* cells were allowed to grow until the absorbance measured was 0.31 at 540 nm in the Coleman Junior Spectrophotometer. Cell density was 4.8×10^6 cells/ml.

To measure the density a serial dilution technique was employed. Three tubes, each containing 9.9 ml of sterile 0.85% saline, were prepared. Into tube I, 0.1 ml of *E. coli* IV cell suspension was introduced and this tube was properly mixed. From tube I, 0.1 ml of cell suspension was introduced into tube II, followed by proper mixing. From tube II, 0.1 ml of cell suspension was introduced into tube III, again followed by mixing. These tubes were allowed to stand at room temperature. After every 24 hours of the starvation period, from each tube plate count determinations were made. Results of the plate counts are shown in Table II.

TABLE II

COLONY COUNTS OF WILD TYPE DURING STARVATION FOR ISOLATION OF STARVATION-RESISTANT MUTANTS

Hours Starvation	Tube No.	Volume plated (ml)	Colony Count	Viable Cells/ml	% Decrease
0	2	0.1	48	4.8×10^6	-
24	2	1.0	62	6.2×10^5	87
48	1	0.1	85	8.5×10^4	98
72	1	0.5	38	7.6×10^3	99.8
96	1	1.0	49	4.9×10^3	99.9
120	1	1.0	21	2.1×10^3	99.96

Colonies counts were average of 5 replicate plates.

After 72 and 96 hour starvation periods, each colony appearing smaller than the average wild type E. coli IV colony was selected and slants were made. Different numbers or letters were assigned to each slant. After 120 hours starvation, each colony appearing on the plate was assigned a number or letter and a slant was made of each. To be sure that each culture was pure, they were restreaked on TSA plates. A total of 52 different culture slants were made.

Determination of Growth Rates of Isolated Strains

All 52 of the isolated cultures were separately inoculated into flasks containing 20 ml of fresh glucose minimal medium and incubated at 37°C. With each experiment one wild type E. coli IV was also inoculated. The specific growth rate for each strain was calculated as described previously.

Of the 52 isolated cultures, only 18 possessed slower specific growth rates than the wild type E. coli IV. Results are summarized in Table III. Of the 18 slow growth rate strains it was observed that only four strains, E. coli X3, E. coli X4, E. coli 15 and E. coli P were stable. The unstable strains would begin growing after a very long lag phase with a slow growth rate. However, in the middle of log phase growth, they would begin growing as fast as the wild type E. coli IV. One explanation for this phenomenon could be that they possessed a point mutation and they subsequently reverted back to normal wild type. Since selection pressure favors a normal growth rate instead of a slow growth rate, a strain which has reverted to wild type would be favored. Figures 3-9 show growth curves for the wild type, the four stable

TABLE III

SPECIFIC GROWTH RATES OF WILD TYPE AND SLOW GROWING MUTANTS

Strain	Specific Growth Rate, hr^{-1}
Wild type	0.71
X3	0.19
15	0.27
X4	0.40
P	0.31
2	0.42
7	0.39
X1	0.53
9	0.51
10	0.37
X7	0.36
12	0.39
X9	0.39
X10	0.53
X12	0.37
X14	0.12
X19	0.13
X20	0.31
X21	0.33

Figure 3. Measurement of the Specific Growth Rate of the Wild Type, *E. coli* IV

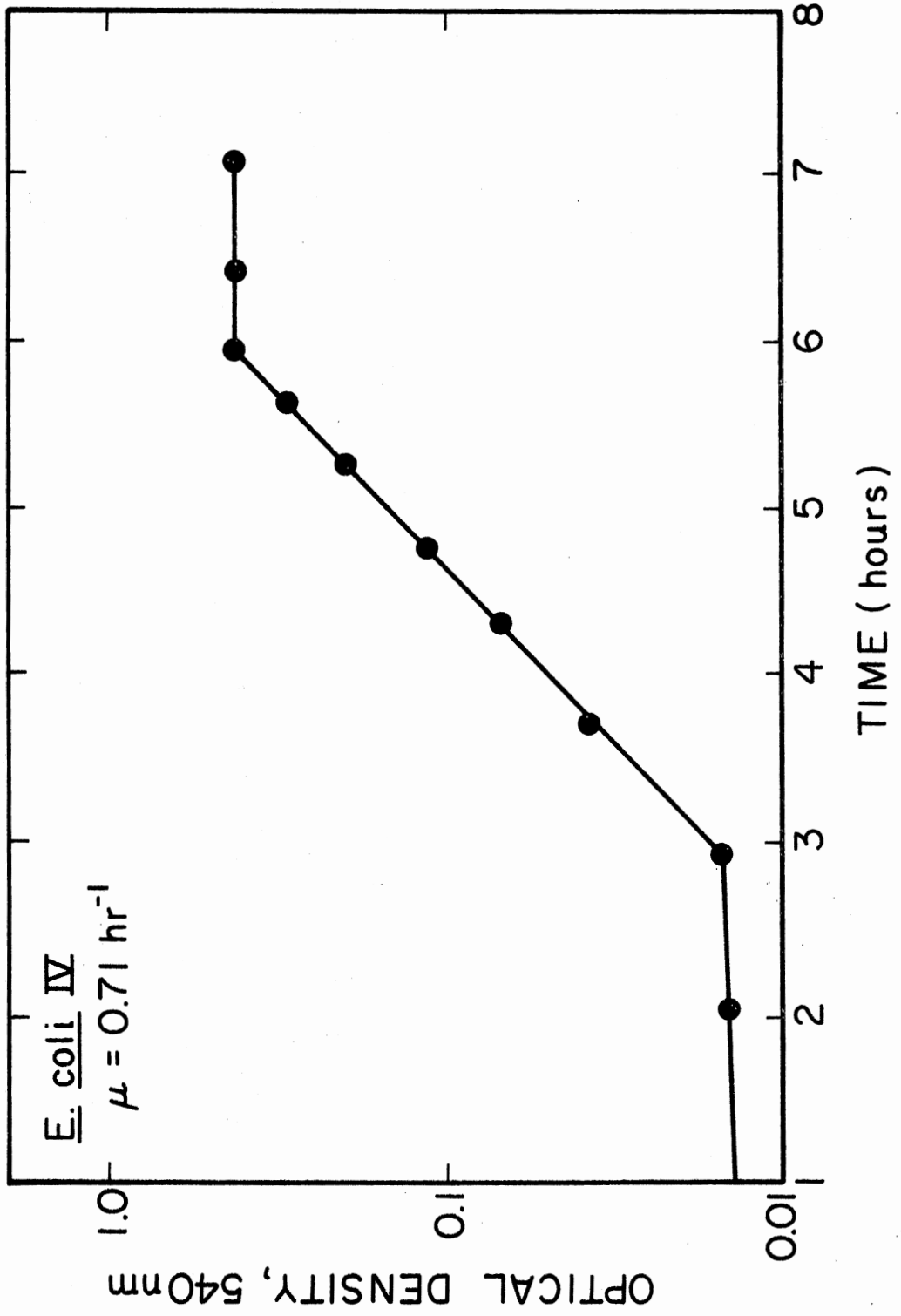

OPTICAL DENSITY, 540nm
1.0
0.1
0.01
E. coli IV
$\mu = 0.71\ hr^{-1}$
1
2
3
4
5
6
7
8
TIME (hours)

Figure 4. Measurement of the Specific Growth Rate of *E. coli* 15

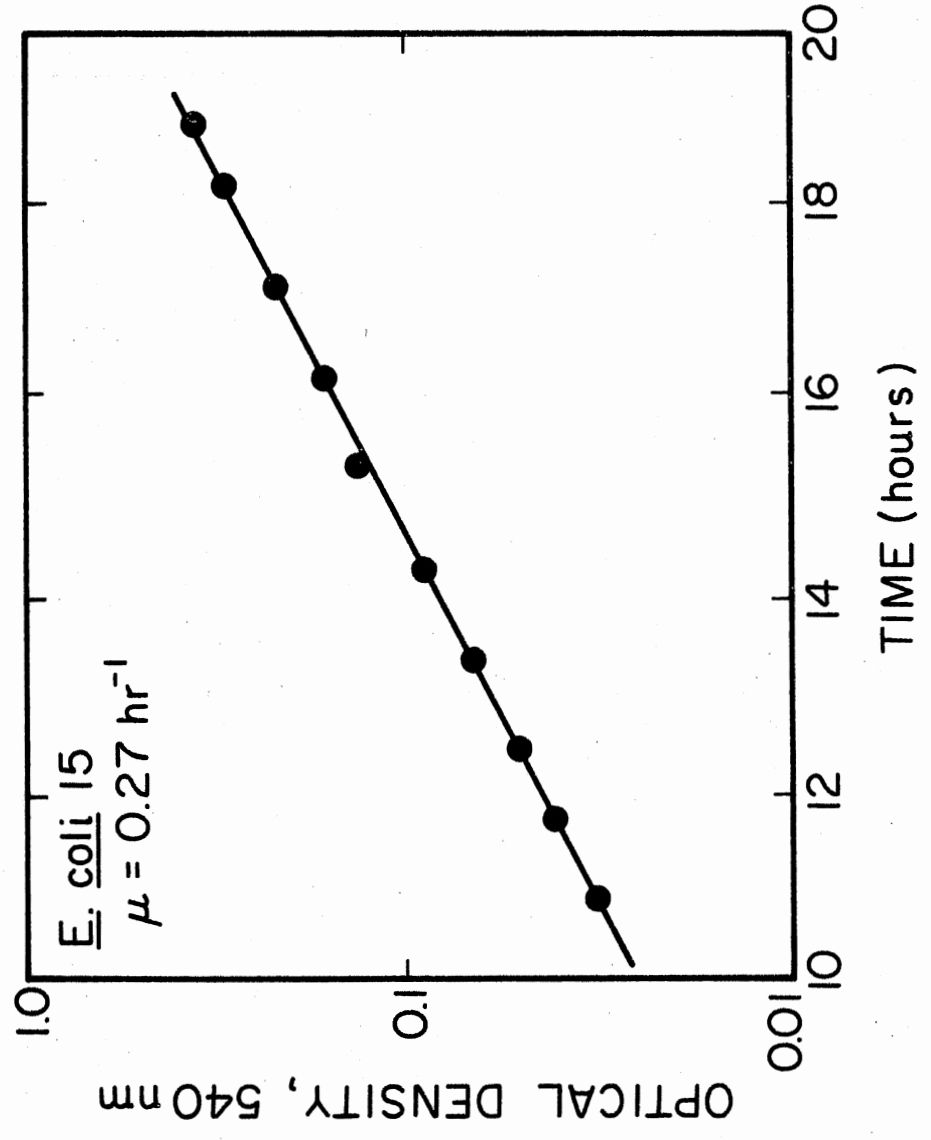

E. coli 15
μ = 0.27 hr⁻¹
1.0
0.1
0.01
10
12
14
16
18
20
TIME (hours)
OPTICAL DENSITY, 540 nm

Figure 5. Measurement of the Specific Growth Rate of *E. coli* X3

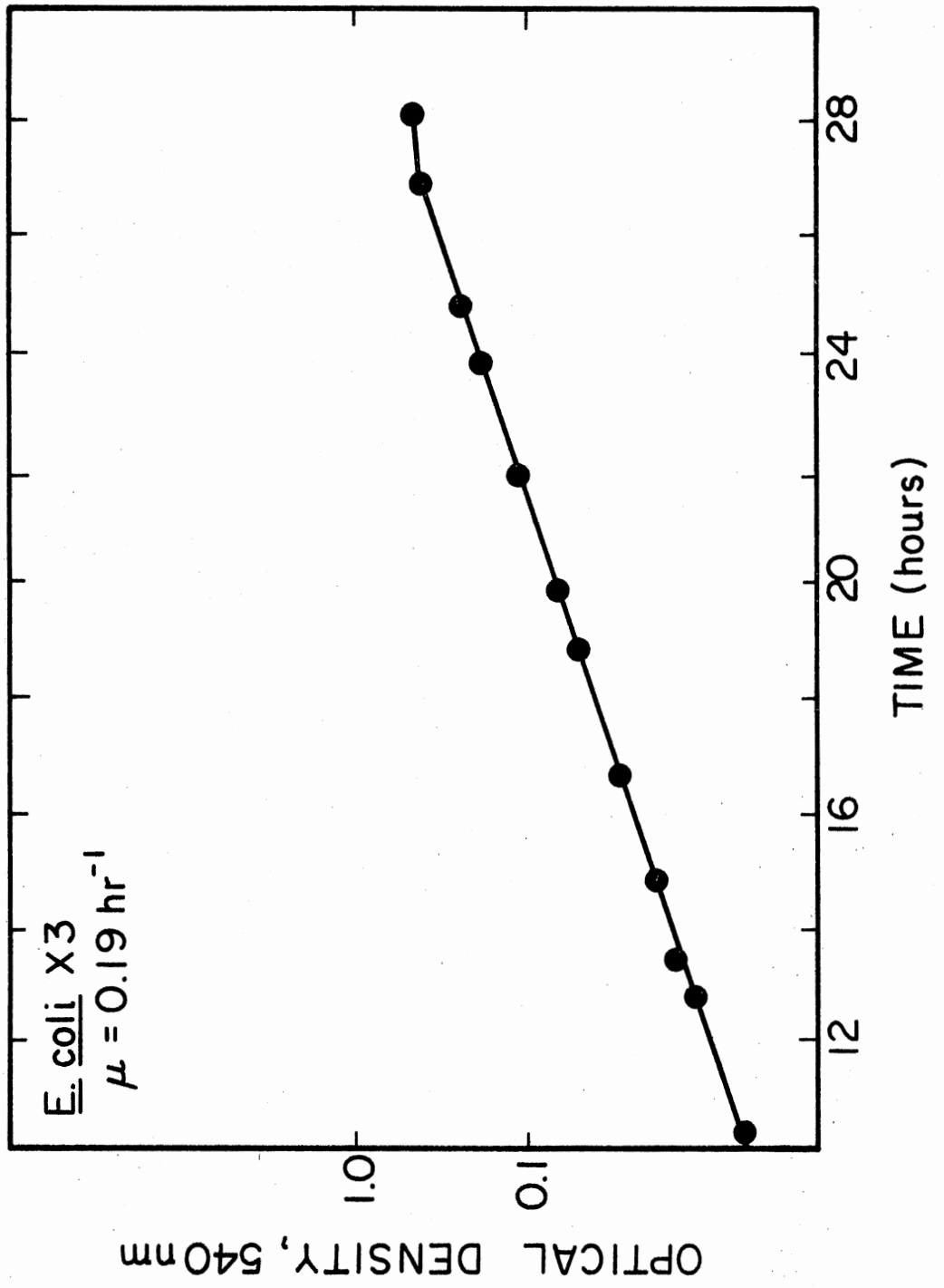

E. coli X3
μ = 0.19 hr⁻¹
1.0
0.1
OPTICAL DENSITY, 540nm
12
16
20
24
28
TIME (hours)

Figure 6. Measurement of the Specific Growth Rate of *E. coli* X4

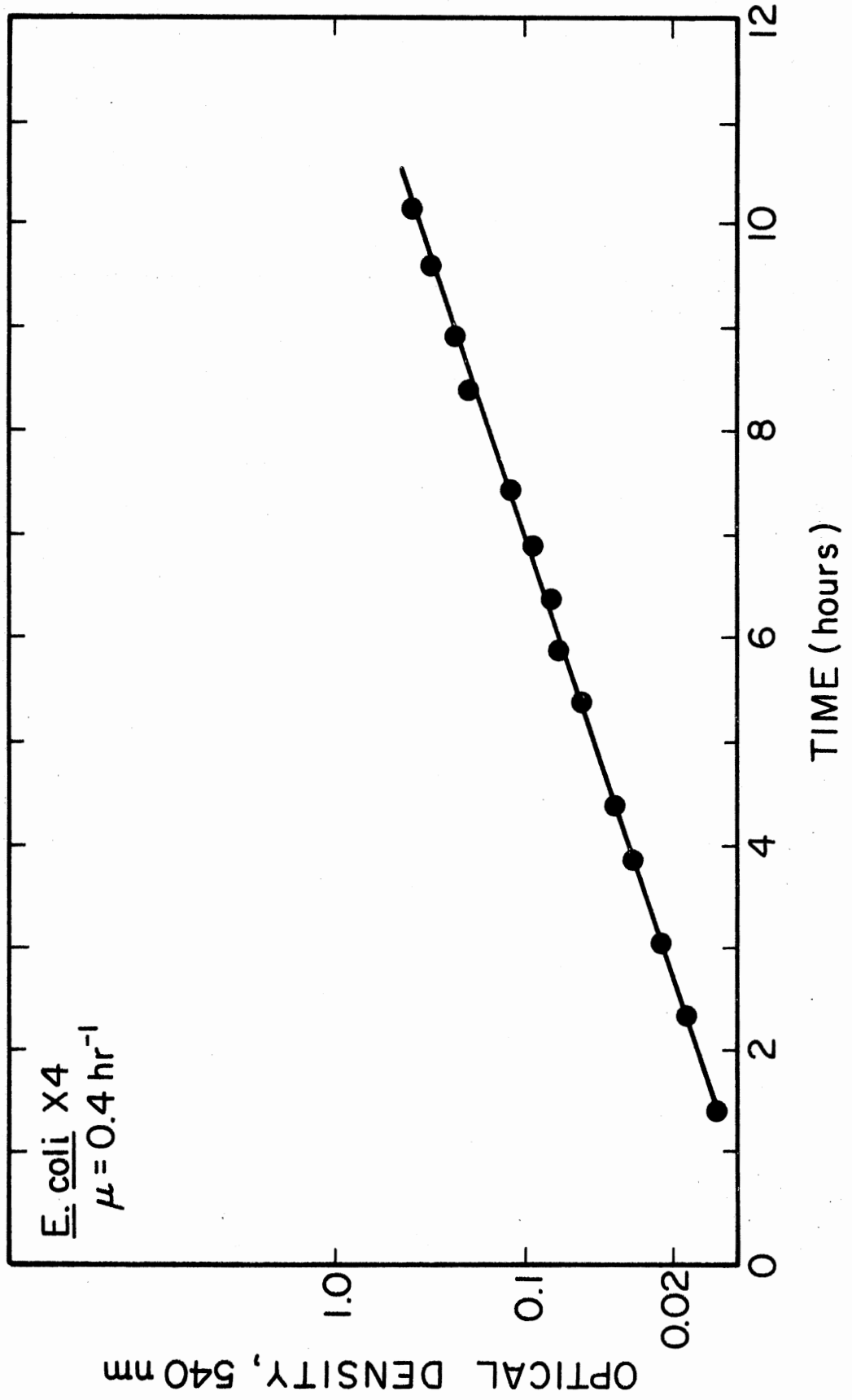

E. coli X4
μ = 0.4 hr⁻¹
OPTICAL DENSITY, 540 nm
1.0
0.1
0.02
0
2
4
6
8
10
12
TIME (hours)

Figure 7. Measurement of the Specific Growth Rate of *E. coli* P

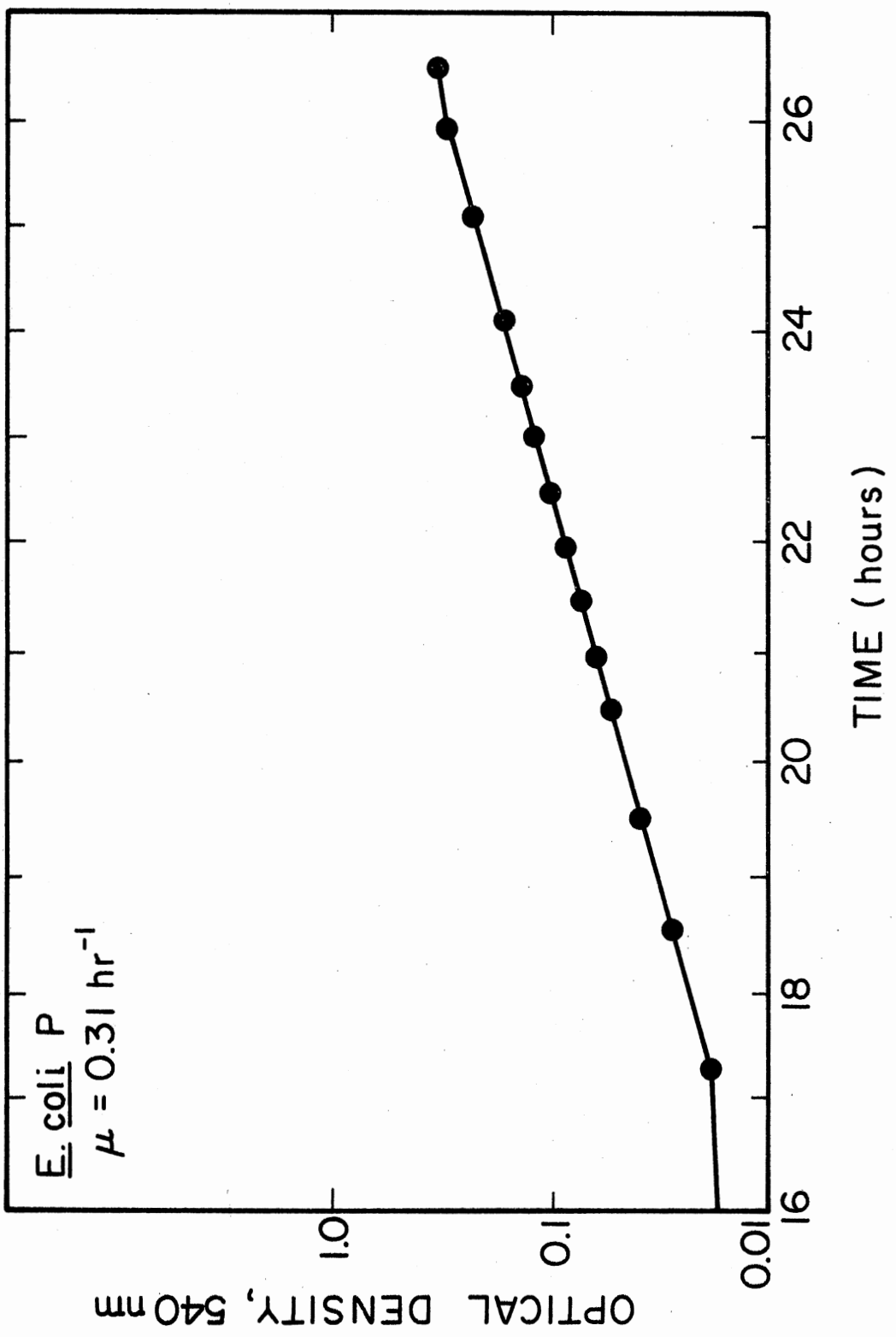

E. coli P
μ = 0.31 hr⁻¹
OPTICAL DENSITY, 540 nm
1.0
0.1
0.01
16
18
20
22
24
26
TIME (hours)

Figure 8. Measurement of Growth of *E. coli* X21 with Respect to Time, Showing the Change in the Specific Growth Rate of the Microorganisms During Log-Growth Phase

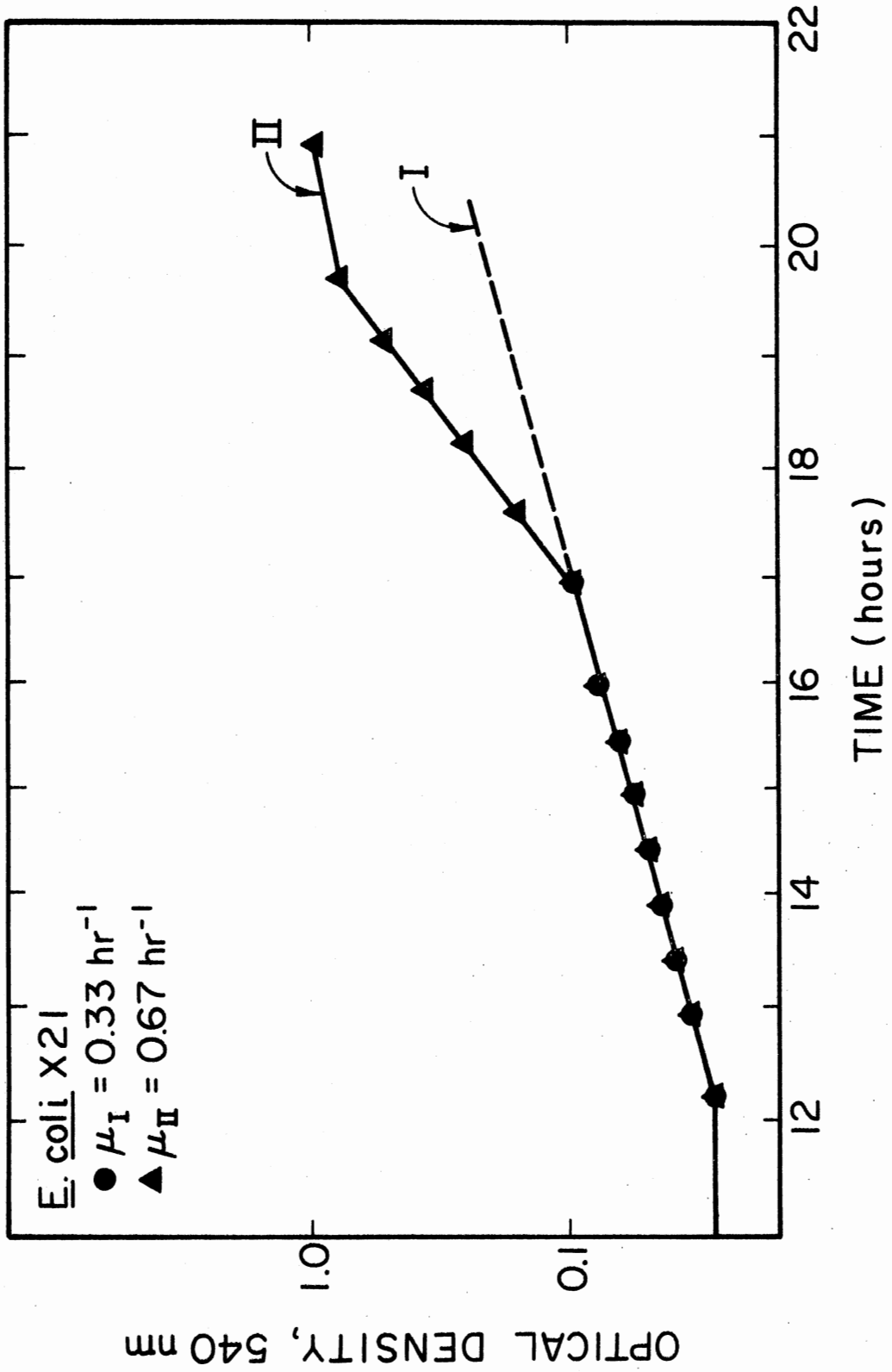
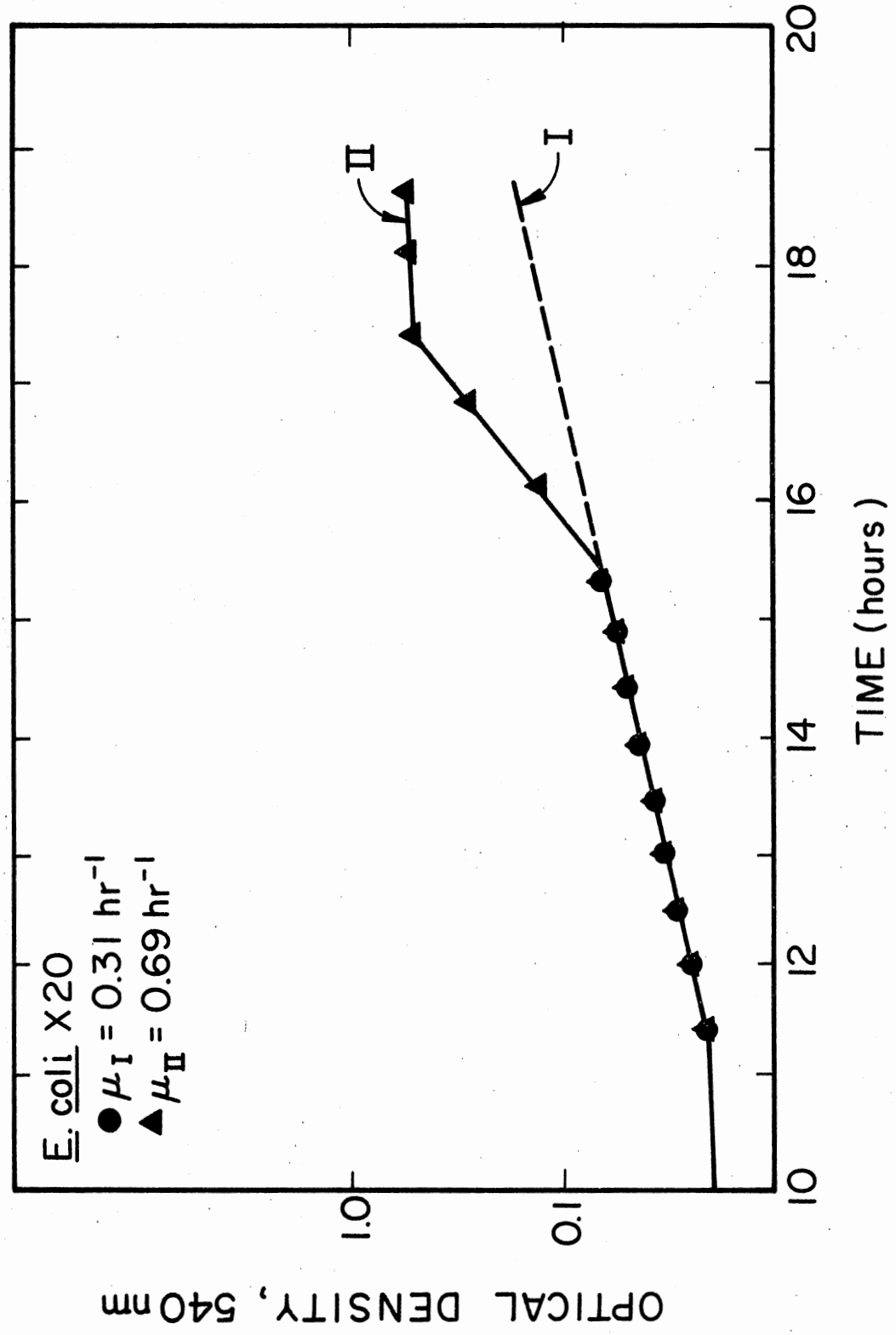

E. coli X21
● μ_I = 0.33 hr^{-1}
▲ μ_{II} = 0.67 hr^{-1}
I
II
OPTICAL DENSITY, 540 nm
1.0
0.1
12
14
16
18
20
22
TIME (hours)

Figure 9. Measurement of Growth of E. coli X20 with Respect to Time, Showing the Change in the Specific Growth Rate of the Microorganism During the Log-Growth Phase


E. coli X20
● μ_I = 0.31 hr^{-1}
▲ μ_{II} = 0.69 hr^{-1}
I
II
1.0
0.1
10
12
14
16
18
20
TIME (hours)
OPTICAL DENSITY, 540nm

strains (Figures 4-7) and two typical reverting strains (Figures 8 and 9).

Determination of the Stability of the Slow Growth Rate Mutants

To demonstrate that the slow specific growth rates of strains E. coli X3, E. coli X4, E. coli P, and E. coli 15 were the result of mutations which were stable, the following study was undertaken. A 0.5 ml volume of a cell suspension (approximately 10^9 cells/ml) of E. coli X4, E. coli X3, E. coli 15, E. coli P and the wild type E. coli IV was inoculated into 20 ml of glucose minimal medium in separate sidearm flasks. Growth was followed on the spectrophotometer at 540 nm. At the end of the first growth cycle, 0.5 ml of cell suspension of each mutant and wild type from the first completed growth cycle was inoculated into a fresh 20 ml of glucose minimal medium in a sidearm flask. Again growth was followed, and for mutants still growing slowly the same procedure was repeated. Specific growth rates were calculated for each cycle of growth. The graphs are shown in Figures 10-14 and results are summarized in Table IV. It was concluded from these results that E. coli X3 and E. coli 15 were much more stable than E. coli X4 and E. coli P. Further work in this study was carried out with mutants E. coli X3 and E. coli 15.

To be certain that each mutant was E. coli with a different specific growth rate, many biochemical tests were performed. To supplement these tests Gram stains and wet mounts were observed. Results are summarized in Table V.

Figure 10. Stability of Specific Growth Rate of *E. coli* 15 Through Three Consecutive Cycles of Growth

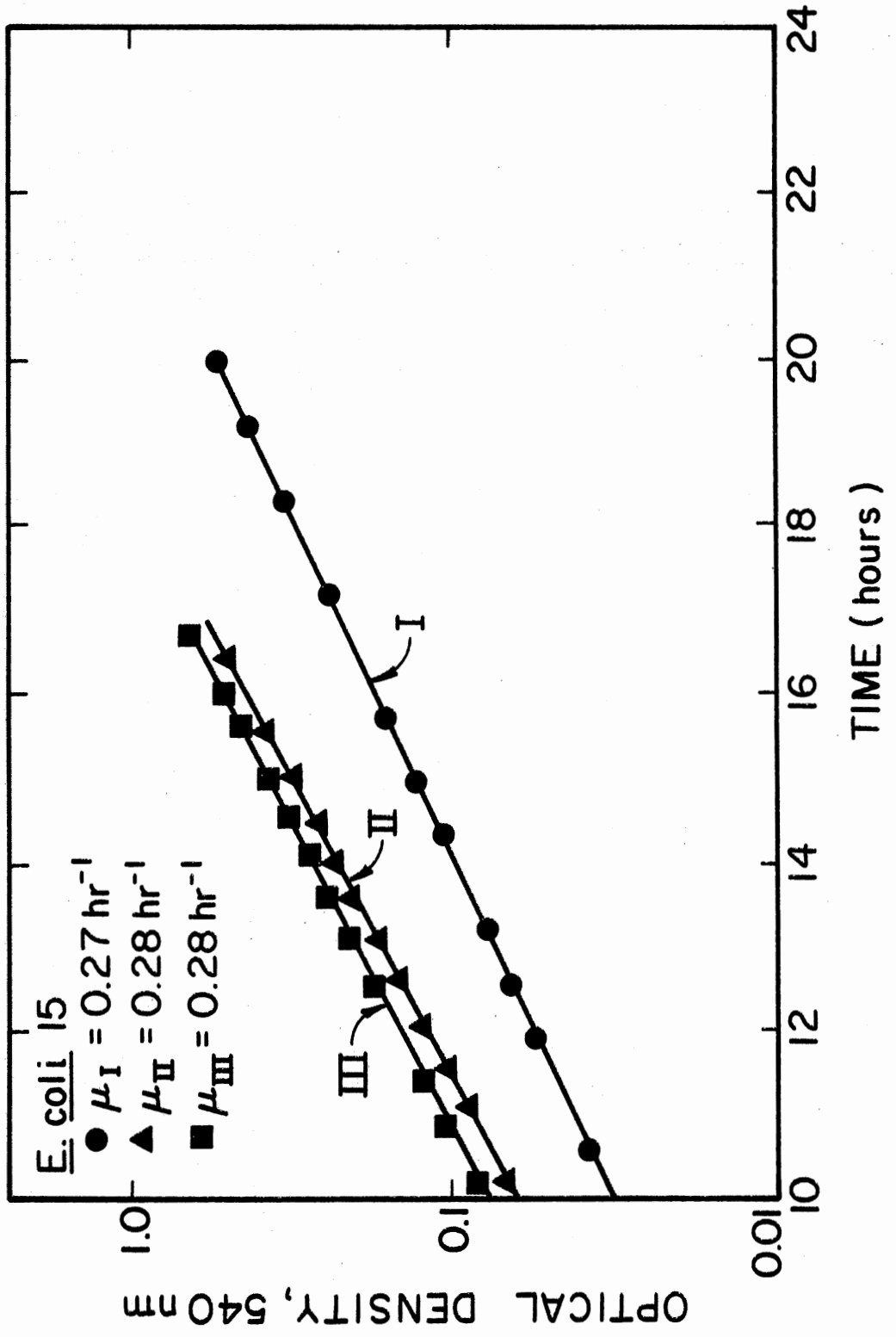

E. coli 15
● μ_I = 0.27 hr^{-1}
▲ μ_{II} = 0.28 hr^{-1}
■ μ_{III} = 0.28 hr^{-1}
I
II
III
OPTICAL DENSITY, 540 nm
1.0
0.1
0.01
10
12
14
16
18
20
22
24
TIME (hours)

Figure 11. Stability of Specific Growth Rate of *E. coli* X3 Through Three Consecutive Cycles of Growth

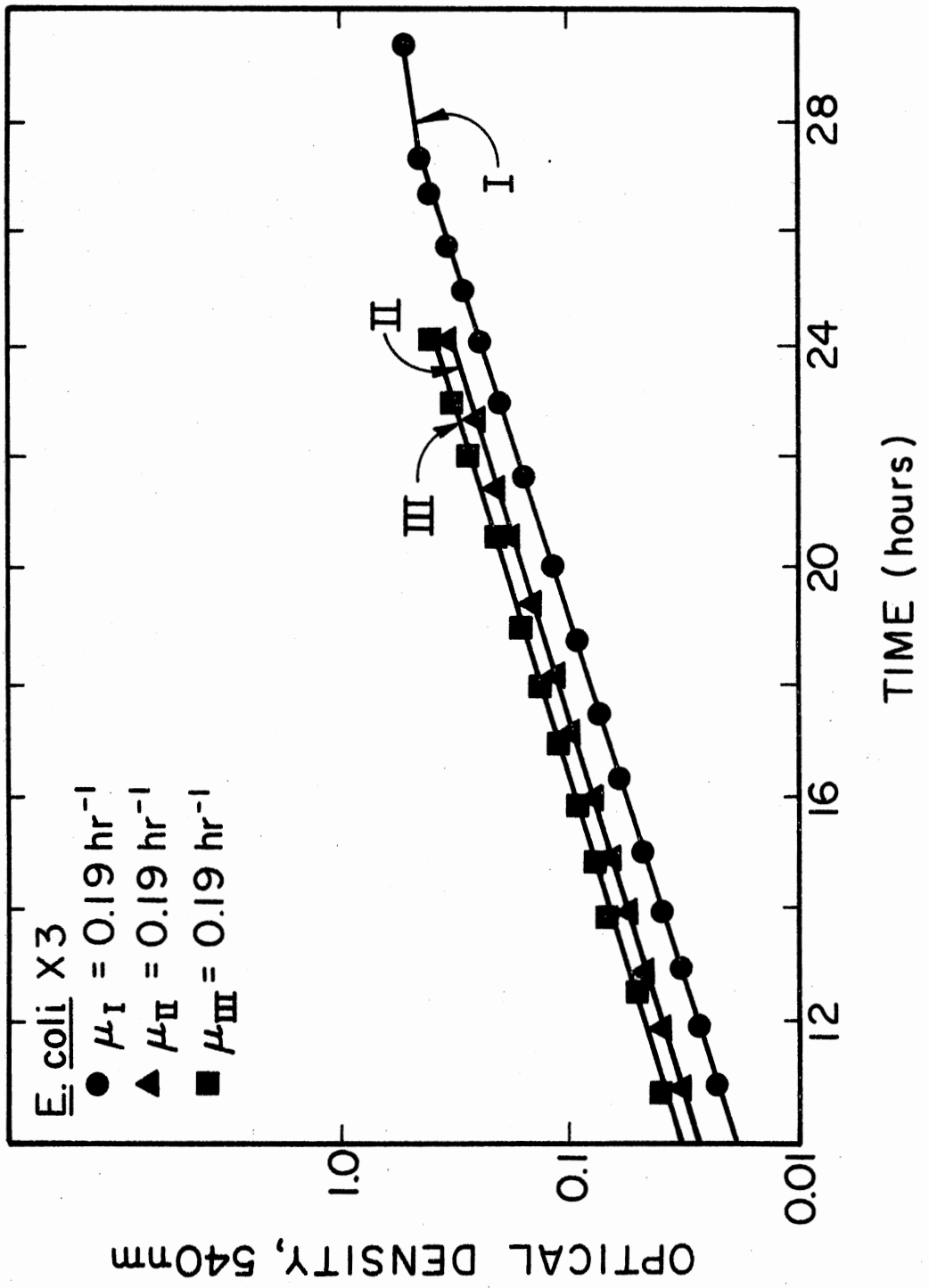

E. coli X3
● μ_I = 0.19 hr^{-1}
▲ μ_{II} = 0.19 hr^{-1}
■ μ_{III} = 0.19 hr^{-1}
I
II
III
OPTICAL DENSITY, 540nm
1.0
0.1
0.01
12
16
20
24
28
TIME (hours)

Figure 12. Stability of Specific Growth Rate of E. coli P Through Two Consecutive Cycles of Growth

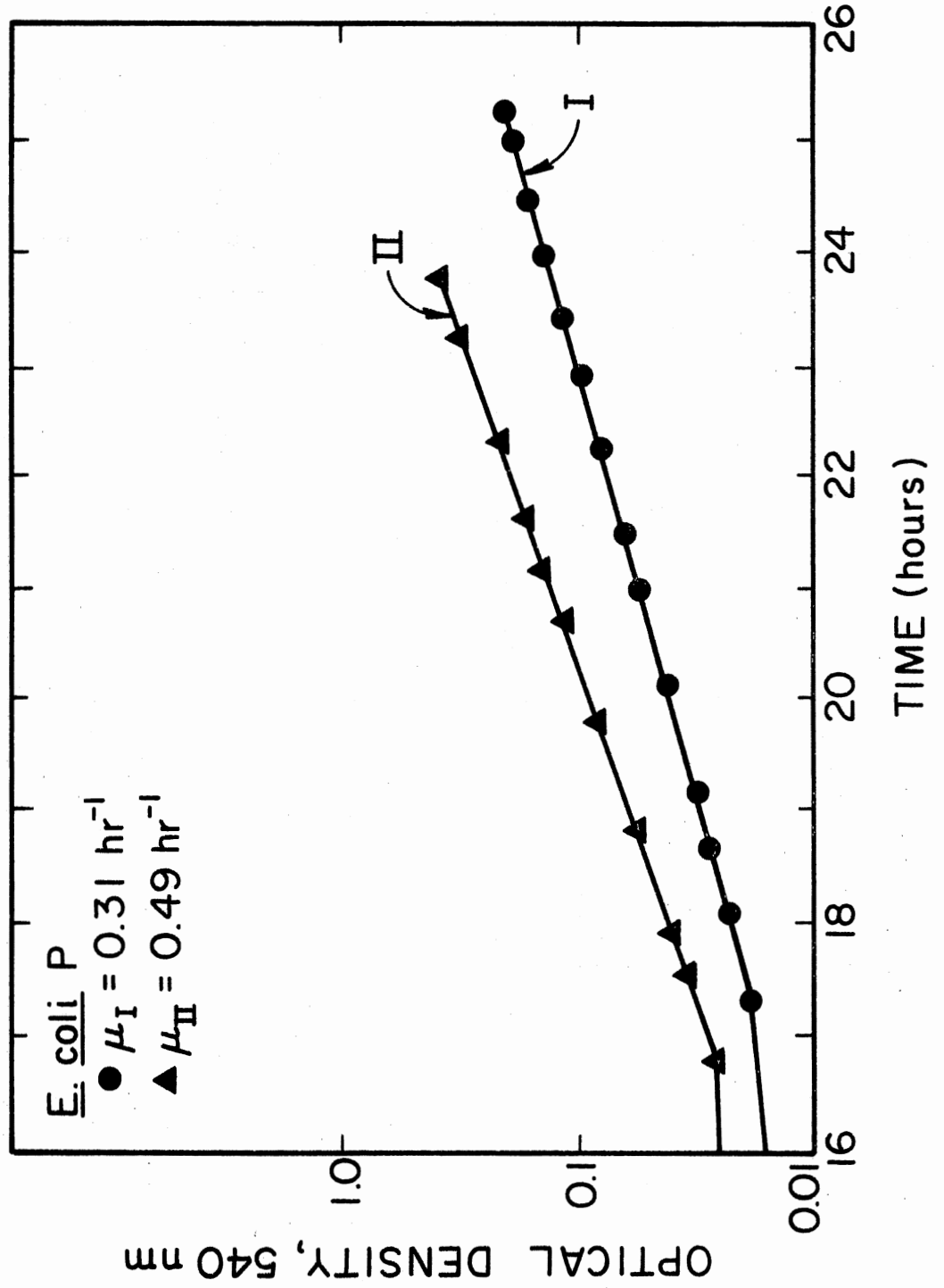

E. coli P
● μ_I = 0.31 hr^{-1}
▲ μ_{II} = 0.49 hr^{-1}
I
II
OPTICAL DENSITY, 540 nm
0.01
0.1
1.0
16
18
20
22
24
26
TIME (hours)

Figure 13. Stability of Specific Growth Rate of E. coli X4 Through Two Consecutive Cycles of Growth

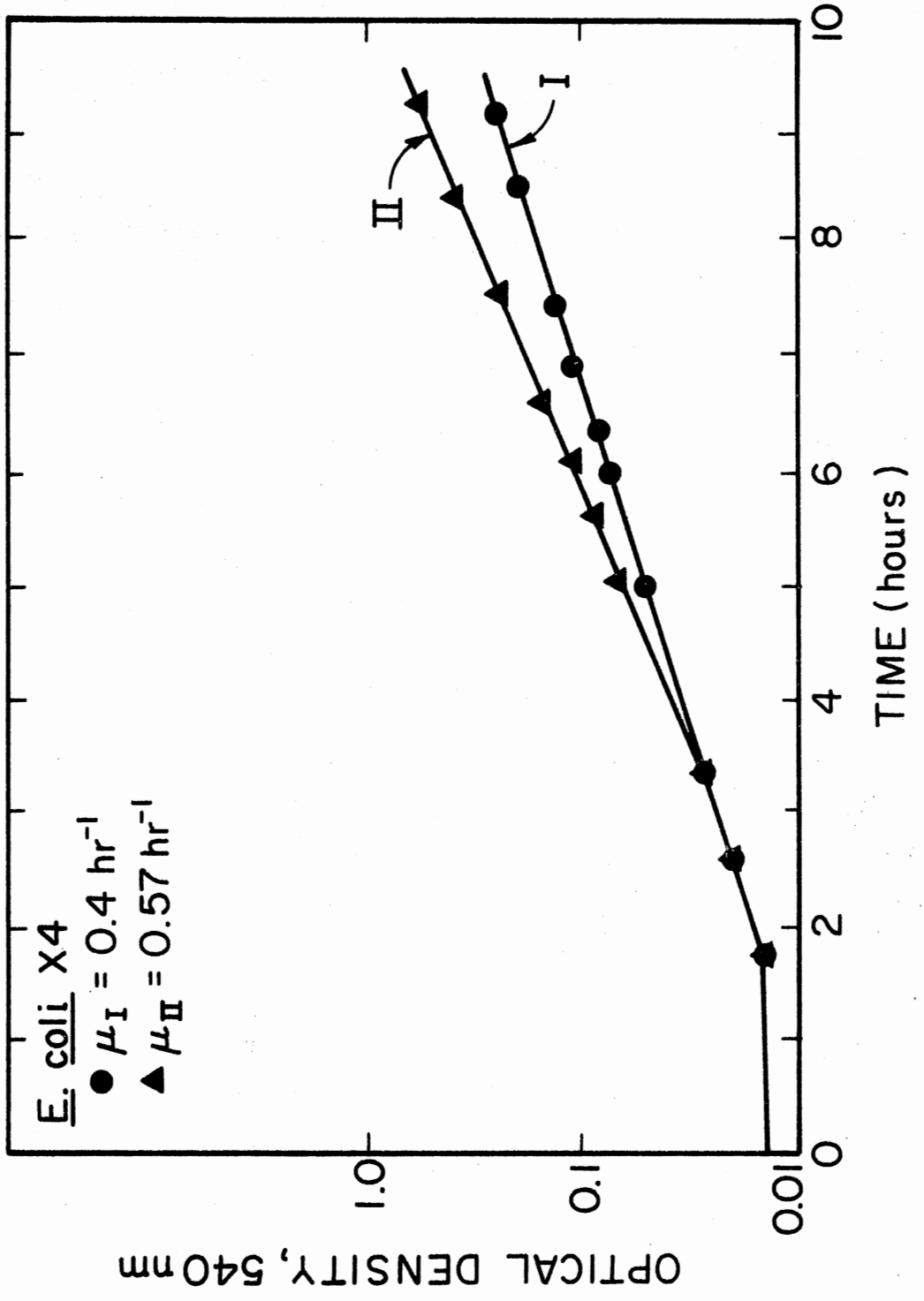

E. coli X4
● μ_I = 0.4 hr^{-1}
▲ μ_{II} = 0.57 hr^{-1}
I
II
OPTICAL DENSITY, 540nm
0.01
0.1
1.0
0
2
4
6
8
10
TIME (hours)

Figure 14. Stability of Specific Growth Rate of E. coli IV Through Two Consecutive Cycles of Growth

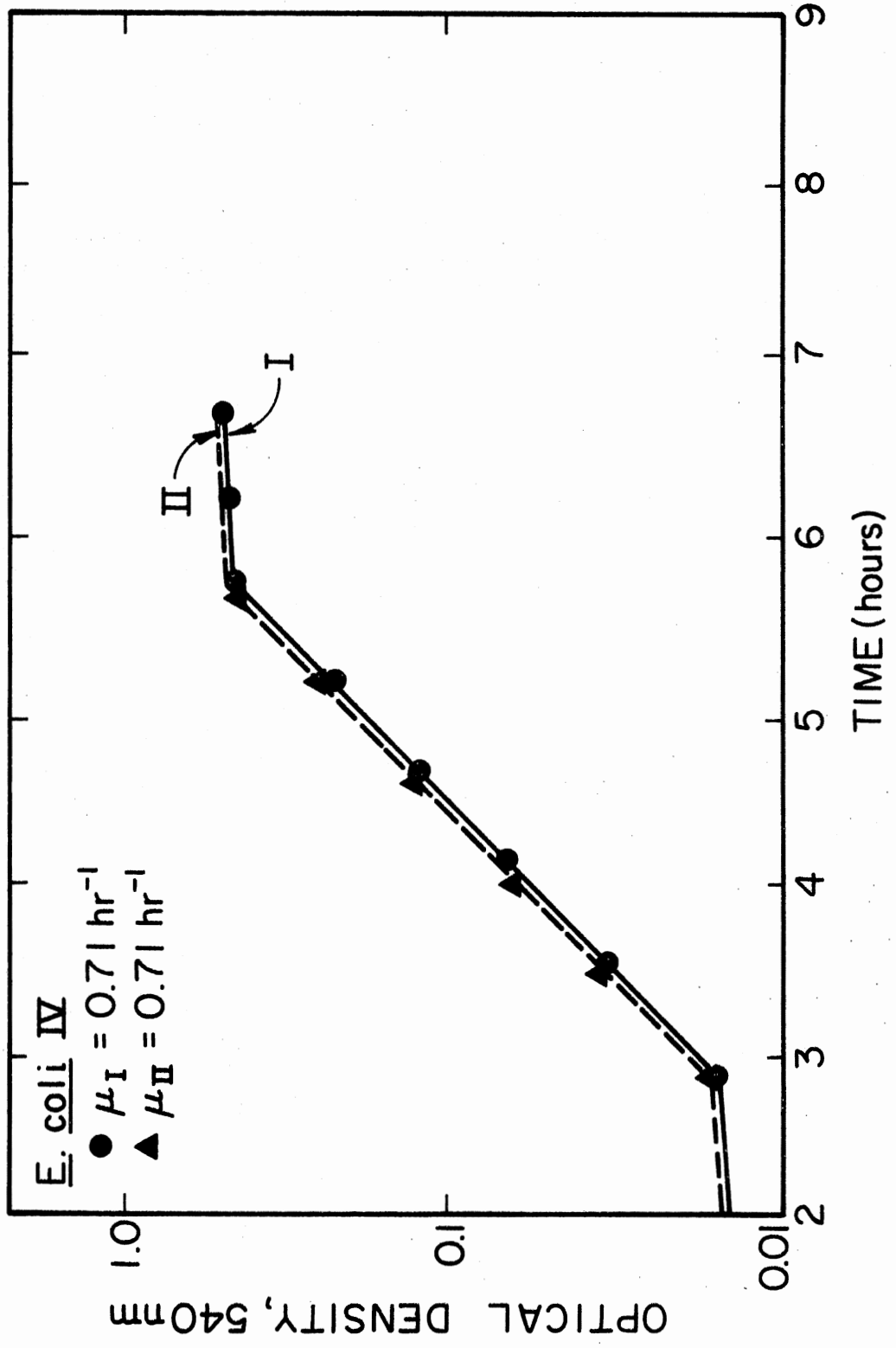

E. coli IV
● μ_I = 0.71 hr^{-1}
▲ μ_{II} = 0.71 hr^{-1}
I
II
OPTICAL DENSITY, 540nm
1.0
0.1
0.01
2
3
4
5
6
7
8
9
TIME (hours)

TABLE IV

DETERMINATION OF THE STABILITY OF SPECIFIC GROWTH RATES IN BATCH

Organism	Specific Growth Rates, hr^{-1}		
	Cycle I	Cycle II	Cycle III
E. coli P	0.31	0.49	-
E. coli 15	0.27	0.28	0.28
E. coli X3	0.19	0.19	0.19
E. coli X4	0.40	0.57	-
E. coli IV	0.71	0.71	-

TABLE V

RESULTS OF BIOCHEMICAL TESTS OF MUTANTS AND WILD TYPE

Test	*E. coli* IV	*E. coli* X3	*E. coli* X4	*E. coli* 15	*E. coli* P
Glucose	Acid/gas	Acid/gas	Acid/gas	Acid/gas	Acid/gas
Lactose	Acid/gas	Acid/gas	Acid/gas	Acid/gas	Acid/gas
Mannitol	Acid	Acid	Acid	Acid	Acid
Indole	+	+	+	+	+
Methyl Red	+	+	+	+	+
Citrate	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-
Litmus Milk	Acid curd	Acid curd	Acid curd	Acid curd	Acid curd
Urea	-	-	-	-	-
NO_3 to NO_2	+	+	+	+	+

All organisms Gram-negative rods and motile in broth medium.

Determination of the Effect of Nutrition Upon the Growth Rate of Mutants

To ensure that the slow growth rate of mutants E. coli 15 and E. coli X3 was not due to the lack of any required nutrient, enriched media were used in these studies. Previous studies utilized only glucose minimal medium.

Specific growth rates of mutants E. coli 15 and E. coli X3 and wild type E. coli IV were measured in nutrient broth, nutrient broth with 0.5% glucose, nutrient broth with 0.5% yeast extract and nutrient broth with 0.5% glucose and 0.5% yeast extract, and glucose minimal medium with 0.5% yeast extract. To observe the specific growth rate of each organism in each medium, acclimated cells of each organism from each medium were used for inoculation. Specific growth rate was measured and calculated as described previously. Graphs are shown in Figures 15-19 and results are summarized in Table VI. For further studies mutant E. coli 15 was used since it had a slower specific growth rate than the wild type in all media tested.

Determination of Starvation Resistance

Each organism, E. coli 15, the wild type, E. coli IV, and the carbenicillin-resistant strain, E. coli IVc, was grown in glucose minimal medium under aerobic conditions until the stationary phase for each organism was reached. The same serial dilution technique used in the original isolation was employed for each organism, except that instead of saline, distilled water was used. After each 24 hours, a plate count determination was made. Results are shown in Figure 20.

Figure 15. The Measurement of Growth of Microorganisms *E. coli* IV, *E. coli* 15 and *E. coli* X3 with Respect to Time in Nutrient Broth with 0.5% Glucose

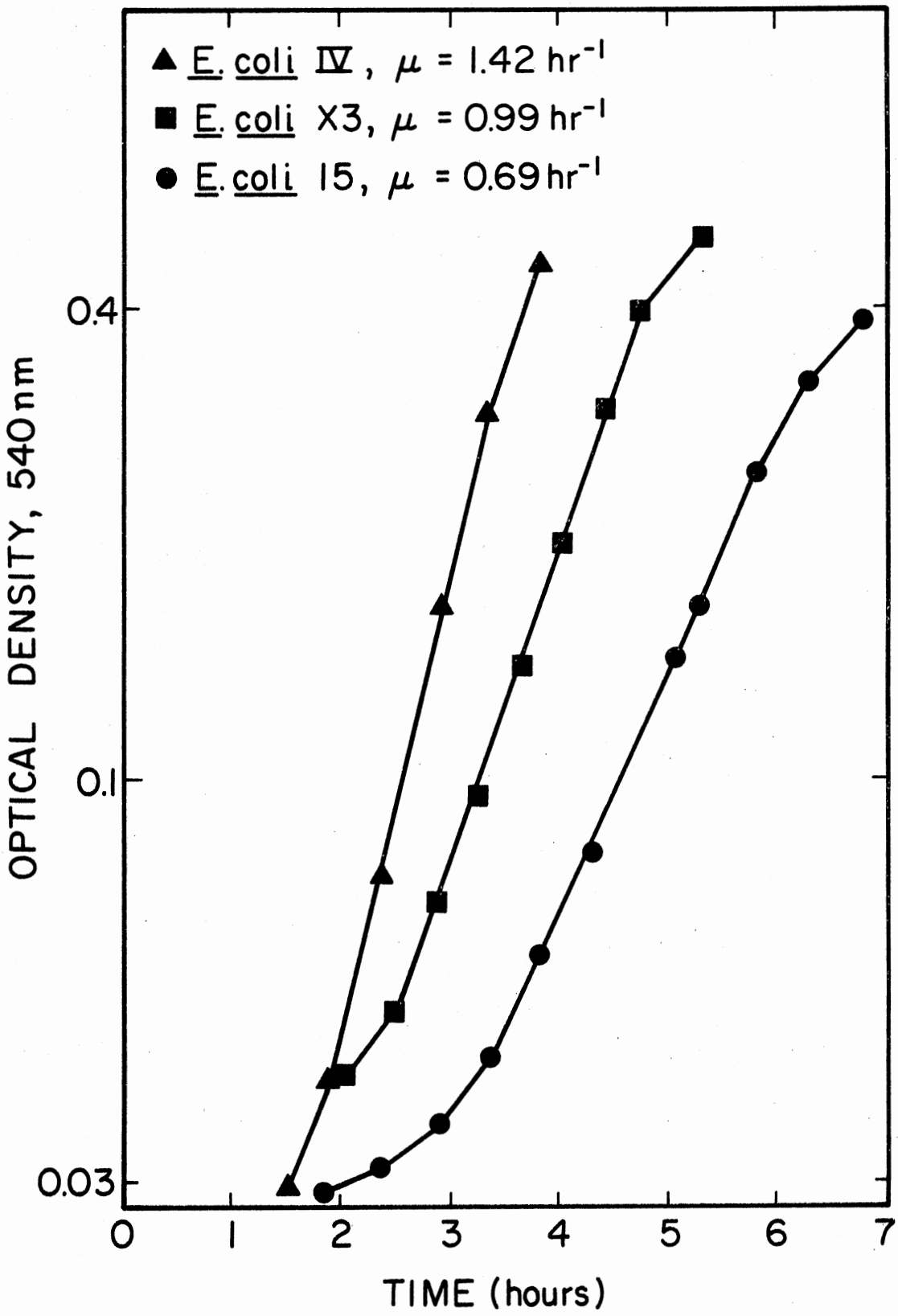

▲ E. coli IV, μ = 1.42 hr⁻¹
■ E. coli X3, μ = 0.99 hr⁻¹
● E. coli 15, μ = 0.69 hr⁻¹
OPTICAL DENSITY, 540nm
0.4
0.1
0.03
0
1
2
3
4
5
6
7
TIME (hours)

Figure 16. The Measurement of Growth of Microorganisms *E. coli* IV, *E. coli* 15 and *E. coli* X3 with Respect to Time in Glucose Minimal Medium with 0.5% Yeast Extract

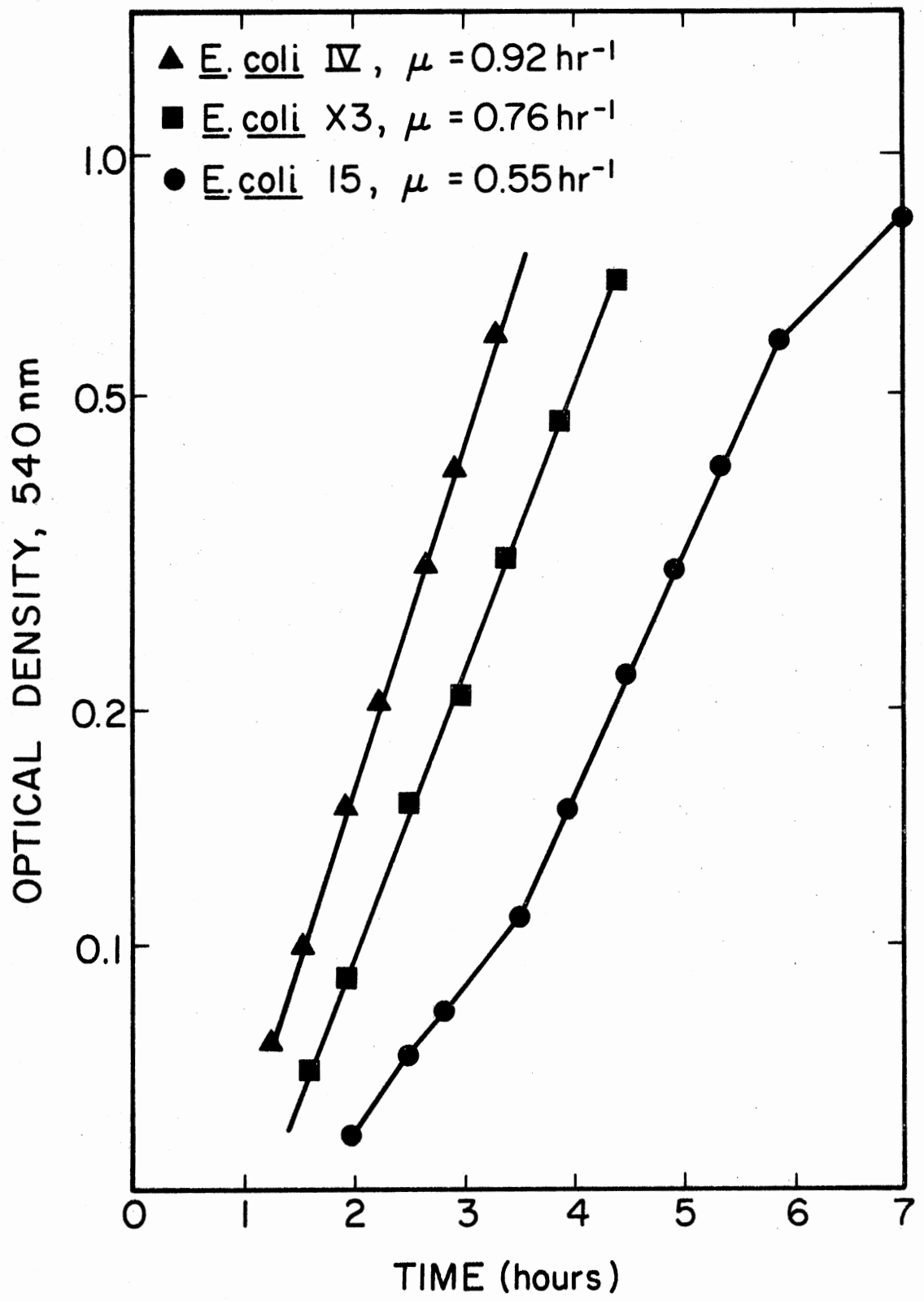

▲ E. coli IV, μ = 0.92 hr⁻¹
■ E. coli X3, μ = 0.76 hr⁻¹
● E. coli 15, μ = 0.55 hr⁻¹
OPTICAL DENSITY, 540 nm
1.0
0.5
0.2
0.1
0
1
2
3
4
5
6
7
TIME (hours)

Figure 17. The Measurement of Growth of Microorganisms *E. coli* IV, *E. coli* 15 and *E. coli* X3 with Respect to Time in Nutrient Broth with 0.5% Yeast Extract

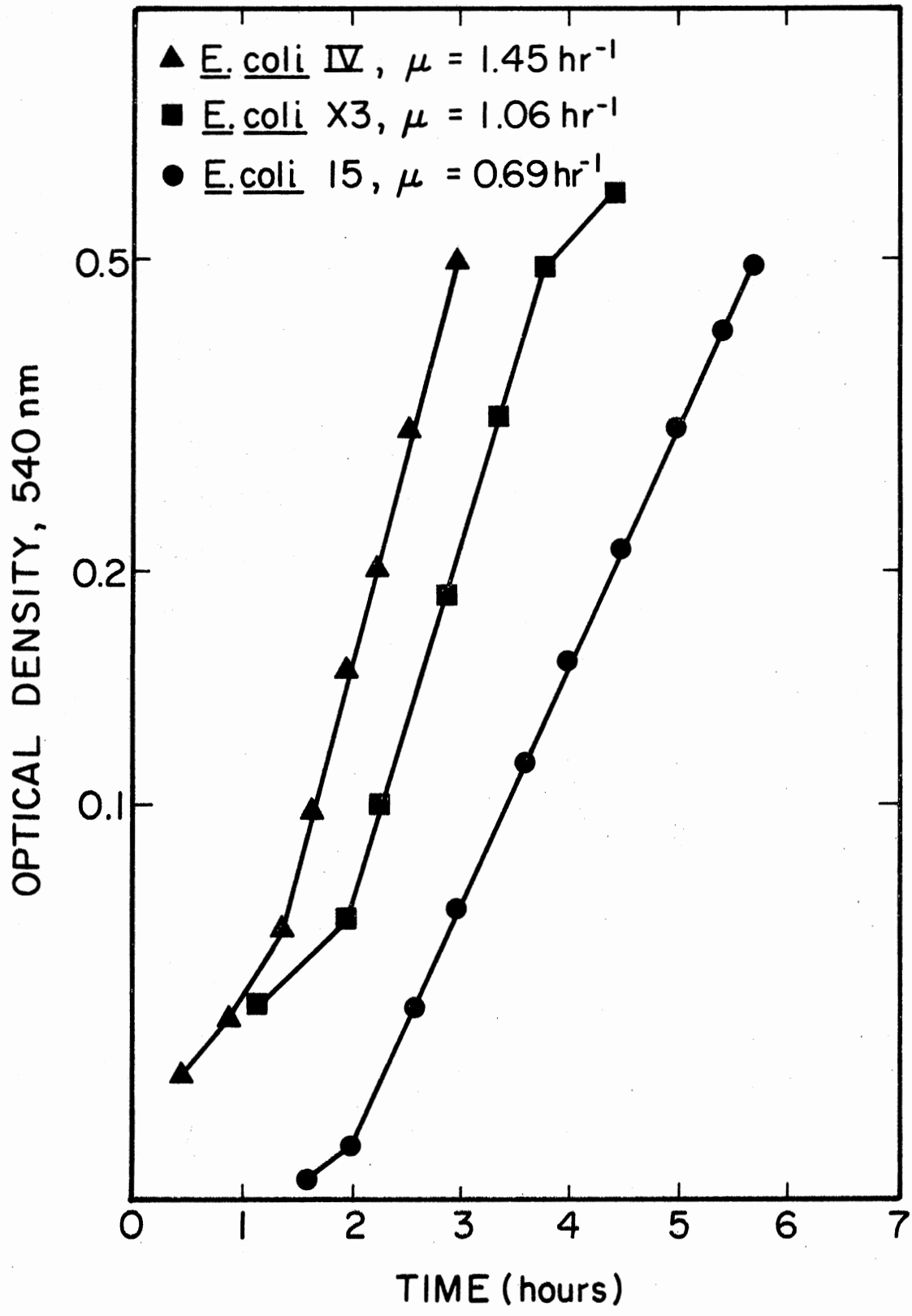

▲ E. coli IV, μ = 1.45 hr⁻¹
■ E. coli X3, μ = 1.06 hr⁻¹
● E. coli 15, μ = 0.69 hr⁻¹
OPTICAL DENSITY, 540 nm
0.5
0.2
0.1
0
1
2
3
4
5
6
7
TIME (hours)

Figure 18. The Measurement of Growth of Microorganisms *E. coli* IV, *E. coli* 15 and *E. coli* X3 with Respect to Time in Nutrient Broth with Glucose and Yeast Extract, 0.5% Each

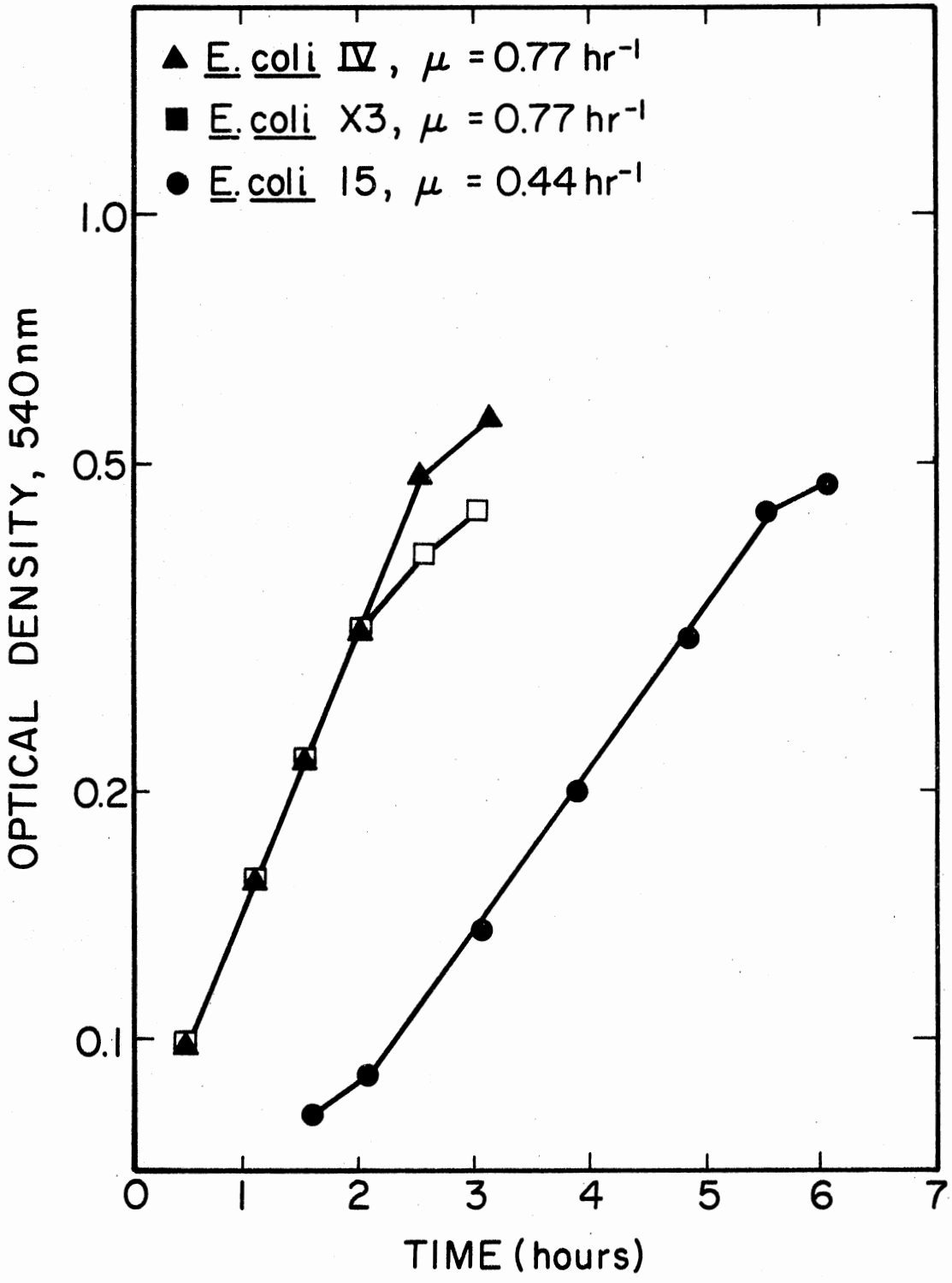

▲ E. coli IV, μ = 0.77 hr⁻¹
■ E. coli X3, μ = 0.77 hr⁻¹
● E. coli 15, μ = 0.44 hr⁻¹
OPTICAL DENSITY, 540nm
1.0
0.5
0.2
0.1
0
1
2
3
4
5
6
7
TIME (hours)

Figure 19. The Measurement of Growth of Microorganisms *E. coli* IV, *E. coli* 15 and *E. coli* X3 with Respect to Time in Nutrient Broth

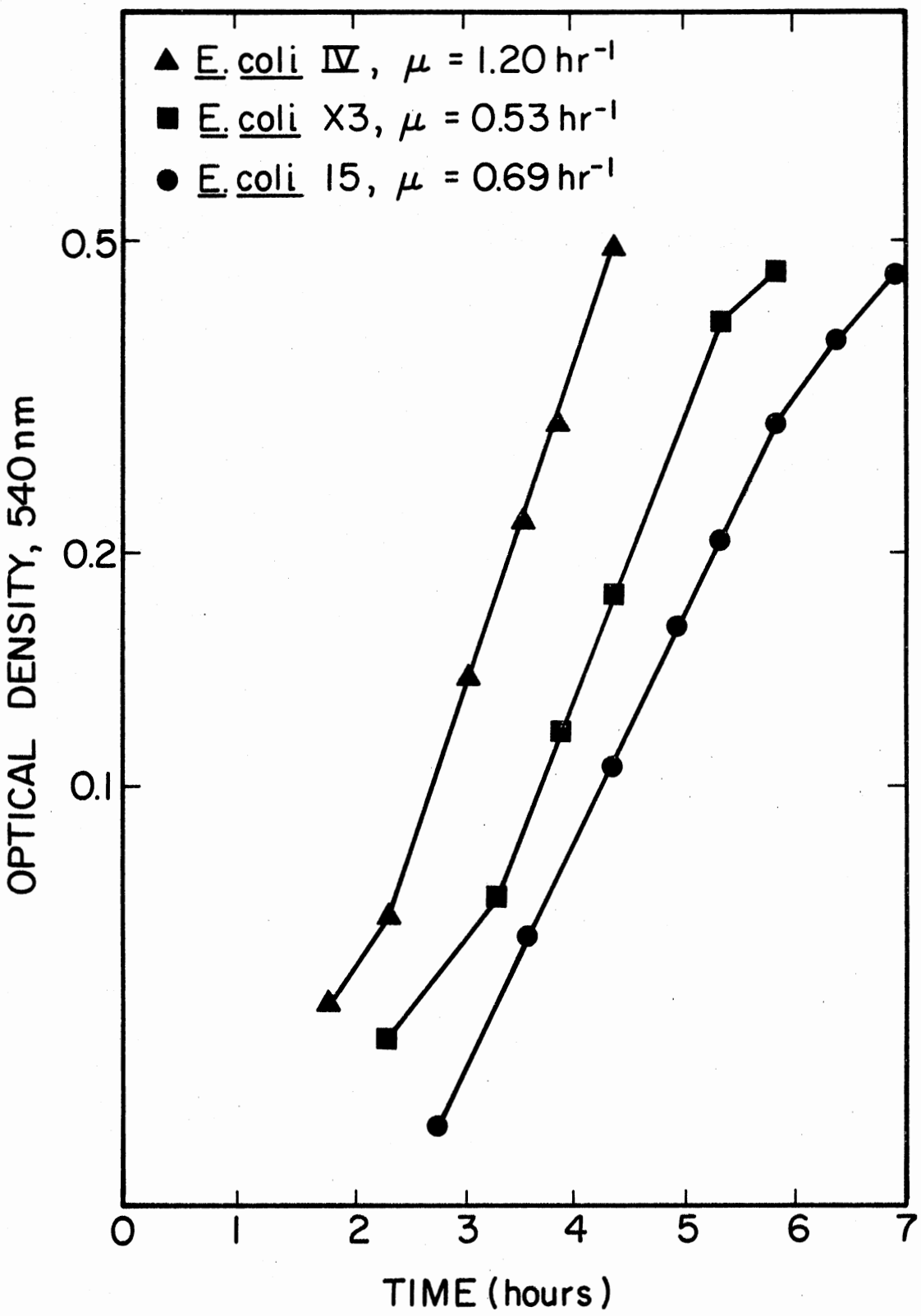
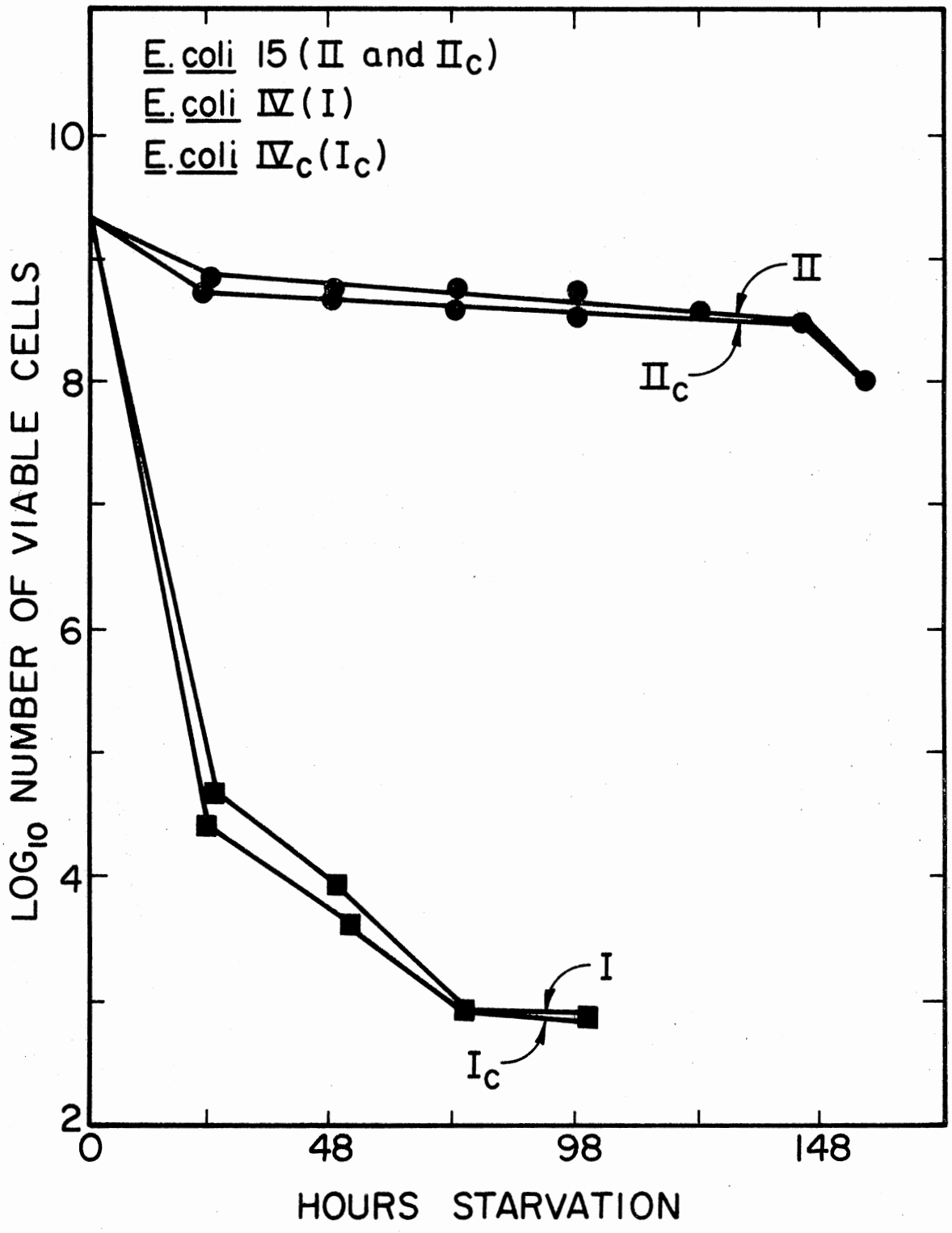

▲ E. coli IV, μ = 1.20 hr⁻¹
■ E. coli X3, μ = 0.53 hr⁻¹
● E. coli 15, μ = 0.69 hr⁻¹
OPTICAL DENSITY, 540 nm
0.5
0.2
0.1
0
1
2
3
4
5
6
7
TIME (hours)

TABLE VI

SPECIFIC GROWTH RATES OF MUTANTS AND WILD TYPE IN NUTRITIONALLY DIFFERENT MEDIA

Medium	*E. coli* IV hr^{-1}	*E. coli* 15 hr^{-1}	*E. coli* X3 hr^{-1}
Glucose minimal	0.71	0.27	0.19
Nutrient broth	1.2	0.693	0.533
Nutrient broth with 0.5% glucose	1.42	0.693	0.99
Nutrient broth with 0.5% yeast extract	1.45	0.693	1.06
Nutrient broth with 0.5% each glucose and yeast extract	0.71	0.44	0.71
Glucose minimal with 0.5% yeast extract	0.924	0.55	0.76

Figure 20. Viability of *E. coli* IV, *E. coli* IVc and *E. coli* 15 in Distilled Water


E. coli 15 (II and IIc)
E. coli IV (I)
E. coli IVc (Ic)
LOG10 NUMBER OF VIABLE CELLS
10
8
6
4
2
0
48
98
148
HOURS STARVATION
II
IIc
I
Ic

Water was employed instead of saline because it represents the most extreme starvation condition. It has been observed in the literature that post-log phase cells (stationary phase cell) are better able to survive in starving condition than are log-phase cells. Therefore, in this experiment post-log phase cells were used to allow the wild type cells to exhibit their maximum resistance to starvation.

Determination of the Growth Characteristics of Mutant *E. coli* 15 and Wild Type *E. coli* IV

It has been observed by many workers that the substrate-based yield of bacteria is dependent upon specific growth rate. At higher growth rates a higher yield is obtained and at lower growth rates a lower yield is obtained. To determine the substrate-based yields of mutant *E. coli* 15 and wild type *E. coli* IV, yield determinations were performed in different kinds of media. Results are summarized in Table VII.

Growth constants K_s and μ_m were also determined for mutant *E. coli* 15 and wild type *E. coli* IV. Specific growth rates of each organism were measured in glucose minimal medium, with glucose concentrations of 100, 200, 400, 600, 800, and 1000 mg/l. Double reciprocal plots of specific growth rate versus initial substrate concentrations were drawn as shown in Figure 21. The reciprocal of the intercept was defined as μ_m. The K_s was determined from the slope and μ by multiplying the two. Each pair of growth constants was utilized for calculating the curves shown in Figures 22 and 23. The constants were substituted into the Monod equation and the calculated curve was drawn as shown for both organisms. Table VIII shows the effect of varying substrate concentration on specific growth rate as obtained experimentally and by calculation.

TABLE VII

YIELD VALUES OF WILD TYPE AND MUTANT IN DIFFERENT MEDIA

Medium	Yield, mg/mg COD	
	E. coli IV	*E. coli* 15
Glucose minimal	0.49	0.27
Nutrient broth	0.51	0.29
Nutrient broth with 0.5% glucose	0.49	0.33
Nutrient broth with 0.5% yeast extract	0.51	0.31
Nutrient broth with 0.5% each glucose and yeast extract	0.51	0.29
Glucose minimal 0.5% yeast extract	0.48	0.31

All the batch yields were an average of 3 to 9 separate determinations.

Figure 21. Variation of Specific Growth Rate, μ, with Initial Substrate Concentration, S_o

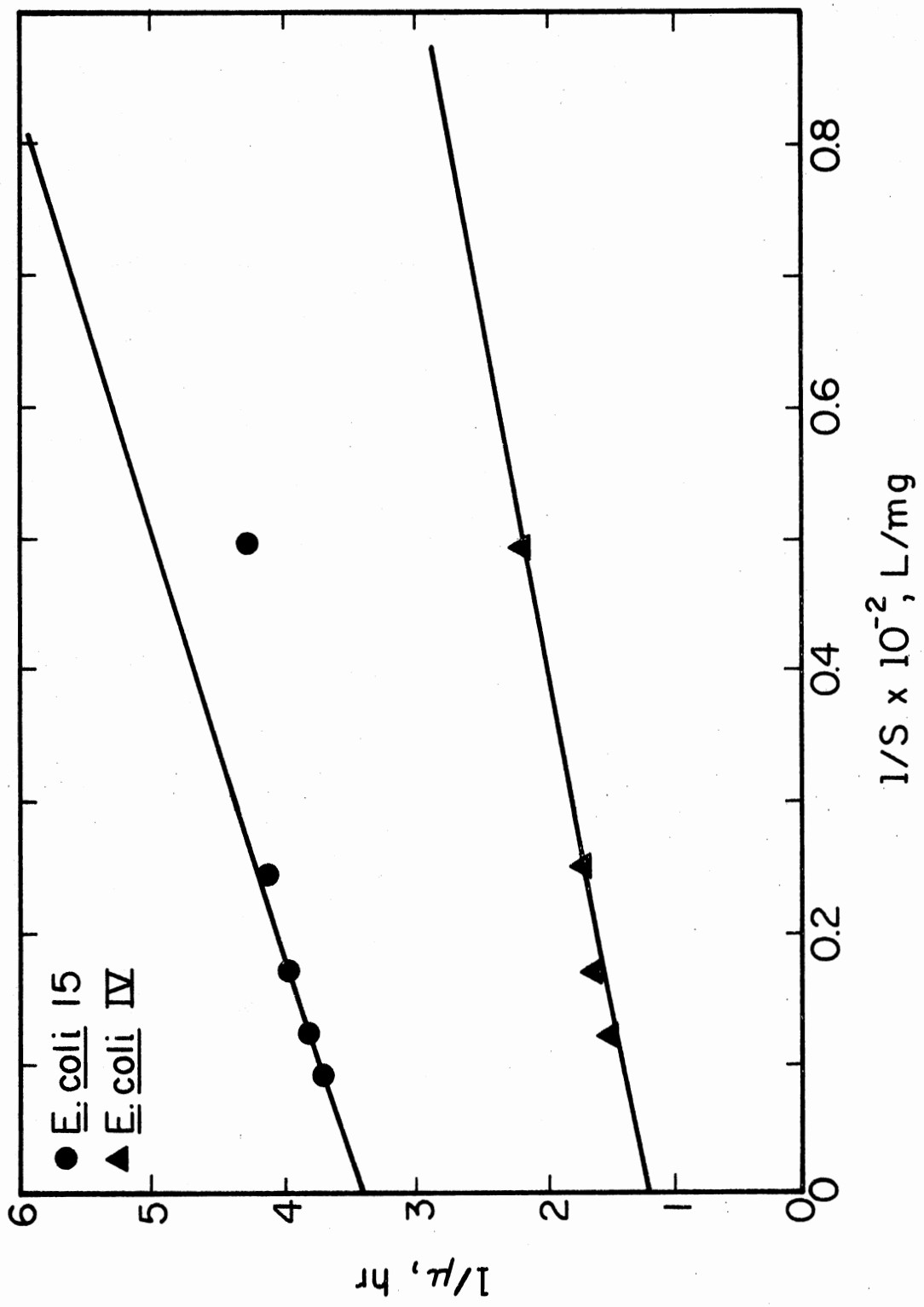

● E. coli 15
▲ E. coli IV
1/μ, hr
1/S x 10⁻², L/mg
0
1
2
3
4
5
6
0.2
0.4
0.6
0.8

Figure 22. Comparison of Calculated Monod Plot with Experimental Data for *E. coli* IV

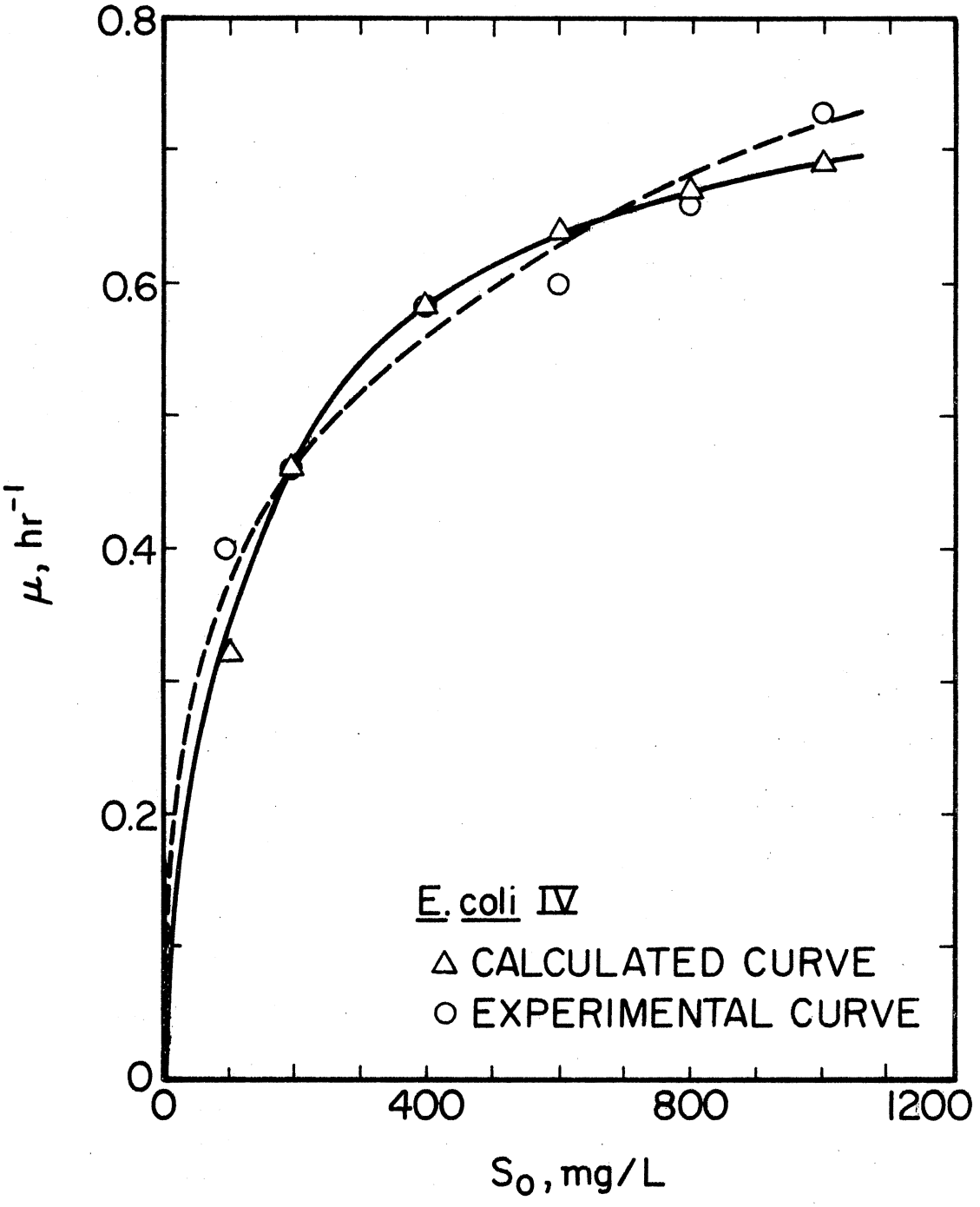

0.8
0.6
0.4
0.2
0
μ, hr⁻¹
0
400
800
1200
S₀, mg/L
E. coli IV
△ CALCULATED CURVE
○ EXPERIMENTAL CURVE

Figure 23. Comparison of Calculated Monod Plot with Experimental Data for *E. coli* 15

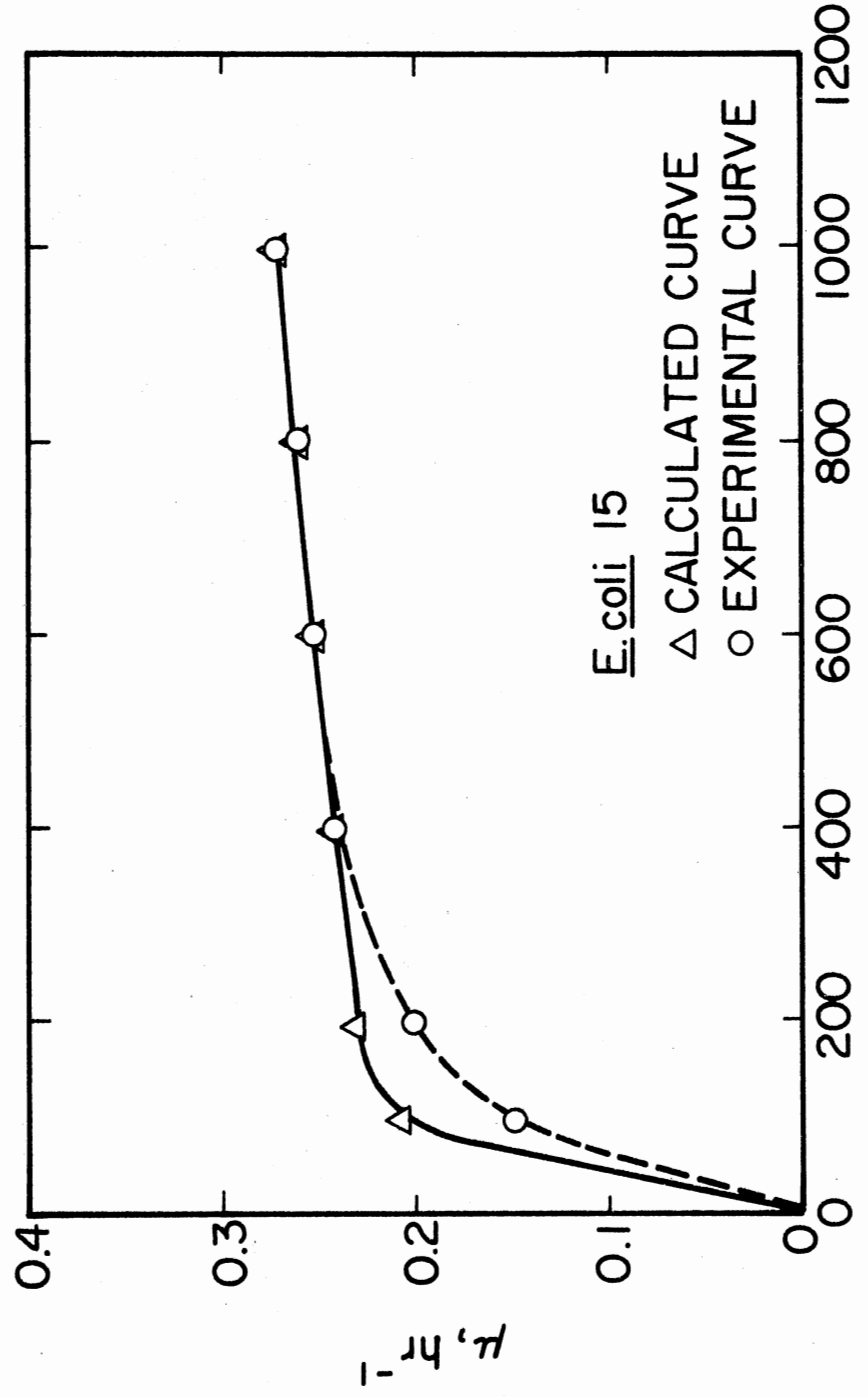

E. coli 15
△ CALCULATED CURVE
○ EXPERIMENTAL CURVE
μ, hr⁻¹
0.4
0.3
0.2
0.1
0
0
200
400
600
800
1000
1200
S₀, mg/L

TABLE VIII

COMPARISON OF OBSERVED SPECIFIC GROWTH RATES OF WILD TYPE AND MUTANT WITH VALUES CALCULATED USING GROWTH RATE CONSTANTS DETERMINED EXPERIMENTALLY

Initial Substrate Concentration, mg/l	Specific Growth Rate, hr^{-1}			
	E. coli 15		*E. coli* IV	
	Obs.	Calc.	Obs.	Calc.
100	0.21	0.15	0.40	0.32
200	0.23	0.20	0.46	0.46
300	0.24	0.24	0.58	0.58
600	0.25	0.25	0.60	0.64
800	0.26	0.26	0.66	0.67
1000	0.27	0.27	0.73	0.69

Calculated values for specific growth rate were obtained by substituting the experimentally determined values of μ_m and K_s for each organism into the Monod equation

$$\mu = \frac{\mu_m S}{K_s + S} .$$

Values used were:

E. coli IV, $\mu_m = 0.81\ hr^{-1}$, $K_s = 145$ mg/l,
E. coli 15, $\mu_m = 0.29\ hr^{-1}$, $K_s = 90$ mg/l.

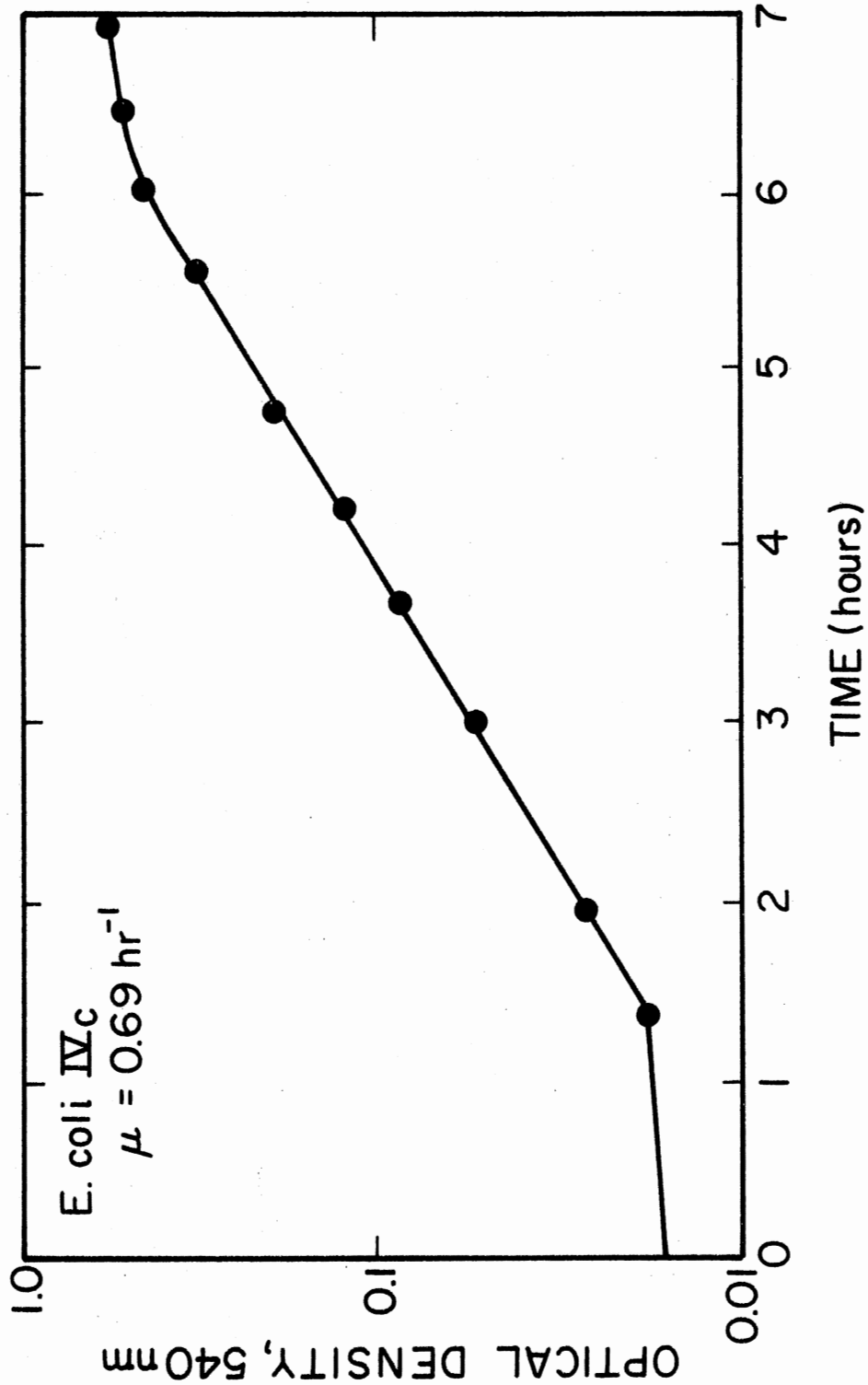
Continuous Flow Studies

In a once-through continuous flow system, the specific growth rate is equal to the dilution rate (Herbert et al., 1956). To determine the effect of dilution rate on selection of the mutant E. coli 15 or the wild type E. coli IV from a mixture of the two, a chemostat was inoculated with approximately equal numbers of cells of each organism.

The continuous flow equipment and setup was described in Materials and Methods. The medium used for this study was glucose minimal medium containing 1000 mg/l glucose as sole source of carbon and energy. Two dilution rates were used, 0.05 and 0.125/hr. For these studies, it was necessary to devise a method of making differential counts of the wild type and mutant in mixtures of the two.

When a cell suspension of E. coli IV and E. coli 15 was diluted and spread on glucose minimal medium agar plates, colonies of E. coli IV appeared after 48 hours of incubation at 37°C. They were white, round, flat and 1.0 to 1.5 mm in diameter. Colonies of E. coli 15 could not be observed until 72 hours and at that time they were very small (pin point) round colonies. To allow differential counting, a carbenicillin-resistant mutant was derived from wild type E. coli IV as described previously. The carbenicillin-resistant mutant was referred to as "E. coli IVc" in this study. To be certain that E. coli IVc had the same characteristics as wild type E. coli IV, maximum specific growth rate and yield were measured in glucose minimal medium. The maximum specific growth rate of E. coli IVc was 0.69/hr and yield was 0.49 mg/mg. Comparable figures for the wild type E. coli IV were 0.71/hr and 0.49 mg/mg, respectively. Figure 24 shows the growth rate curve for E. coli IVc. The colony size of E. coli IVc on glucose minimal

Figure 24. Measurement of the Specific Growth Rate of the *E. coli* IVc


E. coli IVc
μ = 0.69 hr⁻¹
OPTICAL DENSITY, 540nm
1.0
0.1
0.01
0
1
2
3
4
5
6
7
TIME (hours)

medium plates was approximately 1.0 mm in diameter after 48 hours incubation at 37°C.

On TSA plates both the organisms, E. coli IVc and E. coli 15 grew in 24 hours. It was difficult to distinguish them by colony size. When equal volumes of cell suspension of each organism E. coli IVc and E. coli 15 were spread on glucose minimal medium plates and on TSA plates, they yielded approximately the same number of colonies on each medium. The results are summarized in Table IX. To distinguish the mutant E. coli 15 from the wild type E. coli IV in mixed suspensions, three different types of plates were used, TSA, glucose minimal medium and glucose minimal medium with 10 μg/ml of carbenicillin. On TSA plates, a colony count represents the total number of microorganisms. The colony count on glucose minimal plates after 48 hours incubation at 37°C represents only the wild type E. coli IVc from the mixed culture. To make certain the glucose minimal medium plates represented only the wild type E. coli IVc, a colony count on glucose minimal medium plates with 10 μg/ml carbenicillin was also made.

E. coli IVc and E. coli 15 were grown separately in 20 ml of glucose minimal medium at room temperature until cell density was 10^8-10^9 cells/ml. To insure that approximately equal numbers of cells of each organism were inoculated into the chemostat, volumes of cell suspension were calculated from the optical density readings. Plates counts were made to determine actual initial concentrations of viable cells. Depending upon the optical density, 1.0 to 1.5 ml of cell suspension of each organism was inoculated into an already filled and aerated chemostat. The organisms were allowed to grow at room temperature at the experimental growth rates.

TABLE IX

COLONY COUNTS OF CELL SUSPENSIONS ON DIFFERENT MEDIA

Organism	No. Colony on TSA (cells/ml)	No. Colony on Glucose Minimal (cells/ml)	No. Colony on Glucose Minimal with Carbenicillin (cells/ml)
E. coli IVc	120	115	111
E. coli IVc	119	111	107
E. coli IVc	106	101	96
E. coli IVc	114	104	100
E. coli 15	86	81	-
E. coli 15	81	75	-
E. coli 15	90	81	-
E. coli 15	117	111	-

For the type of continuous flow apparatus used in this study, it was found experiments were best completed within 7-9 days. It was observed, especially at the low dilution rate, that due to evaporation of medium in the chemostat an unsteady dilution rate resulted. Also, tubing through the pump started to leak if it was used more than 9-10 days continuously. Normally, steady state was reached after 24-48 hours. It was determined by optical density readings and confirmed by direct plate count.

At each particular dilution rate, colony counts were done every 24 hours. Replicate samples of diluted cell suspension from the chemostat were spread on TSA plates and on glucose minimal medium plates without and with 10 μg/ml concentration of carbenicillin. TSA plates gave the total cell count in the chemostat containing E. coli IVc and E. coli 15, while glucose minimal medium plates with and without carbenicillin at 48 hours gave total counts of E. coli IVc only. By subtracting the concentration of E. coli IVc from the total cell count, the concentration of mutant E. coli 15 in the chemostat was determined. Results are summarized in Tables X and XI for the slow and fast dilution rates.

At the end of each chemostat experiment, yield was measured for the continuous flow system as explained in Materials and Methods and 0.5 ml of the mixed cell suspension was transferred to 20 ml of glucose minimal medium in a 250 ml flask. To determine the batch yield for the mixed population, organisms were allowed to grow at room temperature on the shaker until growth ceased. At the end of the growth period, yield was measured for this batch culture. Specific growth rate was calculated

TABLE X

DETERMINATION OF THE PERCENTAGES OF WILD TYPE AND MUTANT IN MIXED CULTURE IN THE CHEMOSTAT AT STEADY STATE AT A DILUTION RATE OF 0.125 hr^{-1}

Hours	Viable Cells/ml x 10^{-9}		
	TSA	Glucose Minimal	Glucose + Carbenicillin
24	0.42	0.29	0.23
48	1.21	0.74	0.68
72	1.68	1.29	1.27
96	1.67	1.23	1.23
120	1.61	1.20	1.19
144	1.58	1.25	1.23
168	1.62	1.24	1.21
Steady State Average	1.63	1.24	1.22

Initial inoculum:
1.06 x 10^9 cells *E. coli* IVc
0.91 x 10^9 cells *E. coli* 15

Concentrations at steady state:
75% *E. coli* IVc
25% *E. coli* 15

TABLE XI

DETERMINATION OF THE PERCENTAGES OF WILD TYPE AND MUTANT IN MIXED CULTURE IN THE CHEMOSTAT AT STEADY STATE AT A DILUTION RATE OF 0.05 hr^{-1}

Hours	Viable cells/ml x 10^{-9}		
	TSA	Glucose Minimal	Glucose + Carbenicillin
24	1.16	0.51	0.47
48	2.27	0.94	0.82
72	2.26	0.91	0.79
96	2.27	0.93	0.78
120	2.46	0.91	0.77
144	2.40	0.94	0.80
Steady State Average	2.29	0.924	0.787

Initial inoculum:
1.20 x 10^9 cells *E. coli* IVc
1.23 x 10^9 cells *E. coli* 15

Concentrations at steady state:
40% *E. coli* IVc
60% *E. coli* 15

for the mixed population as described previously. Results are summarized in Table XII.

The lower yields and slower specific growth rates of the mixed population in batch cultures inoculated from the chemostat presents an interesting results. According to a previous study in this laboratory, Jones (1974) found with a pure culture of E. intermedia that the yields in batch cultures were the same as yields in the chemostat at high and low dilution rates, respectively, even though specific growth rate in the batch experiments was considerably higher than those in the chemostat at either dilution rate. The same observations were made by Gaudy et al. (1974) working with a mixed microbial population. In the present study, specific growth rates in batch culture with excess substrate and mixed population of mutant E. coli 15 and wild type E. coli IV were considerably lower than the maximum specific growth rate for the wild type. The yields observed in batch were lower than the yields recorded in the chemostat. In batch cultures of the mutant E. coli 15 alone, there was a very long lag period, while the wild type lag period was only 2-4 hours. During growth studies in batch flasks of mixed populations inoculated from the chemostat, it was observed that specific growth rate (especially corresponding to the low dilution rate) and yields were much the same as those of a pure culture of mutant E. coli 15. However, the lag period was much shorter. There could exist a relationship between these two organisms such that wild type E. coli may provide a nutrient or nutrients to the mutant E. coli 15, therefore allowing mutant E. coli 15 to shorten its lag period in the mixed population. The same relationship may exist between these two organisms in the continuous flow system at a higher dilution rate. Hence, the organisms attain a steady

TABLE XII

EFFECT OF SPECIFIC GROWTH RATE ON CELL YIELD DURING CONTINUOUS GROWTH AND BATCH GROWTH

	$D = 0.05\ hr^{-1}$	$D = 0.125\ hr^{-1}$
Continuous flow yield, mg/mg	0.40	0.46
Batch yield, mg/mg	0.27	0.37
Specific growth rate, batch, hr^{-1}	0.27	0.39

Continuous flow yield values are the average of six determinations.

Batch yield values are the average of three determinations.

Batch cultures were inoculated with the steady state mixed culture from the chemostat at each dilution rate: at $D = 0.05\ hr^{-1}$, 40% *E. coli* IV, 60% *E. coli* 15; at $D = 0.125\ hr^{-1}$, 75% *E. coli* IV, 25% *E. coli* 15.

state level at higher dilution rate instead of the mutant washing out of the system and leaving the wild type to predominate.

To determine whether lower yield values and slower growth rates of the mixed population in batch inoculated from the chemostat were programmed by continuous flow dilution rates, or alternatively by some special characteristic of one or both of the bacterial types, the following experiment was carried out.

Two sets of batch flasks containing glucose minimal medium were inoculated from overnight cultures of E. coli IV and E. coli 15 in different populations. One set of flasks contained as inoculum 68% E. coli IV and 32% E. coli 15 as found in the chemostat at steady state for a dilution rate of 0.125/hr; i.e., E. coli IV, 75% and E. coli 15, 25%. The other set of flasks contained as inoculum 29% E. coli IV and 71% mutant. This represented approximately the same proportion of the two organisms as at the steady state level in the chemostat for a dilution rate of 0.05/hr; i.e., E. coli IV, 40% and E. coli 15, 60%. Absorbance was measured for each set of flasks at 540 nm in the Coleman Junior spectrophotometer. Specific growth rate was calculated for each set of flasks and at the end of growth, yield was measured. Results of this experiment are summarized in Table XIII.

These experimental results suggested that in mixed populations of E. coli IV and E. coli 15, the mutant E. coli 15 partially inhibited the growth of the wild type. The partial inhibition of wild type E. coli IV by the mutant could be due to competition for nutrients or due to production of a toxic metabolite by the mutant. To determine whether this inhibition was due to some defective metabolic pathway of glucose

TABLE XIII

SPECIFIC GROWTH RATES AND YIELDS OF MIXED CULTURES OF MUTANT AND WILD TYPE IN DIFFERENT PROPORTIONS

Initial Cell Concentration	μ, hr^{-1}	Yield, mg/mg
29% *E. coli* IVc, 71% *E. coli* 15	0.28	0.29
68% *E. coli* IVc, 32% *E. coli* 15	0.49	0.43

All the batch yield values are the average of three separate determinations.

All the plate counts are the average of three replicate plates.

possessed by E. coli 15 leading to production of a glucose metabolite, the following experiment was carried out. Acclimated cells of wild type E. coli IV and mutant E. coli 15 were mixed in different proportions in 20 ml volumes of nutrient broth and nutrient broth with 0.5% yeast extract. Neither of these media contain sugar as carbon and energy source. Absorbance for each culture was measured at 540 nm on the Coleman Junior spectrophotometer. Specific growth rate was calculated for cells in each medium and, at the end of growth, yield was measured. Results are summarized in Table XIV and graphs are shown in Figure 25. In both the media without glucose, specific growth rates and yields of the mixed populations were very similar to the values obtained with a pure culture of the mutant in corresponding media. Therefore, the inhibitory characteristics of mutant E. coli 15 were not attributable to production of glucose metabolite due to a defective pathway of glucose metabolism. To seek additional evidence as to whether the inhibitory quality of mutant E. coli 15 was due to the production of a toxic metabolite in the growth medium of the mixed population, the following experiment was run.

Mutant E. coli 15 was allowed to grow in glucose minimal medium until it approached late logarithmic growth phase. Ten ml of spent medium was filtered through a Millipore filter. The filtrate was mixed with 10 ml of fresh glucose minimal medium and 0.25 ml of 2% glucose solution, and a 24-hour culture of E. coli IV was used as inoculum for this and the two control media described below. Specific growth rates were measured and calculated and yields were determined for each medium. Two different types of control media were prepared:

1. Wild type E. coli IV was grown in glucose minimal medium.

TABLE XIV

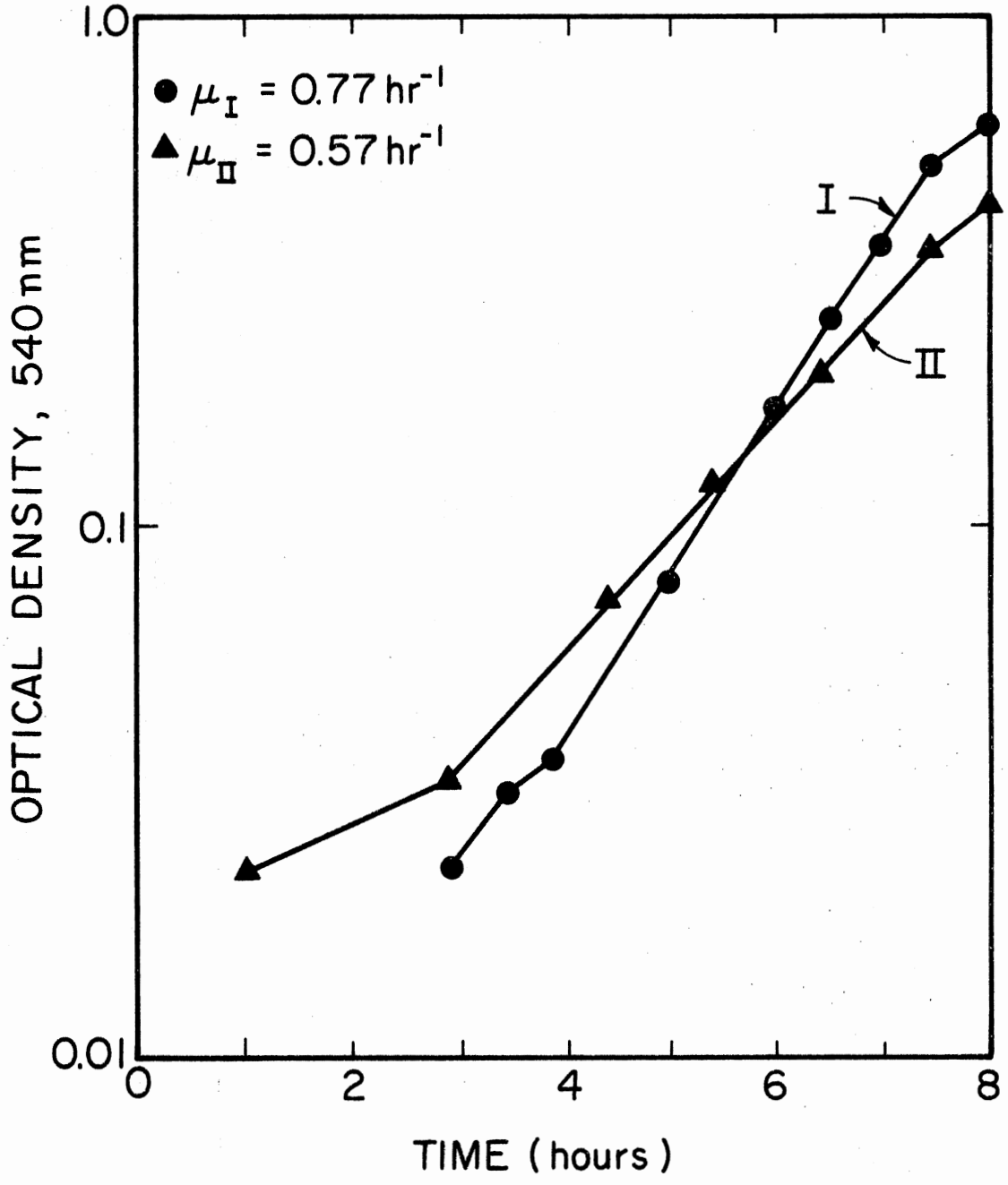
SPECIFIC GROWTH RATES AND YIELDS OF MIXED CULTURES OF MUTANT AND WILD TYPE IN COMPLEX MEDIA

Initial Cell Concentration	Medium	μ, hr^{-1}	Yield mg/mg
28% *E. coli* IVc 72% *E. coli* 15	Nutrient broth	0.57	0.27
32% *E. coli* IVc 68% *E. coli* 15	Nutrient broth + yeast extract	0.77	0.33

All the batch yield values are the average of three separate determinations.

All the plate counts are the average of three replicate plates.

Figure 25. Effect on Specific Growth Rate of Mixed Culture of Mutant and Wild Type in (I) Nutrient Broth with Yeast Extract, (II) Nutrient Broth


1.0
0.1
0.01
OPTICAL DENSITY, 540 nm
● μ_I = 0.77 hr^{-1}
▲ μ_{II} = 0.57 hr^{-1}
I
II
0
2
4
6
8
TIME (hours)

At the end of late log phase spent medium filtrate was prepared as mentioned above. Ten ml of filtrate was mixed with 10 ml of fresh glucose minimal medium and 0.25 ml of 2% glucose solution.

2. Ten ml of fresh glucose minimal medium, 10 ml of saline (0.85%) and 0.25 ml of 2% glucose solution were mixed.

Results of this experiment are summarized in Table XV and graphs are shown in Figure 26.

TABLE XV

EFFECT OF SPENT MEDIUM OF MUTANT ON THE GROWTH OF WILD TYPE

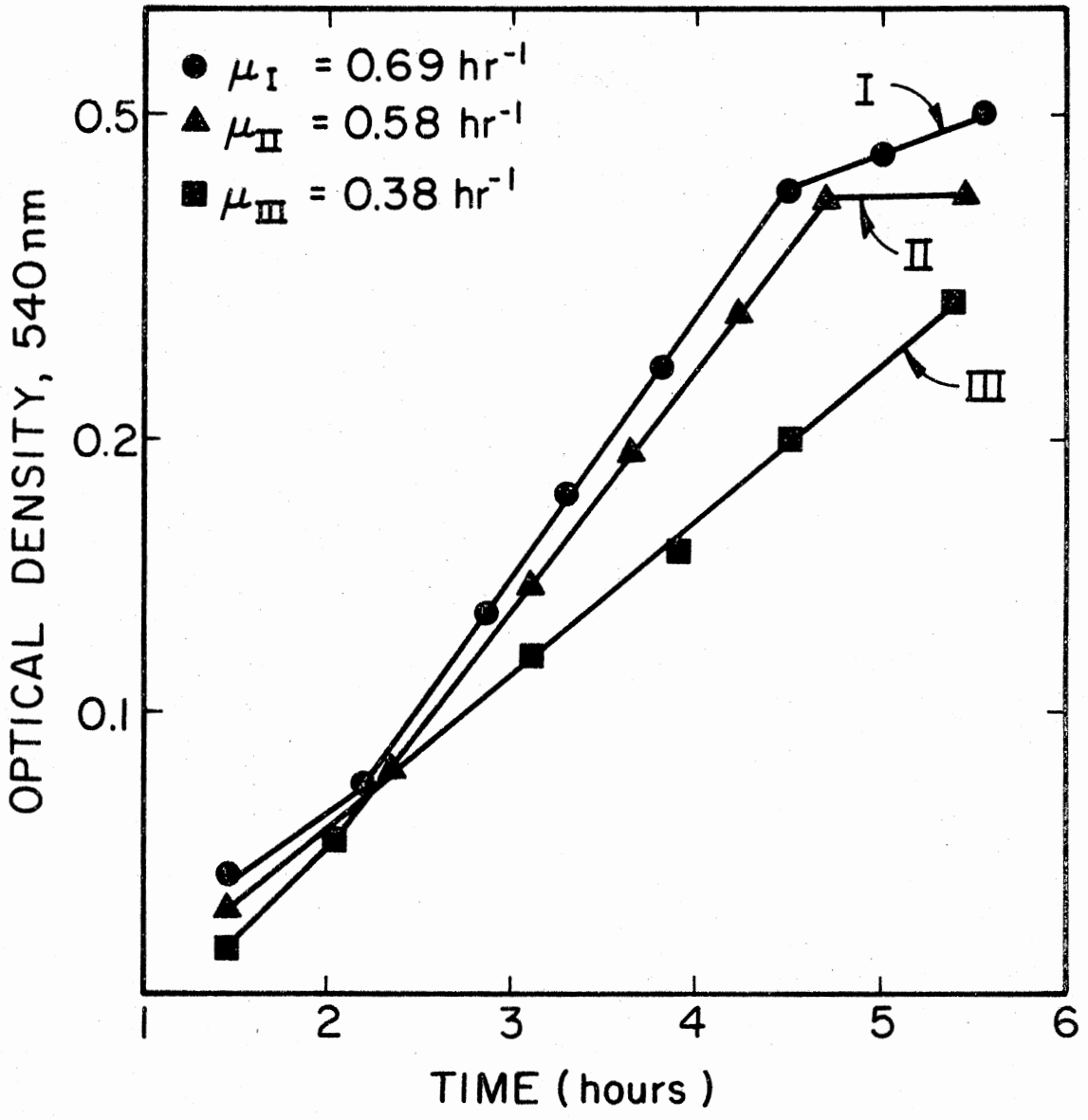
Media	Specific Growth rate, hr^{-1}	Yield mg/mg
Test	0.38	0.36
Control I	0.69	0.42
Control II	0.58	-

Test medium: 10 ml of filtered supernatant of mutant E. coli 15 + 10 ml of glucose minimal medium + 0.25 ml of 2% glucose solution.

Control medium I: 10 ml of filtered supernatant of wild type E. coli IV + 10 ml glucose minimal medium + 0.25 ml of 2% glucose solution.

Control medium II: 10 ml of glucose minimal medium + 10 ml of 0.85% saline + 0.25 ml of 2% glucose solution.

Figure 26. Effect on the Growth of Wild Type of the Spent Medium of the Mutant



The media differed in the following components: I, 10 ml filtered supernatant of wild type culture; II, 10 ml of 0.85% saline, III, 10 ml of filtered supernatant of mutant. All media contained 10 ml of glucose minimal medium (1000 mg/l glucose) and 0.25 ml of 2% glucose solution.

CHAPTER IV

DISCUSSION

Harrison and Lawrence (1963) showed that "starvation resistant" mutants could be obtained from batch culture populations and these were different in several ways from the "wild type". In particular their maximum exponential growth rate was slower and logarithmic and stationary phase populations showed little difference in their survival characteristics, in contrast to the wild type in which logarithmic phase populations were much more sensitive to starvation.

Employing an E. coli strain instead of A. aerogenes as the test organism and using the same method employed by Harrison and Lawrence (1963), it was possible to isolate many mutants of E. coli with maximum specific growth rates slower than wild type. All of these mutants had a long lag phase in comparison to the wild type. However, most of these mutants were unstable. Many were able to revert back to a wild type specific growth rate during log phase growth. This could be explained by assuming the mutant arose from a simple point mutation which could easily revert to the wild type. Selection pressure should favor the normal or fast growing strain. Of 18 slow specific growth rate mutants, only four proved stable and all these stable mutants had different specific growth rates. This could be due to mutation at different sites in the same gene or mutation in different genes.

It was found that two of the mutants, E. coli 15 and E. coli X3, as well as the wild type had constant specific growth rates and that these specific growth rates were constant through several growth cycles using the previous growth cycle as source of inoculum. Specific growth rates of mutants E. coli X4 and E. coli P increased on transfer to fresh medium. Mutant E. coli 15 and E. coli X3 both had slow maximum specific growth rates in glucose minimal medium compared to the wild type.

When mutants E. coli 15 and E. coli X3 and wild type E. coli IV were grown in nutrient broth, nutrient broth with 0.5% glucose, nutrient broth with 0.5% yeast extract and nutrient broth with 0.5% glucose and 0.5% yeast extract, in each case the growth rate of each organism was faster than in glucose minimal medium. Increases in specific growth rates in these nutrient-enriched media were due to the presence of all the nutrients required by growing bacteria. In the enriched media all the bacteria had shorter lag periods than in the glucose minimal medium. The specific growth rate of mutant E. coli X3 was faster than that of mutant E. coli 15 in all media except nutrient broth, even though the specific growth rate of mutant E. coli X3 was slower than that of E. coli 15 in glucose minimal medium. In nutrient broth with glucose and yeast extract, the specific growth rate of mutant E. coli X3 was the same as that of wild type E. coli IV. In all media, specific growth rates of mutant E. coli 15 were approximately half of the specific growth rates the wild type. Observation of these specific growth rates makes it clear that mutant E. coli 15 is a slow growth rate mutant. E. coli X3 may represent a leaky nutritional mutant. It was also observed that the specific growth rate of the mutant E. coli X3 almost approached the specific growth rate of wild type E. coli IV in medium enriched with

yeast extract. Yeast extract might provide the nutrient(s) that enables mutant E. coli X3 to grow at close to the normal rate. It was observed that specific growth rates of E. coli 15, E. coli X3 and wild type E. coli IV were lower in medium containing nutrient broth with glucose and yeast extract than the specific growth rates of these same organisms in nutrient broth with glucose and nutrient broth with yeast extract. One explanation could be that when yeast extract, glucose and nutrient broth are autoclaved together they may form toxic compounds, which could retard the growth of the organisms. However, the same results were obtained when nutrient broth, yeast extract and glucose solutions were autoclaved separately, cooled and mixed in proper concentrations, and the experiment repeated.

Mutant E. coli 15 has a slower specific growth rate than wild type in corresponding media. This mutant may have a mutational defect at some point in transcription or translation or in a generalized transport mechanism. The failure of added nutrients to alter the differential in mutant and wild type growth rates indicates that the defect is not nutritional. Differences between the mutant E. coli 15 and the wild type were observed only in colony size on glucose minimal medium plates and in measured growth constants. All biochemical tests, wet mounts and Gram stains gave identical results.

The yield for a pure culture should be constant for a specific set of conditions. Any change in the environment of the culture may also have an effect on the yield. It is noteworthy that whenever yield is reported to be affected by some physical parameter the specific growth rate has also been affected (Mennett, 1971). This suggests a correlation between the two. An important concept has evolved; i.e., that as

the specific growth rate increases, cell yield also increases. The reverse situation is also found (i.e., decreased growth rate results in a decreased yield). Postgate and Hunter (1962) showed this effect with A. aerogenes using carbon, nitrogen, phosphorus and magnesium as limiting nutrients in several series of experiments. Herbert (1958) showed a similar effect using A. aerogenes and Torula utilis; Schulze and Lipe (1964) using E. coli performed the same type of experiment. They used glucose as limiting carbon and energy source and were able to show that at a μ of 0.06/hr, a yield of 0.44 mg/mg was obtained, while at a μ of 0.66/hr, the value obtained for the yield was 0.54 mg/mg. Since mutant E. coli 15 has a slower specific growth rate than wild type E. coli IV, it should also have a lower yield if the relationship between μ and yield is always applicable. Examinations of yield data (Table VII) in different media support this relationship. Yield of mutant E. coli 15 varies with media from 0.27 mg/mg to 0.31 mg/mg and yield of the wild type varies from 0.47 mg/mg to 0.51 mg/mg. The common reason given for variation of yield with growth rate is the maintenance energy requirement, i.e., the utilization of exogenous substrate to maintain the status quo. The slow growing populations would require higher proportions of the substrate for maintenance; therefore, less amounts of substrate are channelled to synthesis of fresh cells. Another theory postulated was the endogenous decay of cells. That is, a higher amount of autodigestion of cells occurs at lower growth rates. Up to now, it has not been proven conclusively which, if either, of these two theories is correct. The decrease in yield may be a manifestation of one or both phenomena acting together, or may be due to other causes.

It was also experimentally observed that other growth constants of the wild type and mutant were different. These were determined from a series of batch flask cultures with media containing different concentrations of glucose. The specific growth rates were determined for each flask and double reciprocal plots were made for specific growth rate versus substrate concentration. The μ_m and K_s of the wild type were 0.81/hr and 145 mg/l respectively, while the μ_m and K_s of the mutant were 0.29/hr and 90 mg/l respectively. Jones (1974) also showed, in different types of experiments, that these "growth constants" apparently were not constant when the cells were growing at different rates. Jones (1974) used a chemostat to grow cells at different growth rates. At the end of each continuous flow experiment, he inoculated a series of flasks with cells grown in the chemostat and determined the growth constants, μ_m, K_s and Y, for those cells in batch. He found that the dilution rate at which the cells had been grown in the chemostat influenced the values of the growth constants. All three growth constants had lower values for cells which had been grown at a lower specific growth rate in the chemostat.

It has been observed by many workers that populations harvested from the stationary phase of growth are often less fragile than those from the experimental phase. Such cells have been shown to be more resistant to cold shock (Sherman and Albus, 1923; Meynell, 1958; Gorrill and McNeil, 1960), heat shock (Lemcke and White, 1959), decompression (Fraser, 1951), desiccation (Lemcke, 1959), freezing and drying (Fry and Greaves, 1951) as well as starvation (Strange et al., 1961).

Harrison and Lawrence (1963) made use of this difference in resistance between log and postlog phase cells to deleterious

circumstances to explain the starvation resistance of their mutants. Of three starvation-resistant mutants of A. aerogenes examined, all converted to heat resistance more rapidly than did wild type cells. According to them, mutant cell suspensions during starving condition can lose up to 20% of their dry weight with no apparent loss in the number of viable cells. On the other hand this same loss in mass by a suspension of wild type cells occurs concomitantly with a loss of viability of well over 90%. Harrison and Lawrence postulated that since most cells in nature exist in low nutrient environments a selection of slow growing cells may offset the selection of fast growing cells during the periods of an active proliferation.

It was observed in the present study that, when stationary phase cells of wild type E. coli IV were starved in distilled water, within 24 hours most of the wild type cells were non-viable (90%). Similarly when stationary phase cells of the mutant E. coli 15 were starved in distilled water, it remained viable after six days of starvation. In this study purposely stationary phase cells were used since they are supposed to be more resistant to starvation than are log phase cells. Since stationary phase cells of wild type died so much faster than the mutant in distilled water, the mutant was much more resistant to starvation than the wild type. One of the explanations suggested by Harrison and Lawrence (1963) for the starvation resistance of mutants was that the mutant could survive a longer time in buffer because of its ability to convert from susceptible log phase physiology to resistant postlog phase physiology more rapidly than could the wild type. But this explanation is not sufficient to explain the starvation resistance of the mutant in this study. In this study stationary phase wild type was

employed, and it died much faster than the mutant under starvation conditions.

It was observed in continuous flow studies at a low dilution rate (0.05/hr) that mutant E. coli 15 predominated over the wild type, in a ratio of 60% of E. coli 15 to 40% of E. coli IV. On the other hand at a faster growth rate (0.125/hr) the wild type distinctly predominated over mutant E. coli 15 in a ratio of E. coli IV, 75%, to E. coli 15, 25%.

It was interesting to note that at the higher dilution rate (0.125/hr) before the total number of organisms achieved a steady state level, the wild type increased in the chemostat while the number of mutants decreased. Once the total number of organisms reached a steady state in the chemostat, both the organisms achieved a steady state level. Wild type E. coli IVc did not completely predominate nor did the mutant dilute out as time progressed. Similarly at the lower dilution rate, a larger percentage of mutant (60%) and lower percentage of wild type (40%) remained almost constant after achieving a steady state level with respect to total number of organisms in the chemostat. The mutant did not increase in numbers and wild type did not decrease in numbers as time progressed. This phenomenon cannot be explained fully on the basis of existing knowledge. One of the reasons for this behavior could be the partial inhibition of the wild type by the mutant due to production of a toxic metabolite in the chemostat. This toxic metabolite would not accumulate in the chemostat since it was continuously diluted out. Therefore, possibly it could not inhibit a larger percentage of the wild type. Another reason for the above phenomenon could be the production

of an intermediate metabolite by the wild type that can be utilized by the mutant to increase its specific growth rate. Furthermore it was interesting to note that the total number of organisms was higher in the chemostat at the lower dilution rate (2.29×10^9 cells/ml) than at the higher dilution rate (1.63×10^9 cells/ml). This observation points out that the concentration of substrate in the chemostat was much less at the low dilution rate. Lower concentration of substrate favors the growth of the mutant over the wild type in the mixed population. The mutant has a lower K_s and thus can grow more rapidly than the wild type at lower substrate concentration. Conversely at the higher dilution rate, high concentration of substrate may favor the growth of the wild type over the mutant in the mixed population. If the continuous flow system could have been run at a dilution rate lower than 0.05/hr, it might have been possible to achieve a larger percentage of mutant E. coli 15 in a mixed population (> 60%). This was not possible in this study due to the limitations of the pump employed.

It was observed by Gaudy et al. (1967) in a continuous flow system that, as the dilution rate was increased above the critical value in a mixed microbial population, complete dilute out of the culture was not observed but the system apparently selected for organisms with inherently faster specific growth rates. Gaudy and Srinivasaraghavan (1974) working with mixed cultures showed that such cultures grown in either a once-through chemostat or a cell recycle system have yields determined by the specific growth rate at which they were growing. This observation has been made by a number of investigators (Abbott and Clamen, 1973; Schulze and Lipe, 1964; Herbert, 1958). Similar results were found in this study. At the higher dilution rate (0.125/hr) the

average yield of the mixed population was 0.46 mg/mg and the average yield of the mixed population at the lower dilution rate was 0.39 mg/mg.

A unique feature of the study by Gaudy and Srinivasaraghavan (1974) was the investigation of yield in batch culture using an inoculation from the continuous flow reactors operated at various specific growth rates. They found that the yield in the batch experiment was the same as the yield in the continuous flow system from which the inoculum was taken even though the specific growth rates were much higher in the batch flasks. This experiment proved to be repeatable and was explained on the basis of natural selection. Species with different yields were postulated to predominate at different specific growth rates.

Jones (1974) performed similar experiments using a pure culture of E. intermedia. A once-through chemostat was used to grow a continuous culture of the organism. Batch flasks inoculated from the chemostat operated at a dilution rate of either 1/6 hr^{-1} or 1/24 hr^{-1} presented unexpected results. At each chemostat dilution rate, the corresponding batch reactors resulted in a yield nearly identical to that in the chemostat. The yields in batch flasks inoculated from a chemostat with $D = 1/6\ hr^{-1}$ averaged 0.38 mg/mg as compared to the identical average from chemostat data. Similarly the yield from the batch flasks inoculated with cells grown at $D = 1/24\ hr^{-1}$ averaged 0.33 mg/mg as compared to 0.32 mg/mg in the chemostat. For mixed cultures, this effect can be explained by the selection of low yield species. Selection of species could not occur using a pure culture, although it is conceivable yield mutants may have arisen during the course of the experiment in the chemostat. Hughes (1955) showed that within a given

pure culture there were cells with inheritable differences in growth rates.

Results were obtained in the present study using a mixed population of mutant E. coli 15 and wild type E. coli IV in experiments similar to those done by Jones (1974) with the pure culture. At the end of each run of the continuous flow system at the low dilution rate (0.05/hr) where the mutant predominated over the wild type (by 60% to 40%) and at the higher dilution rate (0.125/hr) where the wild type predominated over the mutant (by 75% to 25%), series of batch flasks were inoculated. Average yield in the chemostat at a dilution rate of 0.05/hr was 0.39 mg/mg and average yield of these cells in a batch flask was only 0.27 mg/mg with a maximum specific growth rate and yield identical to that of the mutant E. coli 15 in pure culture. The average yield in the chemostat at a dilution rate of 0.125/hr was 0.46 mg/mg and the average yield of cells from this chemostat culture in a batch flask was only 0.39 mg/mg with a maximum specific growth rate of 0.37/hr. In both cases the mixed population in batch was inoculated into glucose minimal medium containing excess substrate (1000 mg/l glucose). It was expected that, due to excess substrate in batch, the faster growing wild type would predominate and grow with near maximum specific growth rate. One explanation for the low yield and the low specific growth rate of the mixed popualtion in batch could be that the yield and slow growth rate characteristics are programmed into the cells by the comparatively low dilution rates employed in the chemostat. Another reason for the low yield and slow specific growth rate in batch of the mixed population might be the inhibition of the wild type by the mutant E. coli 15. Results obtained by performing batch experiments with a

mixed population containing different proportions of wild type and mutant, not previously grown in continuous culture, supported the hypothesis that mutant E. coli 15 inhibits the growth of wild type E. coli IV.

Since E. coli 15 has a very low yield it apparently utilizes the substrate inefficiently. This could be due to a defect in the production of energy from the substrate. It might also be due to an effective uncoupling between energy-yielding metabolism and the energy-consuming reactions. A third possibility is incomplete oxidation of the substrate, leading to excretion of metabolic intermediates.

Inhibition of growth of E. coli IV by the mutant E. coli 15 in a mixed population was observed in nutrient broth and nutrient broth with yeast extract. In both media specific growth rates and yields of the mixed population were near the yield values and specific growth rates of a pure culture of E. coli 15 in corresponding media. Therefore, the effect of the mutant upon the wild type was not specifically related to glucose metabolism.

Inhibition of E. coli IV by E. coli 15 may have been caused either by production of a toxic metabolite or by competitive utilization of substrate by mutant E. coli 15 that inhibited and slowed down the growth of the wild type. Harrison and Lawrence (1963) observed, when starvation resistant mutants were mixed with the wild type in spent medium, that starvation resistant mutants grew at the expense of the wild type simply due to more successful competition for cell lysis products by mutants. Results obtained in this study (Table XV) indicate that inhibition of wild type by the mutant might be due to production

of one or more toxic metabolites. A large difference in specific growth rates in control (0.69/hr) and in test (0.38/hr) media was observed, but the values of yields in both the media were similar. It was difficult to conclude definitely since yields in test and control media were close. A definite conclusion for the mechanism of inhibition requires further work. In any case, the effect on growth rate of the wild type cannot be wholly attributed to competition for substrate, since it occurs in the absence of mutant cells and can be caused by addition of spent medium from a mutant culture. The lesser magnitude of the effect of spent medium, as compared to the presence of the actual cells could indicate that: (1) both competition for substrate and production of inhibitory metabolites occur; or (2) the inhibitory material is unstable and must be continuously produced for maximum effect; or (3) the inhibitory material was not present at maximum concentration at the time the spent medium was harvested.

CHAPTER V

SUMMARY AND CONCLUSIONS

Isolation of slow growth rate mutants of E. coli was possible using the method described by Harrison and Lawrence (1963). The mutant used in this study had a slower maximum specific growth rate, lower yield and smaller colony size than the wild type. The slow growth rate mutant was also starvation resistant in buffer and distilled water.

Experimental results obtained in this study, i.e., that organisms with a slow growth rate have low yield, correlate well with the work of Jones (1974) with a pure culture of E. intermedia and the work of Gaudy and Srinivasaraghavan (1974) with mixed microbial populations. More importantly, the results of the present study offer evidence in support of a possible explanation of the data obtained by these investigators. Evidence of selective pressure exerted by dilution rate on a mixed population of wild type and mutant in a continuous flow system was presented. The higher dilution rate selected greater numbers of the high yield, fast growth rate organism in the mixed population, while the slow dilution rate selected for a greater proportion of the low yield, slow growth rate organism. This experimental result correlates well with the theory proposed by Jones (1974) for his pure culture and by Gaudy and Shrinivasaraghavan (1974) for mixed microbial cultures.

The growth rates and yield values of a mixed population of mutant and wild type in proportions of $\geq$ 60% and $\leq$ 40%, respectively, in batch

with excess substrate were similar to the values for the mutant in pure culture. Experimental results in this study ascertained that the mutant inhibited the growth of the wild type in a mixed population. The inhibition of growth of wild type by the mutant in mixed culture may be due to either or both of the following:

1. Successful competition for nutrients by the mutant in the mixed population.
2. Production by the mutant of metabolites toxic to the wild type.

Experimental results lean more toward the second explanation for inhibition of growth of the wild type in mixed culture. Still, to allow a definite conclusion on the mechanism of inhibition more work is needed.

The site of the mutation in the mutant was not ascertained. However, several possible explanations were suggested in an attempt to account for the characteristics of the mutant, i.e., low yield, slow growth rate, and ability to survive for a considerable time in starving conditions.

1. The mutant may be defective or inefficient in generalized transport mechanisms.
2. There may be effective uncoupling between energy-yielding metabolism and the energy-consuming reactions.
3. There may be a defect in the production of energy from substrate.
4. There may be a mutation at some point affecting translation or transcription.

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VITA 2

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