CHARACTERIZATION OF A *Rel A* MUTANT OF *Escherichia coli*

By

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Dedicated to my Father and Mother

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Escherichia coli

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CHAPTER I

LITERATURE REVIEW

During the removal of organic material from wastewater in a biological treatment process, the interactions between the microbial flora are important. However, before fully understanding these interactions, the single species and their characteristics must be known. Also, since the efficiency of a process is affected not only by its design and operation but also by the growth characteristics of the individual components of the biomass, an investigation into these characteristics should help in understanding operational performance.

The relationship between the specific growth rate (μ) of an organism and the concentration of the growth-limiting substrate (S) was first introduced by Monod (1942). The specific growth rate was defined as the rate of increase per unit of organism concentration $1/X(dX/dt)$. This was shown to be proportional to the substrate concentration, up to a limiting saturation value, as presented in the Monod equation:

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

μ_m is the maximum value of the specific growth rate; i.e., further increases in initial substrate concentration cause no further increase in μ. K_s is the substrate concentration at which $\mu = 1/2\ \mu_m$. In a given set of conditions for a particular bacterial strain, μ_m and K_s

are constant. The equation has been shown to fit experimental data for both pure and heterogeneous populations (Gaudy et al., 1967).

Monod also developed the concept of substrate-based yields. He showed the relationship between growth and utilization of substrate from which he developed the yield constant, which is defined over any finite growth period as

$$Y = \frac{\text{dry weight of bacteria produced}}{\text{weight of substrate utilized}}$$

Postgate and Hunter (1962) showed that as the specific growth rate increased cell yield also increased, and vice versa. This has also been shown by Herbert (1958) and Schulze and Lipe (1964). The relationship has been shown with mixed cultures in once-through chemostats, batch, and cell recycle systems and also with pure cultures in the same systems. The explanation for this phenomenon has been centered around the concepts of endogenous metabolism and maintenance.

Monod (1942) proposed that theoretically there should be a minimum substrate concentration which would allow only survival or maintenance of cells. Evidence was presented in support of this theory by Dawes and Ribbons (1964) and McGrew and Mallette (1961). The current concept of maintenance energy is that, in the absence of exogenous substrate, an endogenous substrate must be present to provide energy for maintenance. After exhaustion of the endogenous substrate, if no exogenous energy source is present, the cell dies. The cellular processes which require maintenance energy are those involved with synthesis of unstable macromolecules, osmotic regulation, maintenance of a constant intracellular pH, and motility.

Gaudy and Srinivasaraghavan (1974) discovered with a heterogeneous population that growth in a cell recycle system at very low specific growth rates resulted in yields that increased when the specific growth rate was increased. Yet, when the recycle was changed into a once-through system with higher specific growth rates, the yield remained the same as in the recycle system. Even when a batch system was inoculated from the cell recycle system, the yield remained the same. They proposed that a selection of species had occurred which resulted in a low yield population replacing the higher yield species.

Jones (1974) showed that growth of a pure culture of Escherichia coli in a chemostat at different dilution rates affected the yield characteristics of the cells. Cells from the different dilution rates were shown to have two different yield values which remained the same when the cells were inoculated into batch flasks with excess substrate. Under these conditions, according to prevalent theory, μ_m should be obtained and the true yield should be observed. Jones proposed several possible explanations of the unexpected effect of previous growth rate on yield, one of which was selection of a mutant with a lower growth rate. He isolated a mutant of this type by subjecting the culture to starvation.

Damani (1977), using the same procedure, isolated a number of slow growth rate, low yield mutants of Escherichia coli and selected one particularly stable mutant for further study. The mutant had a low growth rate and low yield as compared to the wild type in both complex and defined media. Its K_s for glucose was also lower than that of the wild type. It was starvation-resistant in normal saline and distilled water, and it predominated over the wild type when grown in mixed

culture in a continuous flow system at a slow dilution rate. It was also observed that the mutant inhibited the growth of the wild type in mixed culture in batch. It was further shown that the medium in which mutant cells had been grown decreased both the yield and the specific growth rate of the wild type.

The present work was undertaken for the purpose of further characterizing the mutant studied by Damani by determining the nature of the mutation and the reason for the inhibition of the wild type by medium in which the mutant had been grown. The fact that both the growth rate and the yield of the mutant were affected suggested that the mutation was one which affected efficiency of substrate utilization. Such a mutation might involve a defect in energy-yielding reactions or a loss of control of one or more biosynthetic pathways. The latter possibility seemed more likely in view of the inhibition of the wild type by the culture fluid of the mutant, since loss of control might lead to excretion of a biosynthetic product accumulated by the cell. One such product which may be produced in excess and excreted in the absence of the usual metabolic controls would be an amino acid. Certain amino acids are known to be inhibitory to some strains of E. coli and of several other bacteria.

The fact that amino acids can inhibit growth was first shown by Gordon and McLeod (1926). Later, Gladstone (1939) showed that strains of Bacillus anthracis did not grow when low concentrations of either leucine or valine were added to the medium. However, a small amount of leucine would cancel the effect of valine, and vice versa.

Porter and Meyers (1945) described the inhibition of Proteus morganii by threonine, norvaline, or norleucine. Several amino acids

prevented the effects of threonine and norvaline, but only methionine prevented the effects of norleucine.

Beerstecher and Shive (1946) reported that growth of an E. coli strain was inhibited by tyrosine, and that phenylalanine reversed this effect. They proposed that tyrosine prevented the biosynthesis, rather than the utilization, of phenylalanine. Thus, tyrosine controlled the biosynthesis of phenylalanine and related metabolites.

The inhibition of one strain of E. coli by D-serine was described by Davis and Maas (1949); this effect was reversed by glycine or alanine and to lesser degrees, by histidine and leucine.

Tatum (1949) showed that isoleucine could reverse the bacteriostatic effect of minimal media containing valine with E. coli K-12.

The growth of Streptococcus bovis in a minimal medium plus arginine could be inhibited by adding small amounts of isoleucine and larger amounts of leucine, threonine and norleucine. This was reported by Washburn and Niven (1948). The inhibition produced by these amino acids could be reversed by addition of valine, glutamic acid, methionine and cystine, respectively. They also showed that equal amounts of phenylalanine and tyrosine inhibited the growth of S. bovis, but did not produce this effect when added singly. The inhibition could be reversed by using small amounts of tryptophan, glutamic acid, or cysteine. Thus, it was proposed that isoleucine, leucine, threonine and norleucine stop the synthesis of valine while tyrosine and phenylalanine block the synthesis of tryptophan. Either block prevents the reproduction of the organism.

Rowley (1953a) found, with a strain of E. coli K-12 placed in a minimal medium, that no growth occurred with the addition of DL-valine

or L-valine. This inhibition of growth was reversed with the addition of DL-threonine or DL-leucine. He also showed that the inhibitory effect was reversed by nutrient broth.

Later, Rowley (1953b) did a very complete study of 356 strains of E. coli for inhibition of growth by various amino acids. All inhibitions due to amino acid which he found could be reversed by addition of other amino acids. He proposed that with an addition of valine to a culture of E. coli, there was an accumulation of the precursor keto-valine and that this prevents the conversion of keto-isoleucine to isoleucine.

Ramakrishnan and Adelberg (1964) isolated valine-resistant strains of E. coli K-12. Conjugation experiments showed that the valine-resistant locus is closely linked to genes controlling isoleucine-valine biosynthesis. The mutation from valine sensitivity to valine resistance altered an operator locus causing the depression of threonine deaminase, dihydroxy acid dehydrase and transaminase B. It was also noted that valine-resistant strains excreted excess isoleucine into the medium. This suggests that one of the depressed enzymes is rate-limiting for the synthesis of isoleucine, but not for valine in E. coli K-12.

Guardiola and Iaccarino (1971) reported on a mutant strain of E. coli K-12 that showed resistance to valine inhibition. This was due to a mutation in the active transport of valine. Thus, the amino acid did not enter the cell. They identified two loci on the chromosome, brn Q and brn P, which caused this effect.

Recently, DeFelice et al. (1979) reviewed antagonistic effects between related amino acids in E. coli. Studying valine in detail,

they proposed that the phenomenon of valine inhibition is due to regulation of the biosynthesis of branched chain amino acids. They believed that organisms have not selected biological functions to bypass this antagonism because those conditions, i.e., the presence of a severe excess of one amino acid and not of its relatives, do not normally occur in nature.

Szentirmai and Umbarger (1968) stated that amino acids may play an inhibitory role in one of four ways: "by interfering with the transport of amino acids into the cell; by mimicking the effect of natural amino acids as an endproduct inhibitor; by preventing formation of enzymes necessary for the formation of the natural amino acids; or by interfering with its incorporation into protein."

Not only are certain individual amino acids inhibitory to growth, but various types of small peptides may also play a similar role. Simmonds et al. (1951) first showed that the peptide glycyl-leucine was inhibitory with strains of E. coli. Later, Meislir and Simmonds (1963) showed that aged cultures were more susceptible to the peptide than were young cultures, because the peptide was hydrolyzed more slowly in older cultures, even though uptake was the same.

Von der Haar and Umbarger (1972) showed that inhibition of E. coli K-12 by the dipeptide glycyl-L-leucine could be reversed by isoleucine and by intermediates between threonine and isoleucine in the biosynthetic pathway. Specifically, the glycyl-L-leucine inhibits threonine deaminase by binding at the isoleucine site on the enzyme, reducing the growth rate of the cells.

The synthesis of amino acids in a wild type strain of E. coli growing in minimal medium must proceed in an orderly and balanced manner if

growth is to take place at the maximum rate of which the organism is capable under the conditions imposed upon it. The synthesis of protein depends upon the availability of all the required amino acids, and the synthesis of RNA, upon which protein synthesis depends, is also linked to the synthesis of protein. If an amino acid required for protein synthesis is not available, protein synthesis ceases and the synthesis of transfer and ribosomal RNA also ceases. Strains which exhibit this control of RNA synthesis by protein synthesis are called stringent to distinguish them from mutants lacking such control. Relaxed mutants continue to synthesize tRNA and rRNA even though protein synthesis is prevented by lack of an essential amino acid.

Since the isolation of relaxed mutants occurred (Fill and Friesen, 1968), many investigations have probed into the mechanism by which protein synthesis is coupled to stable RNA synthesis, but it has not been elucidated. One problem encountered during these investigations had been that of testing for the relaxed response. A rather tedious procedure of assaying for nucleoside incorporation into RNA was required. However, Uzan and Danchin (1976) developed a procedure for quick identification of the Rel A mutant. They used a minimal medium that was supplemented with serine, methionine, and glycine, which permits growth of the Rel A^+ phenotype but not that of the Rel A^-. The theory behind this procedure has yet to be uncovered.

When this investigation was initiated, it was realized that the identification of the nature of the genetic lesion of the mutant might be quite difficult, especially since its long-term stability, even under conditions highly selective for reversion, indicated that it had suffered either a multisite deletion or multiple mutations. The

explanation of the basis of the inhibition of the wild type parent by the mutant also promised to be difficult since no clues were available from previous work with the mutant. However, it was decided that the investigation should be undertaken because of the possible significance in both basic and applied use of continuous flow growth conditions of the selection of such mutants. The approach decided upon was one of testing as many as possible of the hypotheses which could be proposed to account for the observed characteristics of the mutant. The experimentation reported herein has not resulted in solution of either of the problems toward which it was directed. However, a number of logical explanations have been eliminated. Also, one genetic difference between the mutant and wild type has been identified.

CHAPTER II

MATERIALS AND METHODS

The Organisms

The strain of E. coli used was isolated from mixed liquor taken from the activated sludge sewage treatment plant in Oklahoma City, Oklahoma, by Jones (1977). The wild type was designated as E. coli IV. The mutant, E. coli 15, was isolated by Damani (1977) from serial dilutions of the wild type subjected to starvation conditions in 0.85 percent saline.

Biochemical Tests

In order to check for purity of the test organisms and to determine possible differences between mutant and wild type, various biochemical tests were performed. Glucose, lactose, nitrate, citrate, and tryptone broths were used. Bromthymol blue was used as the pH indicator for the fermentation tests. Glucose, lactose, and nitrate broths included Durham tubes for determination of gas. Litmus milk and methyl red-Voges Proskauer (MRVP) media were also used. Incubations were at 37^{o}, and readings were taken at 24 and 48 hours except the nitrate broth, litmus milk and the methyl red portion of the MRVP test, which were red at 5, 2 and 5, and 5 days, respectively. In addition, the Gram strain and microscopic observations were performed. The organisms were also streaked on eosin methylene blue (EMB) lactose agar (Difco)

to check for the characteristic green sheen.

Media

Minimal medium was used through the entire investigation for growth studies. Composition of the medium, per liter of solution, was glucose, 1.0 gm; $(NH_4)_2SO_4$, 0.5 gm; $MgSO_4 \cdot 7H_2O$, 0.2 gm; $FeCl_3 \cdot 6H_2O$, 0.5 mg; $CaCl_2 \cdot 2H_2O$, 7.5 mg; KH_2PO_4, 3.0 gm; Na_2HPO_4, 6.0 gm; yeast extract, 1.0 mg; tap water, 100 ml. All ingredients of the medium were autoclaved separately, cooled, and mixed aseptically.

EMB-lactose agar plates and tryptic soy aga plates were prepared by rehydrating the media (Difco) with distilled water.

Cultivation of the Test Organisms

Each organism was streaked on TSA and incubated for 24 hours at 37^{o}. Two TSA slants were inoculated and incubated for 24 hours at 37^{o}; one was stored at 4^{o} and the other at room temperature.

Batch Studies

Batch experiments were performed to determine specific growth rates of the organisms with different additions to the minimal medium. To measure the growth rate, cells were grown for 24 hours in fresh medium at 37^{o}. Then, 0.1 ml of the cell suspension was inoculated into fresh medium of the same type, with or without added components. Growth was followed on the Coleman Junior Spectophotometer at 540 mm. The specific growth rate was determined by plotting absorbance versus time on semilogarithmic paper. The specific growth rate, μ, was calculated from this graph, using the equation $= 0.693/t_d$, where t_d is the

doubling time. This equation was derived from $dx/dt = \mu X$, which is the form of a first order differential equation. X represents the concentration of cells, while t_d, the doubling time, is determined from the semilogarithmic plot and substituted into the equation. μ is then defined as $\ln 2/t_d = 0.693/t_d$. The batch experiments were performed in 250 ml-sidearm flasks using 20 ml of medium. These were incubated at 37° in a Warner-Chilcott shaking water batch. Absorbance was measured at short time intervals.

Inhibition Studies

Inhibition studies using supernatants of batch organisms were performed as follows: Cells were grown for 24 hours; the culture was then centrifuged in a Sorvall Superspeed Centrifuge Type 88-1 at 3100 rpm for ten minutes. The supernatant was filtered, using a Millipore filter apparatus with a filter pore size of 0.45 μm; ten ml of fresh medium were then added to ten ml of supernatant plus 0.25 ml of a two-percent solution of glucose in a sidearm flask; 0.1 ml of the appropriate cells was then added from a 24-hour culture.

CHAPTER III

EXPERIMENTAL RESULTS

As stated previously, two major lines of investigation were pursued: 1) the identification of genetic differences between the wild type and the mutant, and 2) the basis of the inhibitory effect of the mutant upon the wild type. These will be described separately although both lines of investigation were pursued simultaneiously, and experiments in one category suggested experiments in the other.

Comparison of Mutant and Wild Type Strains

To determine whether the two strains, which had been studied by Damani (1977) were stable with regard to specific growth rate, this characteristic was checked by the method described previously, using glucose minimal medium. The specific growth rates reported by Damani for E. coli IV (wild type) and E. coli 15 (mutant) in glucose minimal medium were 0.71 and 0.27 hr^{-1}, respectively. The growth rate constants were found to be quite stable. The values obtained were 0.69 and 0.27 hr^{-1}, respectively.

Anaerobic Growth Rates

A decrease in both growth rate and yield suggests a decreased efficiency of utilization of the carbon and energy source. All previous determinations of growth rate for E. coli IV and 15 had been made under

aerobic conditions. A defect in the coupling of ATP synthesis to electron transport, such as occurs in unc mutants, could account for a decreased efficiency of energy source metabolism. If this were the explanation for the difference in growth rate between the mutant and wild type, it should affect only aerobic growth because ATP synthesis under anaerobic conditions occurs only by substrate level phosphorylation. Therefore, both cultures were grown under anaerobic conditions to determine their growth rates. A screw-cap tube was filled with nutrient broth containing 10 percent glucose. This was placed in a boiling water bath for ten minutes after which it was cooled rapidly under running water, and 0.1 ml of cells was added. The tubes were incubated in a water bath at 37^{o}. The results, shown in Figure 1, indicate that the cause of the low growth rate of the mutant is not a defect in electron transport or its coupling to ATP synthesis. Under anaerobic conditions, as under aerobic conditions, the specific growth rate of the mutant ($0.33\ hr^{-1}$) was significantly lower than that of the wild type ($0.57\ hr^{-1}$).

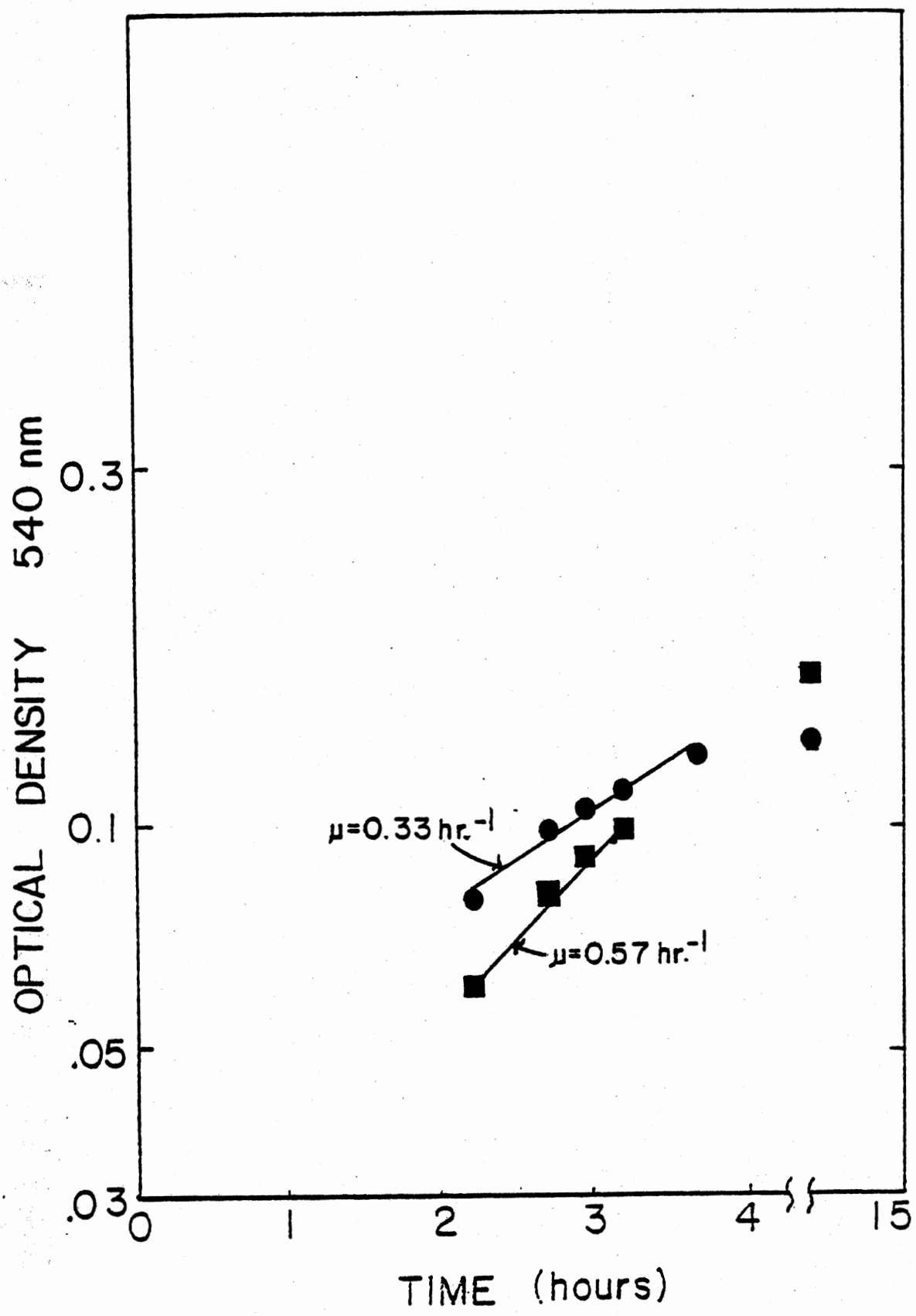
Biochemical Reactions

The standard biochemical tests used in the identification of E. coli were performed upon both the wild type and the mutant to determine whether any differences could be detected. The results of these tests are shown in Table I. No difference was found except that the intensity of the color produced in the test for indole production was visibly less for the mutant than for the wild type. Since indole production has no relation to biosynthetic reactions or energy metabolism, this apparent difference in production of tryptophanase was not

Figure 1. The Specific Growth Rate of *E. coli* IV and *E. coli* 15 When Grown Anaerobically

The medium was nutrient broth plus one percent glucose.

● *E. coli* IV
■ *E. coli* 15


OPTICAL DENSITY 540 nm
0.3
0.1
.05
.03
0
1
2
3
4
15
TIME (hours)
μ=0.33 hr.⁻¹
μ=0.57 hr.⁻¹

considered to be a possible primary cause of the lower growth rate of the mutant.

TABLE I

RESULTS OF STANDARD BIOCHEMICAL TESTS USED IN THE IDENTIFICATION OF *E. coli*

Test	Result	
	E. coli IV	*E. coli* 15
Glucose utilization	acid, gas	acid, gas
Lactose utilization	acid, gas	acid, gas
Nitrate reduction	positive to nitrite	positive to nitrite
Citrate as sole carbon source	negative	negative
Indole production	positive	positive
Voges-Proskauer	negative	negative
Litmus milk	acid curd, slight reduction	acid curd, slight reduction
Gram stain	negative	negative
Cell morphology	short single rods	short single rods

Utilization of Carbon and Energy Sources

The two strains were tested for differences in ability to use a wide variety of compounds as carbon and energy source by the auxanographic method. Minimal medium containing 1.5 percent agar and no source of carbon and energy was inoculated with either *E. coli* IV or

E. coli 15 and poured into Petri plates. The plates were marked in quadrants, and a small amount of each compound to be tested was placed on the surface of the agar in one quadrant. Plates were incubated for 24 hours and were examined for growth in the areas to which the carbon and energy sources had been applied. The results, shown in Table II, detected no difference in catabolic capabilities between the mutant and the wild type.

Serological Tests

Bacto-E. coli Antisera (Difco) are used in the serological identification of strains of E. coli. Strains IV and 15 were tested against somatic, capsular and flagellar antisera by mixing cells and antisera on glass slides and examining for agglutination. Although the antisera identify numerous strains, particularly those associated with intestinal diseases, the antisera do not identify all strains. The strains used in this study did not react with any of the antisera available from Difco.

Control of RNA Synthesis

Possible differences between the mutant and wild type in amino acid metabolism, to be described below, suggested that protein synthesis and therefore RNA synthesis, might be affected differently in the mutant and the wild type. Since the Rel A gene is coupled to RNA synthesis, it seemed possible that a Rel A mutant might exhibit slower growth rates. Hence, strains IV and 15 were subjected to a test for the detection of the Rel A mutation.

Glucose minimal medium was supplemented with 100 mg/ml each of

TABLE II

GROWTH ON CARBON AND ENERGY SOURCES

	E. coli IV	*E. coli* 15
Acetate	+	+
L-arabinose	+	+
Adonitol	-	-
Dulcitol	-	-
D-fructose	+	+
Galactose	+	+
Gluconate	+	+
Malonic acid	+	+
Maltose	+	+
D-mannitol	-	-
D-mannose	+	+
Melibiose	+	+
Melizitose	+	+
Rhamnose	-	-
Ribose	-	-
Raffinose	+	+
Salicin	-	-
D-sorbitol	-	-
L-sorbose	-	-
Succinate	+	+
Trehalose	+	+
D-xylose	+	+
Inulin	-	-
Inositol	-	-
Tryptophan	+	+
Glutamic acid	-	-
Lysine	+	+
Serine	-	-
Phenylalanine	+	+
Leucine	+	+

Key to symbols: (-), no growth
(+), growth

serine, methionine, and glycine, according to Uzan and Danchin (1976). The organisms were streaked on the medium. A Rel A mutant will not grow while normal, Rel A^+ strains will grow. Strain 15 did not grow, while IV did. A second strain of E. coli, a fresh isolage from sewage, also grew. Thus, strain 15 does have a mutation at the Rel A locus.

Inhibition of the Wild Type by the Mutant

Damani (1977) showed that the mutant, E. coli 15, apparently slowed the growth of the wild type, E. coli IV, when the two were grown in mixed culture. This conclusion was based upon the fact that in both batch and continuous flow cultures, the mutant was not overgrown by the wild type as would be expected from the relative specific growth rates of the two strains. Damani further showed that the spent medium of the mutant inhibited growth of the wild type. Before experiments were undertaken to investigate the basis of this inhibition, the experiments performed by Damani were repeated to determine whether the inhibitory relationship between mutant and wild type was stable.

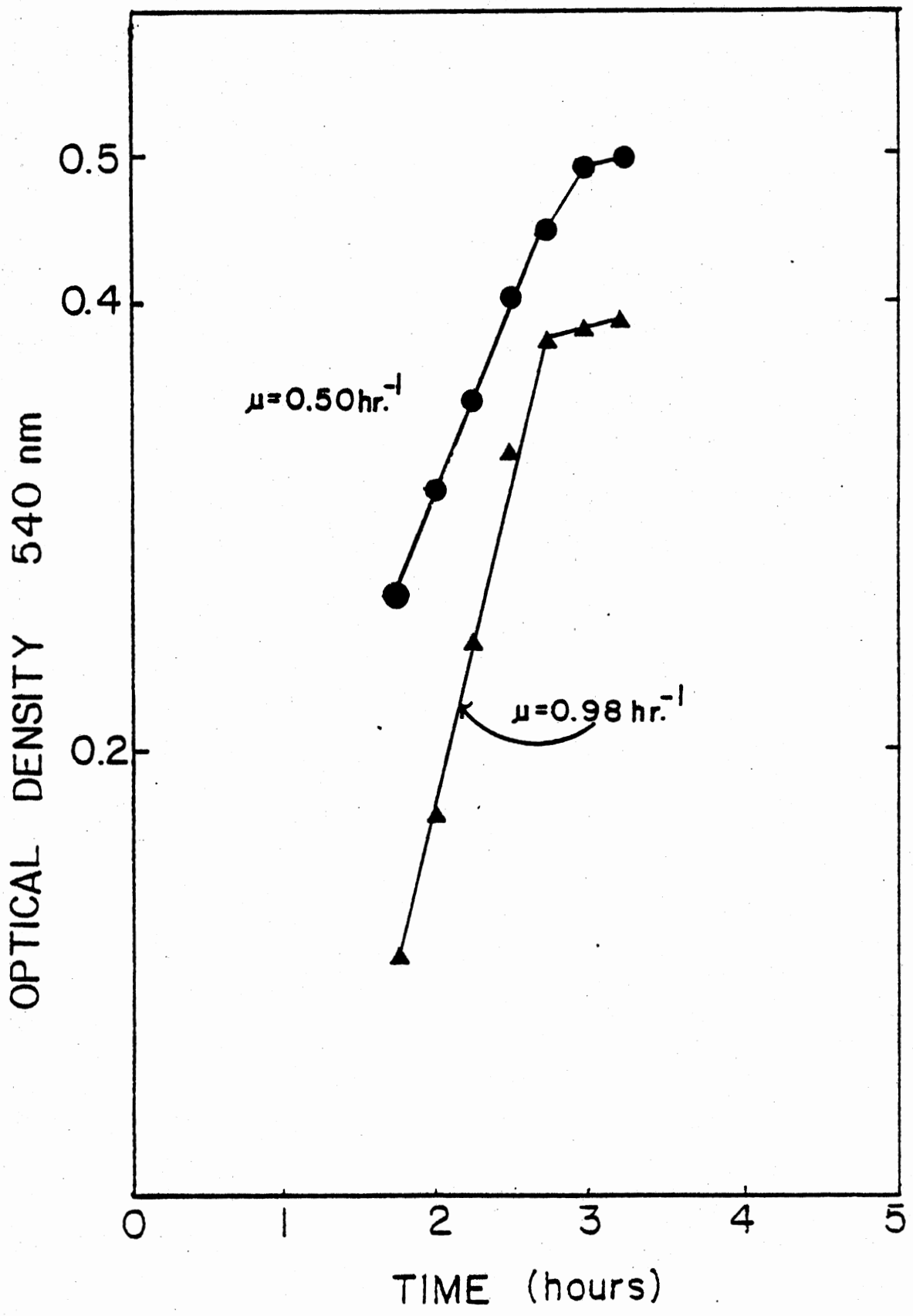
When 10 ml of the supernatant from a culture of the mutant were added to 10 ml of fresh medium plus 0.25 ml of a 2.0 percent glucose solution and inoculated with the wild type strain, the specific growth rate of the wild type was found to be lower than that of the wild type inoculated into 20 ml of fresh medium. Specific growth rates were 0.50 hr^{-1} (Figure 2), and 0.69 hr^{-1}, respectively.

If the wild type supernatant was treated identically and added to the fresh medium with wild type cells, an increase in growth rate (μ = 0.98 hr^{-1}) occurred (Figure 2). Values of specific growth rate observed by Damani were 0.38 hr^{-1} for wild type cells growing in the presence

Figure 2. The Specific Growth Rate of *E. coli* IV With the Addition of Supernatants From *E. coli* 15 and *E. coli* IV

The medium was 10 ml of fresh glucose minimal medium plus 0.25 ml of a two percent glucose solution plus 10 ml of the mutant supernatant.

▲ *E. coli* IV supernatant + *E. coli* 15 cells
● *E. coli* 15 supernatant + *E. coli* IV cells


0.5
0.4
0.2
OPTICAL DENSITY 540 nm
μ=0.50hr.⁻¹
μ=0.98 hr.⁻¹
0
1
2
3
4
5
TIME (hours)

of spent medium from the mutant, and 0.69 hr^{-1} for wild type cells in medium containing supernatant from the same strain.

pH of the Spent Medium

Since the pH of the medium exerts a significant effect upon growth rates, a rather obvious possible explanation for the effect of the spent medium of the mutant upon the growth rate of the wild type was excessive production of acids by the mutant. Both strains were grown in glucose minimal medium and the pH of both spent media was checked. The pH of both was 7.0. Therefore, even if one produced more acids than did the other, the buffer in the medium was sufficient to control the pH and the effect of the mutant upon the wild type could not be explained as a pH effect.

Dialysis of Mutant Supernatant

While the relative growth rates of the mutant and wild type treated with culture supernatants were similar to those observed by Damani, the magnitudes of the effects were not the same. Damani reported a 47 percent decrease in the growth rate of the wild type in the presence of spent medium from the mutant, while only a 28 percent decrease was observed when the experiment was repeated. In Damani's work, no effect of homologous supernatant upon the wild type was observed, while in the present investigation, a 42 percent stimulation of growth rate was observed. It seemed possible that these differences might be due to differences in times of harvesting the culture medium, and it also seemed possible that the material inhibitory to the wild type might reach a maximum concentration at some point during the growth cycle of

the mutant. An experiment was designed to measure the inhibitory effect of the mutant culture throughout its growth cycle by a disc assay method similar to that used for antibiotics. One and one-half percent agar plates were seeded with the wild type organism by placing 0.1 ml of a 24-hour culture on the plate, followed by spreading with a glass rod until the entire surface was covered. A 6.0 mm-diameter disc, soaked in the mutant supernatant was then placed in the center of the plate. Plates were incubated for 24 hours at 37^{o} and observed for zones of inhibition every six hours. As a control, plates seeded with mutant cells were exposed to discs soaked in supernatant from a wild type culture. No inhibition was observed on any of the plates.

The lack of inhibition in the disc assay could have been due to insufficient concentration of the inhibitory material. Therefore, culture supernatants were concentrated by removal of water with Aquacide I (Calbiochem). Dialysis tubing (A. H. Thomas, 12,000 MW minimum retention) containing 20 ml of culture supernatant was covered with Aquacide I and placed in the cold room at $4^{o}C$ for approximately three hours.

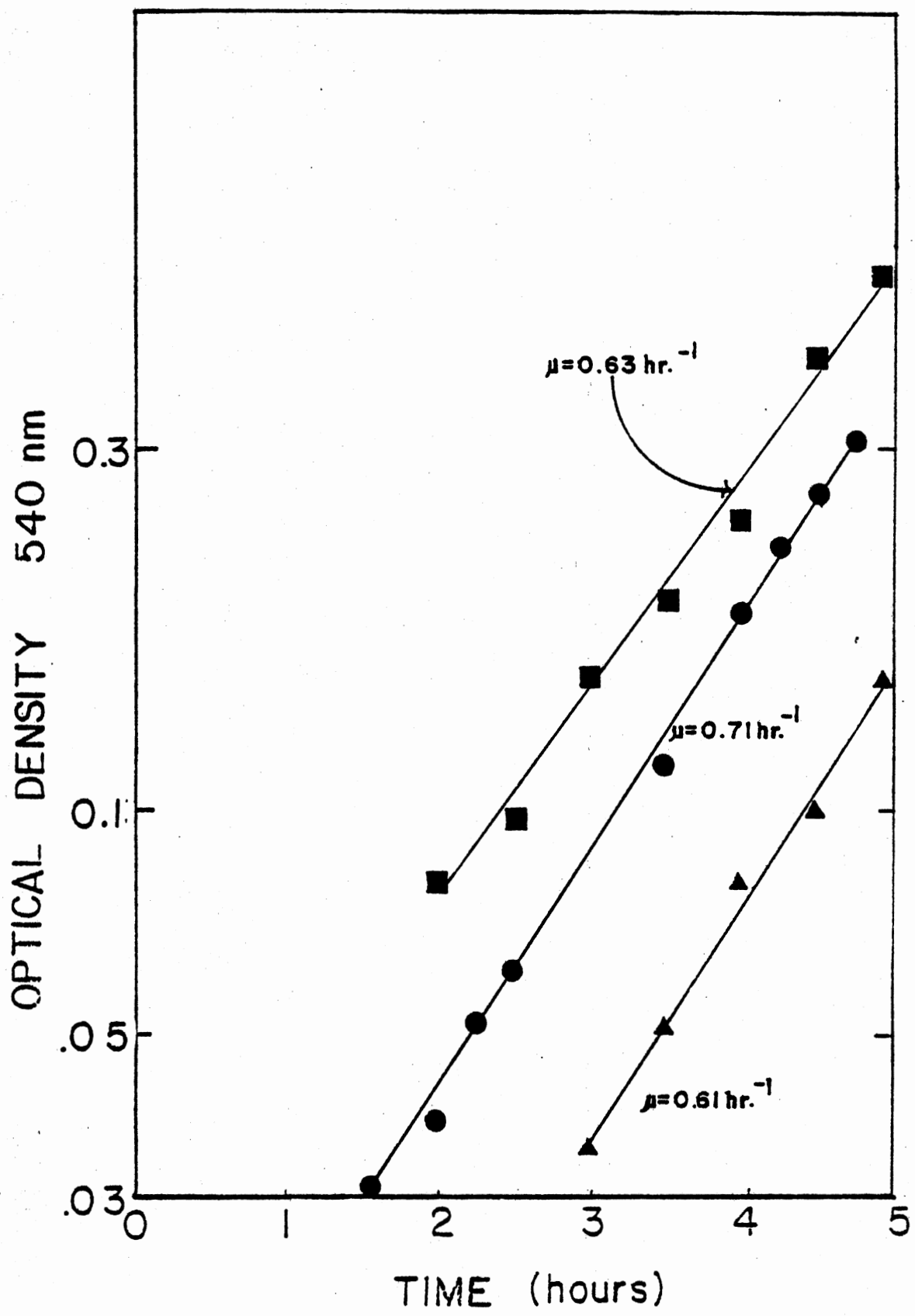
Since small molecules (less than 12,000 MW) would be removed from solution along with the water, the procedure of concentration by dialysis against Aquacide also offered an opportunity to determine whether the inhibitor was of greater or less than 12,000 MW.

Ten ml of dialyzed and concentrated supernatant were added to 10 ml of fresh glucose minimal medium along with 0.25 ml of a two-percent glucose solution. The effect of dialyzed supernatant upon growth rate of the wild type cells is shown in Figure 3. The results were somewhat inconclusive. Dialyzed supernatant from a mutant culture reduced the growth rate constant of the wild type to 0.61 hr^{-1}--less than the

Figure 3. The Effect on Growth Rate of Addition of Dialyzed Supernatant of the Mutant

The medium was 10 ml of glucose minimal medium plus 0.25 ml of a two-percent glucose solution plus 10 m. of dialyzed supernatant.

▲ dialyzed supernatant from a mutant culture plus wild type cells
■ dialyzed supernatant from a wile type culture plus wild type cells
● dialyzed supernatant from a wild type culture plus wild type cells


OPTICAL DENSITY 540 nm
0.3
0.1
.05
.03
0
1
2
3
4
5
TIME (hours)
μ=0.63 hr.⁻¹
μ=0.71 hr.⁻¹
μ=0.61 hr.⁻¹

reduction produced by undialyzed supernatant. Dialyzed homologous supernatant had no effect in one experiment (0.71 hr^{-1}) and had almost the same effect as the mutant supernatant in another experiment (0.63 hr^{-1}). When dialyzed supernatant from a mutant culture was used with mutant cells, the specific growth rate was increased to that of the wild type (0.68 hr^{-1}).

The inhibitory agent was also tested for sensitivity to heat. The centrifuged, filtered supernatant was placed in a boiling water bath for 20 minutes. It was then colled and 10 ml were added to fresh medium along witn 0.25 ml of two percent glucose and the specific growth rates were determined. The results are shown in Figure 4. The wild type control (no supernatant added) had a specific growth rate of 0.77 hr^{-1}. With unheated mutant supernatant, the specific growth rate of the wild type was 0.53 hr^{-1}, while with heated mutant supernatant it was 0.82 hr^{-1}. Thus, the inhibitory material in the mutant supernatant was inactivated by exposure to a temperature of 100°C for 20 minutes.

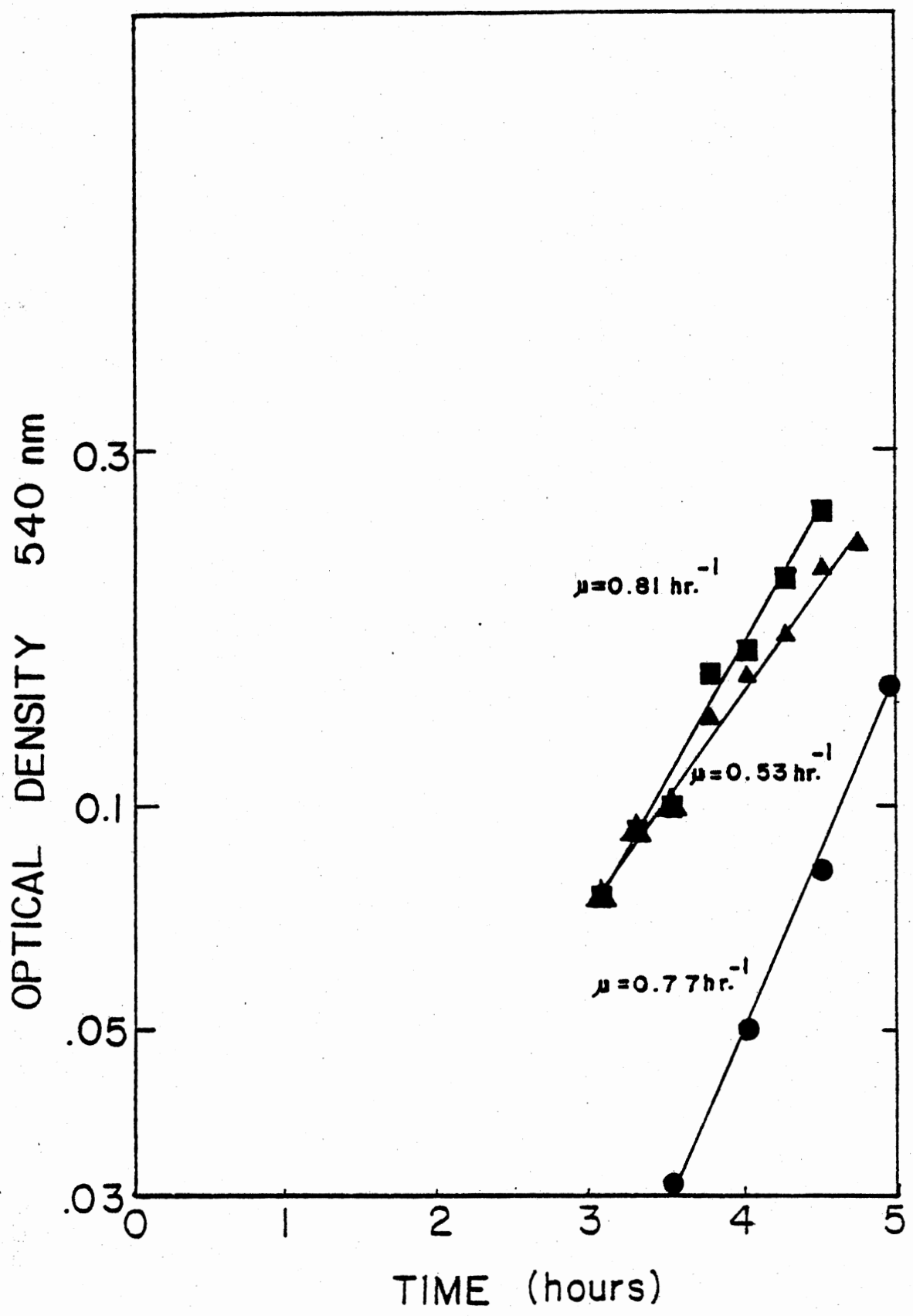
Specificity of Inhibition

In order to determine whether the inhibitory effect of the mutant was a general one or was specific for E. coli, a number of other organisms were tested. These included a related organism, Salmonella, a Gram-positive organism, Bacillus, five Arthrobacter cultures isolated from an activated sludge pilot plant and a fresh isolate of E. coli. The results are shown in Table III. The specific growth rates in the "Experimental" column are those observed when mutant supernatant was added to the culture. Each control received its own supernatant. Of

Figure 4. The Effect of Heat on the Inhibitory Action of the Mutant Supernatant on E. coli IV

The medium was glucose-minimal plus 10 ml of supernatant and 0.25 ml of a two percent glucose solution. The supernatant was heated at 100°C for 20 minutes, cooled to room temperature, and added to the fresh medium.

■ E. coli IV plus heated E. coli 15 supernatant
▲ E. coli IV plus unheated E. coli 15 supernatant
● E. coli IV plus unheated E. coli IV supernatant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TIME (hours)
μ=0.81 hr.-1
μ=0.53 hr.-1
μ=0.77 hr.-1

all the cultures examined, only the E. coli was inhibited by the supernatant from E. coli 15. The growth rate of the Salmonella culture was increased by addition of the supernatant, while the growth of other organisms was unaffected. Thus, the inhibition seemed to be specific for E. coli.

TABLE III

EFFECT OF SPENT CULTURE MEDIUM OF E. coli 15 ON OTHER BACTERIA

Organism	μ, hr^{-1}	
	Experimental	Control
Salmonella	.69	.43
Bacillus	.25	.22
E. coli	.31	.69
Arthrobacter 517	.62	.58
Arthrobacter 314	.48	.50
Arthrobacter 411	.29	.27
Arthrobacter 54	.72	.70
Arthrobacter 34	.48	.42
Arthrobacter 110	.37	.42

Test for Colicin Production

The specificity of the inhibition, as well as the heat sensitivity

of the inhibitor, suggested that the inhibition might be due to production of a colicin by the mutant.

In order to determine colicin production by strain 15, a modified procedure of Clowes and Hayes (1968) was employed. After inoculating plates of minimal medium containing 1.5 percent agar with strains 15 and IV (control), the plates were incubated for 24 hours at 37°. The plates were then sterilized with chloroform vapor for 30 minutes. Following evaporation of the chloroform, 0.1 ml of 24-hour cultures in 0.7 percent agar were layered over the killed bacteria. Strain IV was layered over strain 15, and 15 over IV. At the end of the 24 hours, plates were checked for inhibition zones. No inhibition was observed on either plate, and it was concluded that neither strain produced a colicin effective against the other.

Chromotography of the Mutant Supernatant

While the experiments described above were being carried out in attempts to characterize the inhibitor, another approach was also taken. The supernatant was chromatographed on paper strips in the hope that the inhibitory material could be located by placing the strips on a lawn of wild type cells and examining the lawn for zones of inhibition. The failure of the paper discs soaked in mutant supernatant to produce inhibition indicated that this method would not be successful. The paper strips were exposed to iodine vapor (a general indicator for organic compounds) but no definite spots were detected.

After inconclusive results were obtained with the paper chromatography system, a thin layer chromatography system was employed to look for any compounds that could be ultimately identified as causing

the inhibition of the wild type.

A system employing butanol:acetid acid:water (40:10:50) was prepared, and spent medium of strains 15 and IV were spotted on Silica Gel G plates. After running the plates in the solvent system, the plates were exposed to iodine vapors. Several spots were detected. These spots were also found to be ninhydrin-reactive.

Effects of Amino Acids

Since ninhydrin-positive spots were detected in the culture media of both strains, amino acids known to cause inhibition of *E. coli* were investigated. The most common amino acid known to cause inhibition is valine. Thus, valine and those amino acids synthesized by the same pathway, i.e., leucine, isoleucine, and threonine plus asparate, were used to determine if there were any inhibitory effects. Fifty and 20 µg amounts of the amino acids were added to minimal medium inoculated with strains IV and 15. Results are shown in Table IV.

None of the amino acids affected the growth rate of the wild type. Aspartic, threonine, and valine had no effect on the growth rate of the mutant, but both leucine and isoleucine increased its growth rate. Leucine had a greater effect than did isoleucine.

The effect of added amino acids on the growth rate of the mutant, as well as the detection of ninhydrin-positive material in the supernatant suggested a possible difference in amino acid metabolism between the wild type and the mutant, and the spent media of both strains were analyzed for amino acid content. Ten ml of spent medium were concentrated 100-fold to a final volume of 0.1 ml by lyophilization. An analysis of amino acid composition was performed on an amino acid analyzer

by Dr. R. H. Liao. The amino acids found and their concentrations are shown in Table V. While both culture supernatants contained amino acids, there were differences in some of the amino acids found and those present in both were not present in the same concentrations in several cases. Both culture fluids contained methionine and histidine but both were present in much higher concentrations in the mutant culture fluid than in that of the wild type. Isoleucine and phenylalanine were present at the same levels in both. Aspartic acid, leucine and lysine were found in the wild type culture fluid only, while alanine and tyrosine were found only in the mutant supernatant.

TABLE IV

EFFECT OF INDIVIDUAL AMINO ACIDS ON GROWTH RATE OF MUTANT AND WILD TYPE

Cells	Addition to Medium	μ, hr^{-1}
IV	50 µg aspartic acid	0.67
15	50 µg aspartic acid	0.30
IV	50 µg leucine	0.68
15	50 µg leucine	0.57
IV	50 µg isoleucine	0.69
15	50 µg isoleucine	0.48
15	50 µg isoleucine	0.42
15	20 µg isoleucine	0.42
IV	50 µg valine	0.67
15	50 µg valine	0.33
15	20 µg valine	0.31
IV	50 µg threonine	0.71
15	50 µg threonine	0.27
15	20 µg threonine	0.28

TABLE V

AMINO ACIDS IN SPENT MEDIUM

Strain IV		Strain 15	
Amino Acid	Concentration μ moles/ℓ	Amino Acid	Concentration μ moles/ℓ
Aspartic	0.3 7	Alanine	1.9 9
Methionine	0.6 7	Methionine	5.8 0
Isoleucine	1.5 6	Isoleucine	1.7 9
Leucine	0.4 3	Tyrosine	0.3
Phenylalanine	0.2 3	Phenylalanine	0.4 6
Histidine	0.3 0	Histidine	2.4 0
Lysine	0.0 6		

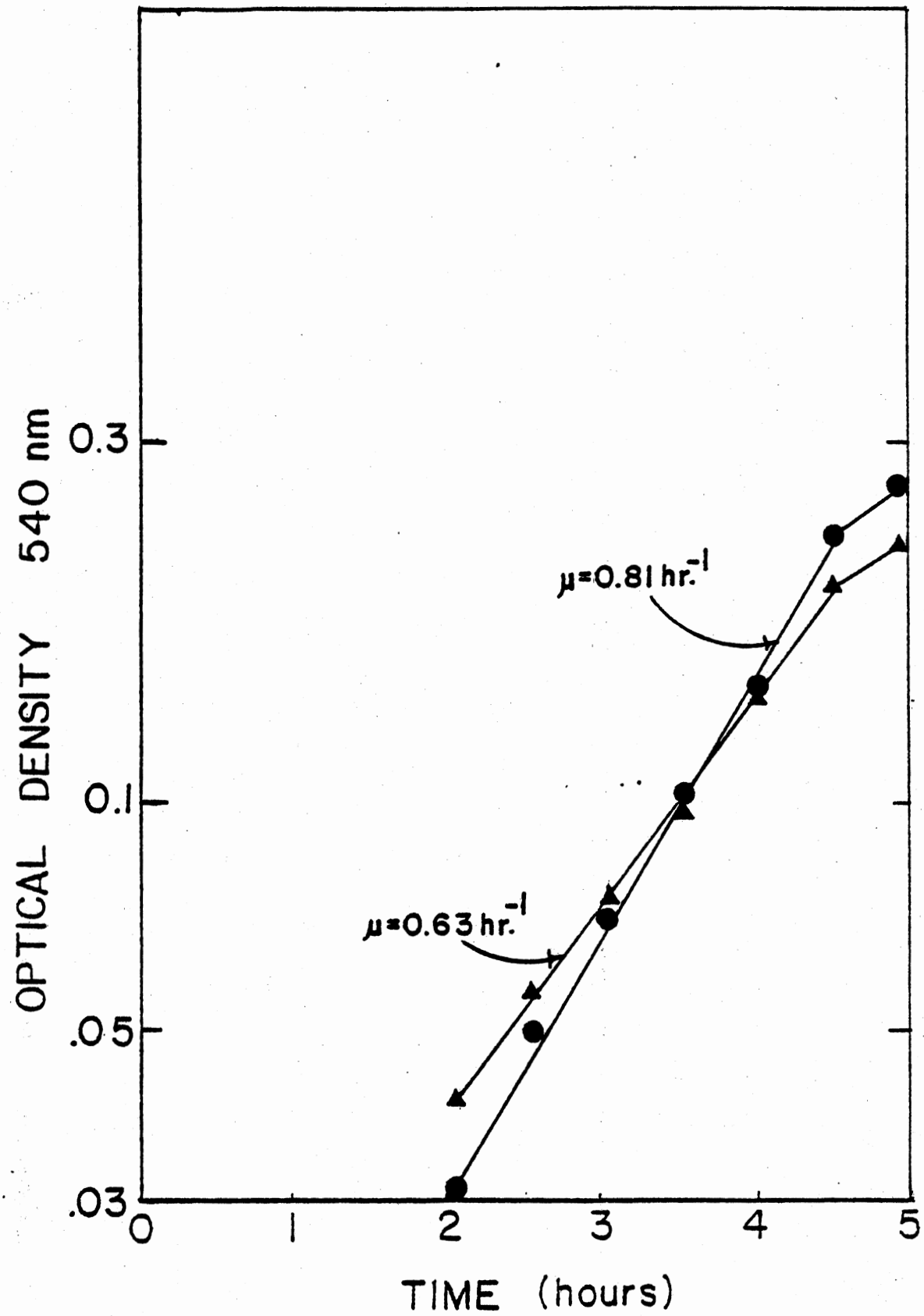
The amino acid analysis was performed by Dr. T. H. Liao, Biochemistry Department, Oklahoma State University.

Several of the amino acids found in the culture fluids had been tested individually in previous experiments. Since interactions of amino acids may be important in their inhibitory effects, the combination of amino acids found in each culture fluid was tested for its inhibitory effect upon both strains. The amino acids were added to glucose minimal medium in the concentrations found in the spent media, and the growth rate of each strain was determined in the presence of both combinations of amino acids. The growth curves are shown in Figures 5 and 6. In both media, the wild type grew at a slightly faster rate than in minimal medium with no additions. Both amino acid

Figure 5. Effect of Amino Acids Found in Supernatant of *E. coli* IV on Growth Rate of Wild Type and Mutant Strains

The medium was glucose minimal plus amino acids found in the supernatant of *E. coli* 15. The concentrations of the amino acids added were identical to the concentrations found in the supernatant.

● *E. coli* IV
▲ *E. coli* 15


OPTICAL DENSITY 540 nm
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0.1
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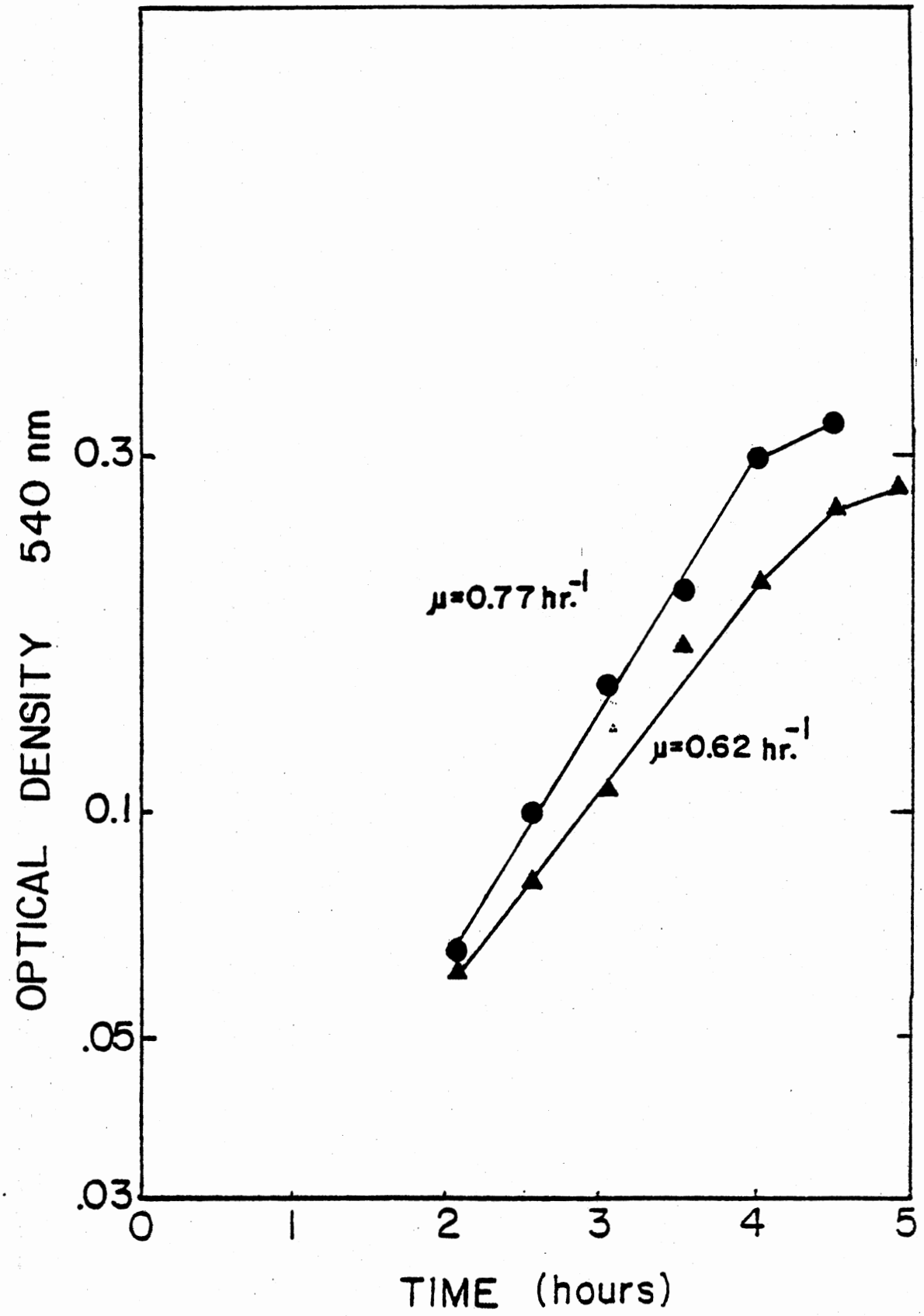
TIME (hours)
μ=0.81 hr.⁻¹
μ=0.63 hr.⁻¹

Figure 6. Effect of Amino Acids Found in Supernatant of E. coli IV on Growth Rate of Wild Type and Mutant Strains

The medium was glucose-minimal plus amino acids found in the supernatant of E. coli IV. The concentrations of the amino acids added were identical to the concentrations found in the supernatant.

● E. coli IV
▲ E. coli 15


OPTICAL DENSITY 540 nm
0.3
0.1
.05
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0
1
2
3
4
5
TIME (hours)
μ=0.77 hr.⁻¹
μ=0.62 hr.⁻¹

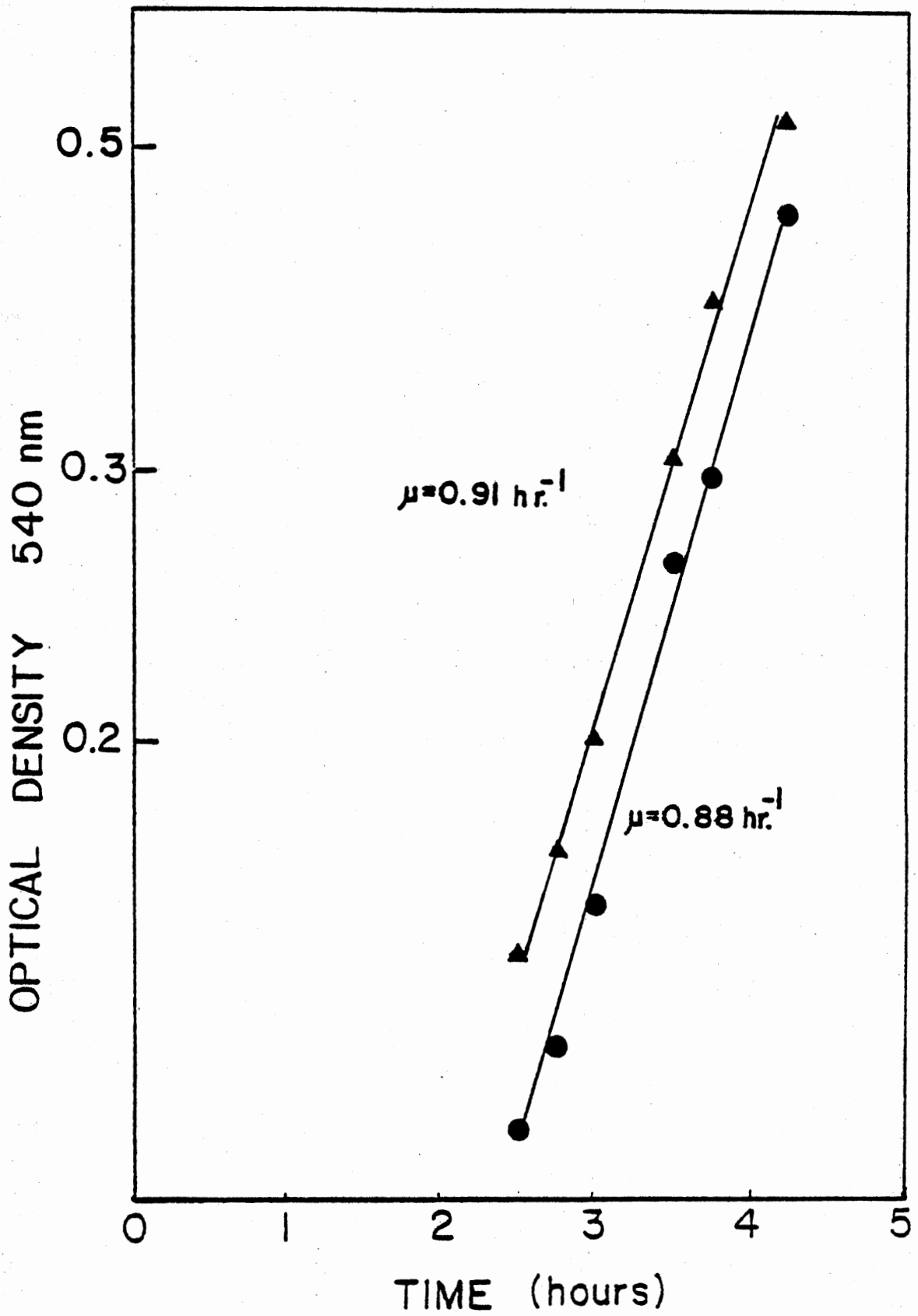
mixtures increased the growth rate of the mutant to approximately that of the wild type in minimal medium. This agrees with the previous findings that dialyzed mutant supernatant increased the growth rate of the mutant to the level of the wild type without additions to the medium.

To determine whether amino acids in high concentration would prevent the production of the inhibitor by the mutant or would reverse its effect, an experiment similar to the previous ones in minimal medium was carried out using nutrient broth. Both strains were grown in nutrient broth; the spent media were centrifuged and filtered through Millipore filters, and 10 ml of spent medium was added to 10 ml of fresh nutrient broth. Each strain was grown in medium containing supernatant from the other. The growth rate constants were very similar, 0.91 hr^{-1} for the wild type and 0.88 hr^{-1} for the mutant (Figure 7). Growth rate constants for the two strains on nutrient broth alone were 1.2 hr^{-1} and 0.69 hr^{-1}, respectively (Damani, 1977). Thus, the wild type was inhibited by the mutant supernatant, while the mutant was stimulated by the wild type supernatant. The degree of inhibition of the wild type was the same as when the experiment was carried out using glucose minimal medium.

Figure 7. The Inhibitory Action of the Supernatants From Nutrient Broth on E. coli IV and E. coli 15

The medium was nutrient broth plus 10 ml of supernatant and 0.25 ml of a two percent glucose solution.

● E. coli 15 plus E. coli IV supernatant
▲ E. coli IV plus E. coli 15 supernatant


0.5
0.3
0.2
OPTICAL DENSITY 540 nm
μ=0.91 hr.⁻¹
μ=0.88 hr.⁻¹
0
1
2
3
4
5
TIME (hours)

CHAPTER IV

DISCUSSION

Damani (1977) isolated a stable slow-growth rate low-yield mutant of E. coli. In continuous flow studies, the mutant predominated over the wild type at a slow dilution rate. The mutant also inhibited the wild type in batch studies. This inhibition was suggested to be the result of production by the mutant of a toxic metabolite which lowered the yield and the specific growth rate of the wild type. The present study was designed to explore further the basis of the mutant's slow growth and its inhibition of the wild type.

In comparing the two strains, anaerobic growth rate studies showed that the mutant grew more slowly than the wild type, 0.33 hr^{-1} vs 0.57 hr^{-1}. This finding rules out the possibility that a defect in electron transport or the coupling of ATP synthesis to electron transport (an unc mutation) could account for the slower growth rate of the mutant strain. Also, in a review by Simoni and Postma (1975) on active transport in bacteria, the phenotype of all unc mutants reported is summarized. It is stated that carbon sources that yield energy primarily through ATP synthesis via oxidative phosphorylation are unable to support growth of unc mutants. Succinate is such a carbon source. Both E. coli 15 and E. coli IV were able to use succinate as a sole carbon and energy source (Table II), thus reinforcing the conclusion that the mutant strain is not uncoupled.

In attempting to find a difference between the two strains, both were tested for ability to utilize a wide range of carbon and energy sources. There was no detectible difference. This rules out transport problems concerning the individual carbon and energy sources investigated.

Damani (1977) grew the strains in a variety of rich media and the mutant always grew more slowly. The present studies show that the mutant can utilize a variety of carbon and energy sources in minimal medium. These data lead to the conclusion that there is no absolute growth factor requirement for the mutant.

Serological testing also detected no difference between the two strains. However, since the strains did not react with any of the antisera used, it is still possible that the strains could be antigenically different but could belong to the group of strains not typed by the antisera provided by Difco.

The results of the amino acid studies did not answer the question as to why the mutant's specific growth rate was slower than the wild type, but did produce some clarification. When the amino acids found in the supernatant of either the mutant or the wild type culture were combined and added to minimal medium, the specific growth rate of the mutant strain approached that of the wild type but did not match it. When individual amino acids were added to minimal medium and the mutant's specific growth rate was determined, only two of the amino acids increased the growth rate--leucine and isoleucine. When these two amino acids increase the growth rate of a strain of E. coli, it is usually an indication that the branched pathway of the pyruvate family of amino acids--leucine, valine, isoleucine and threonine--is involved.

This is usually caused by excess valine that inhibits the growth rate of most strains of E. coli by inhibiting acetohydroxy acid synthase, an enzyme that converts pyruvate into either α-aceto,α-hydroxybutyrate or α-acetolactate. This blocks isoleucine and leucine biosynthesis as well as valine biosynthesis (Umbarger, 1978). Excess valine production could account for the mutant's slower specific growth rate and with over-production of the amino acid it would be excreted from the cell. This could then explain why the mutant supernatant would inhibit the wild type. Excess leucine and isoleucine will overcome this inhibition completely by allowing the cell to grow, depleting the excess valine and thereby removing the block that valine exerts on the enzyme. The data from the experiment with individual amino acid shows that leucine and isoleucine increase the specific growth rate of the mutant. This would be expected if valine were inhibiting acetohydroxy acid synthase. However, excess valine did not have any inhibitory action on the wild type, and valine was not found in the supernatant of either strain, as would be expected if it were being produced in sufficient quantities to block the other amino acid pathways. Thus, although no specific pathway, amino acid or enzyme can be pinpointed, it is evident that the key to understanding the lower growth rate of the mutant lies in the area of amino acid metabolism.

Since a disturbance in amino acid metabolism could easily be related to amino acid synthesis, the finding that the mutant is a relaxed mutant might, when coupled to the hypothesized problem in amino acid synthesis, explain the lower specific growth rate of the mutant.

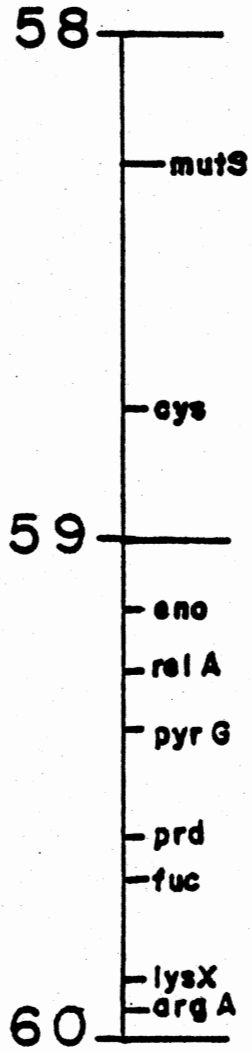
The Rel A gene is expressed as follows: When protein synthesis is restricted after a large decrease in availability of some amino acid,

the synthesis of stable RNA is almost immediately shut down. At the same time, there is an appearance of the nucleoside tetraphosphate ppGpp, which acts as the signal for the shutdown of RNA synthesis. In a relaxed mutant, RNA synthesis proceeds for a long period of time after the shortage of an essential amino acid occurs, while ppGpp is not formed. Relaxed mutants lack the enzyme responsible for synthesis of ppGpp.

Since the mutant is unable to control its synthesis of RNA, it can be seen that the mutant cell is not an efficient biochemical reactor in regard to its use of the energy derived from its environment. Thus, when this is coupled to the apparent problems in amino acid metabolism, it might account for the lowered specific growth rate.

Since the mutant has a defective Rel A locus, it is interesting to look at the genes in proximity to it in order to determine if any could be related to the known characteristics of the mutant. A closeup is shown in Figure 8 of the site on the E. coli chromosome where Rel A is located (Bachman et al., 1976). Since the mutant has been stable for approximately four years, it would be logical to assume that the mutation(s) would be a deletion. Thus, if a particular gene were lost it could not be recovered by the cell, and likewise the function could not be regained. Other types of mutations would be unstable, leading to reversion in the mutant strain. However, this strain apparently does not revert, even at a low rate, since revertants would be strongly selected in any growth situation. The gene eno is close to the Rel A locus. This gene codes for enolase, which catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate. Lack of enolase would prevent anaerobic growth since E. coli ferments sugars

Figure 8. Linkage Map of *E. coli* in the Vicinity of *Rel A*


58
mut S
cys
59
eno
rel A
pyr G
prd
fuc
lysX
arg A
60

via the EMP pathway. Thus, if there is a loss of DNA that includes the Rel A locus, the deletion would not extend into eno.

The pyr G gene produces cytidine triphosphate synthetase. This enzyme produces cytidine triphosphate, which is used by the cell for nucleoside synthesis, choline synthesis, hexose interconversions and regulation of aspartate transcarbamylase. Thus, the absence of this enzyme would also be fatal to the cell. Since it is apparent that the genes on either side of the Rel A gene--eno and pyr G--are necessary for the cell's survival in conditions in which it does grow, the mutation causing the Rel A phenotype could be either a deletion confined within the Rel A locus or one which extends into unmapped genes between Rel A and eno or Rel A and pyr G.

The possibility of multiple mutations has not been eliminated and this is a very viable possibility because of the presence of both the Rel A mutation and an apparent disturbance of some type in amino acid metabolism. It was expected that this problem could be resolved by transduction. However, neither the wild type nor mutant strain was sensitive to P1, the generalized transducing phage for E. coli. The difficulty was apparently not one of restriction because even very high concentrations of phage failed to produce plaques. Attempts to find mutants sensitive to P1 also failed and, since no other generalized transducing phage was available, efforts to carry out transduction were abandoned.

The inhibition of the mutant for the wild type was apparently quite specific since the mutant had no inhibitory activity against a wide variety of organisms tested. Related Gram-negative organisms as well as Gram-positive organisms and five Arthrobacter strains were

tried. Only another *E. coli* isolate from sewage was susceptible to the inhibitory effect of the mutant. The reason for this apparent specificity is unclear, but it was shown not to be due to production of a colicin.

CHAPTER V

SUMMARY AND CONCLUSIONS

The results of the present study are inconclusive as to why the inhibition of the wild type by the mutant strain occurs or why the specific growth rate of the mutant is lower than that of the wild type. However, several possibilities have been eliminated. The possible explanation that colicin activity caused the inhibition has been disproven. The explanation that electron transport or its coupling to ATP synthesis is affected has been eliminated, as shown by anaerobic growth experiments and by utilization of a specific carbon and energy source. The problem of transport has been ruled out for the large number of specific carbon and energy sources tested. There are no detectible differences in standard biochemical tests used in the identification of E. coli that could shed light on the problem. No serological difference between the two organisms was detected. Thus, the exterior of the cells must be nearly identical since the specificity of agglutination reactions is very high. However, it is apparent that amino acid metabolism is disturbed. Leucine and isoleucine, independently, increased the growth rate of the mutant while other amino acids tested had no effect. Only the combination of amino acids found in the supernatant of each strain increased the specific growth rate of the mutant to that of the wild type. Yet, when the mutant was grown in rich medium, the growth rate was below that of the wild type. Further studies in this

area are needed to explain the phenomenon of the low specific growth rate and yield of the mutant.

The suggestion by Damani (1977) that the mutant produces a toxic compound that affects the wild type must be amended to encompass an inhibitor apparently specific for E. coli. This also needs further investigation.

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