PHYSIOLOGY OF L-ASPARAGINASE SYNTHESIS BY *ESCHERICHIA COLI*

DISSERTATION

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By

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A mating between Escherichia coli 4318 ($thi^- leu^- Las^-$ Hfr) and E. coli A-1 ($Met^- Las^+ F^-$) resulted in the formation of prototrophic recombinants having L-asparaginase activities at three distinct levels. The physiology of L-asparaginase synthesis in these recombinants is described. One class of recombinants produced significantly more L-asparaginase than E. coli A-1. L-Asparaginase synthesis in the recombinants was inhibited by the presence of dissolved oxygen in the medium and was transiently repressed by the presence of glucose in the same manner as that observed in the parental strains. L-Asparaginase activity was increased by the addition of oxalacetate as well as other members of the Kreb's cycle.

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CHAPTER I

INTRODUCTION

L-Asparaginase (E.C. 3511) is an amidohydrolytic enzyme which catalyzes the hydrolysis of L-asparagine to its products L-aspartate and ammonia. Interest in this enzyme is primarily due to its antineoplastic activity toward several cell lines (1, 10, 33, 34, 48, 74, 76). The observed antitumor activity is thought to reside in the fact that L-asparaginase depletes the supply of L-asparagine which is essential for growth of various tumor cells. When L-asparagine levels are decreased, tumor cells cannot multiply and soon die. This effect is not seen on normal cells, since these are capable of synthesizing L-asparagine de novo (54, 59, 63, 66, 78).

In 1961, 8 years after Kidd (40, 41) observed that guinea pig serum had antineoplastic activity toward certain transplantable mouse lymphomas, Broome (11, 12, 13) proved that L-asparaginase was responsible for the effect. He based his evidence on the following data.

1) The instability of the enzyme to changes in temperature and pH was found to parallel closely those of the antilymphoma agent of guinea pig serum.
2) L-Asparaginase activity was essentially absent from the serum of newborn guinea pigs and their serum

failed to inhibit the growth of 6 C3HED cells.

3) In the purification procedure for L-asparaginase, only those fractions with enzyme activity possessed antilymphoma activity.

In 1963, Mashburn and Wriston (52, 53) extracted and purified L-asparaginase from E. coli and showed that it had antineoplastic activity similar to that from guinea pig serum. However, L-asparaginase from Bacillus coagulans had no antineoplastic activity when studied under similar conditions (51). This discovery led to an intense search for bacteria other than E. coli which possess L-asparaginase with antineoplastic activity. While most bacteria produce L-asparaginase, only those preparations from Escherichia coli, Serratia marcescens, Erwinia carotovora, Erwinia arroidea, Mycobacterium tuberculosis, Proteus vulgaris, and certain species of Achromobacter, Acinetobacter, Azotobacter, and Pseudomonas have significant antitumor activity (6, 37, 55, 61, 64, 70). Subsequently, a number of reports have appeared in the literature describing the conditions required for maximal synthesis of L-asparaginase in several genera of bacteria. For example, Cedar and Schwartz (17) found higher L-asparaginase activity in E. coli grown anaerobically than when grown aerobically, but Roberts, et al. (66) reported greater yields when E. coli was grown in the presence of air than in the presence of nitrogen. Boeck and Ho (9) stated that the presence of glucose in the growth medium of E. coli was an unconditional requirement for optimum

synthesis of L-asparaginase, while Cedar and Schwartz (17) found that it inhibited synthesis of the enzyme. Other authors have reported amino acids to stimulate the synthesis of L-asparaginase in cultures of E. coli; for example, Netrval (57) found that eighteen amino acids added to the growth medium increased enzyme yields significantly and that when two or more were added simultaneously, the stimulatory effect was additive. Other researchers have tried to find the optimum conditions for production of antineoplastic L-asparaginase by E. coli; these include Bilimoria (7), Boeck et al. (8), Cedar and Schwartz (17), Roberts et al. (66), and Robinson and Beck (69). However, the data concerning the environmental parameters affecting the production of this enzyme remain confusing and even contradictory.

L-Asparaginase therapy, alone or in combination with other drugs, is finding increased success in the management of acute lymphocytic leukemias. In recent clinical trials (33, 48), significant remissions were obtained in more than 50% of patients treated with 1,000 to 3,000 IU/kg body weight per day. Dosages of this magnitude require large amounts of purified enzyme. This is commercially produced by large scale fermentation and purification techniques. Recent reports (8, 17, 30, 37) show that most microorganisms yield only 1.3 to 4.0 IU of L-asparaginase per ml of fermentation medium. Since L-asparaginase is not extracellular and thus

is extracted from whole cells, efficiency in production must be determined by cell density multiplied by the amount of enzyme in each cell. Most large scale fermentation studies have employed corn steep liquor (CSL) as the growth medium (6, 66). While this medium is low in cost, it is time consuming to prepare, high in contaminating microorganisms, inconsistent in quality from one batch to the next, and contains from 25 to 50 percent insoluble particulate debris of unknown composition.

In contrast to the many reports on the physiological conditions that affect L-asparaginase synthesis, little work has been reported on the genetics of E. coli or other bacteria with respect to synthesis of this enzyme. This situation probably prevails because the isolation of strains that produce large amounts of enzyme is made difficult by the lack of suitable media and of adequate techniques for the differentiation of colonies (clones) with genetic blocks in the synthesis of L-asparaginase.

The four major objectives of this research were to (1) determine the optimal culture conditions for maximum synthesis of L-asparaginase by E. coli A-1; (2) develop an inexpensive medium for use in large scale fermentation cultures which is more manageable than the commonly used corn steep liquor; (3) isolate mutants of E. coli which produce greater levels of L-asparaginase than the parental strain, and (4) gain a better understanding of the genetic and

physiological factors which are involved in the synthesis of this enzyme.

CHAPTER II

REVIEW OF THE LITERATURE

History

Although the deamination of L-asparagine by extracts of Escherichia coli was first observed by Tsuji in 1957 (77), significant interest in this area was not expressed until 1961 when Broome (11, 12, 13) discovered the anti-tumor effect of L-asparaginase in guinea pig serum. Kidd (40) later showed that 6 C3HED lymphoma cells treated with L-asparaginase from guinea pig serum were inhibited in the ability to synthesize protein. This was followed by an inhibition of deoxyribonucleic acid (DNA) synthesis resulting in a marked cytotoxic effect. These data, along with several other studies (14, 20, 29, 72), supported the proposed theory that those tumor cells sensitive to the action of L-asparaginase require an exogenous source of L-asparagine.

Purification

A large number of studies have been reported which describe the isolation and purification of L-asparaginase from bacteria from the time of Cedar and Schwartz's (16) discovery in 1968. These authors found that E. coli possesses two L-asparaginases, only one of which has anti-

tumor activity. L-Asparaginase I was found to be a cytoplasmic enzyme, while L-asparaginase II, the enzyme with antitumor activity, was associated with the periplasm and was only synthesized under anaerobic conditions. At about the same time, Campbell et al. (15) described the separation, purification, and activity of two L-asparaginases designated EC-1 and EC-2 from E. coli B. One of these enzymes, EC-2, was found to be a potent antilymphoma agent while EC-1 had no antineoplastic activity. In addition to having different elution profiles (hydroxylapatite chromatography), the two enzymes were unlike in other properties. For example, a difference in the kinetic properties of the two enzymes was shown by the shape of the curves representing activity as a function of pH. While EC-1 had a rapid and progressive decrease in activity below pH 8.4, EC-2 showed very little change in activity over the range from pH 6.0 to 8.4, but had maximal activity at approximately pH 7.0. They found that it was possible to determine the amount of EC-1 or EC-2 in mixtures of the two enzymes by measuring the activities at pH 5.0 and 8.4. The antitumor enzyme, L-asparaginase II, was extracted from E. coli and characterized by Ehrman in 1971 (21). This enzyme was found to behave in a similar manner to the EC-2 enzyme described by Campbell et al. (15) when examined by various biochemical tests. Roberts et al. (67) also showed that E. coli (ATCC 11303) contained two L-asparaginases. Using

diethylaminoethyl cellulose chromatography, the first protein peak to emerge was capable of causing complete regression of the Gardner 6 C3HED lymphosarcoma. This fraction also exhibited optimum activity at a pH of 8.0 and was stable on prolonged incubation with 6 C3HED mouse serum. The second peak had maximum enzyme activity at pH 8.5, was inactivated on incubation with mouse humoral fluids and lacked anti-neoplastic activity.

L-Asparaginase from E. coli HAP has been recently purified and characterized by Liu and Speer (44). Essentially 100% of the L-asparaginase produced by this organism showed anti-neoplastic activity when tested against Gardner's lymphoma carried in 6 C3HED mice. Unlike the strains of E. coli previously described, this microorganism produced a single L-asparaginase enzyme as indicated by diethylaminoethyl cellulose purification. The enzyme had a molecular weight of 139,000 and a final specific activity of 470 IU/mg protein. Amino acid analysis of the purified protein revealed no free sulfhydryl groups. The enzyme was also stable when incubated in body fluids and had a broad pH activity with a maximum at pH 8.0.

In 1976, Roberts (68) reported the purification of a highly potent antitumor enzyme from Pseudomonas aeruginosa having both glutaminase and asparaginase activity. Using both molecular seiving and ion exchange chromatography, he isolated a purified enzyme having an activity of 162 IU/mg

protein. Physical, kinetic, and biological properties of this enzyme were different than those of the antitumor enzyme of E. coli. For example, the purified Pseudomonas aeruginosa 7A glutaminase-asparaginase had an unusually long biological half-life and was effective against solid neoplastic tissue, as well as ascites tumor cells.

Davidson et al. (19) reported the purification and characterization of three enzymes isolated from Citrobacter freundii having some asparaginase activity. One of these enzymes also had substantial glutaminase activity, but this was easily inactivated by the purification techniques employed. The antitumor enzyme with a molecular weight of 140,000, produced significant increases in the survival time of C3H/HED mice carrying 6 C3HED lymphoma tumor.

L-Asparaginase activity has been recently reported in Azotobacter vinelandii, a species somewhat distant in its taxonomic relationship to E. coli (25, 26). The enzyme was purified to near homogeneity by standard methods of enzyme purification, including affinity chromatography. It had an optimum activity at pH 8.6 and an approximate molecular weight of 84,000. Administration of 2,000 IU of this L-asparaginase per kilogram body weight, provided maximum survival of isogenic Wistar rats against Yoshida ascites sarcoma. Increasing dosages beyond this limit resulted in a decrease in survival period. The authors felt that high concentrations of their enzyme preparations were toxic.

In 1968, Wade et al. (80) extracted and purified L-asparaginase from Erwinia carotovora, a common pathogen phylogenetically closer to E. coli than to Azotobacter vinelandii. The purified Erwinia L-asparaginase was shown to be serologically distinct from the E. coli enzyme and to have significant antitumor activity. A year later, Peterson and Ciegler (62) also extracted and purified an antitumor L-asparaginase from Erwinia carotovora. Maximum yields of 1,250 IU of L-asparaginase g/dry weight of cells were obtained in eight hours. Partial purification and concentration of the extracted L-asparaginase yielded a preparation with an activity of 275 IU/mg protein. Electrophoresis revealed only one L-asparaginase, and it exhibited a pH optimum of 7.5 and Km of 3×10^{-3}M. Serological tests with antibodies reactive with L-asparaginases from E. coli and Serratia marcescens proved that the L-asparaginase from Erwinia carotovora was immunologically distinct.

Clinical Studies

Clinical application of E. coli L-asparaginase therapy for cancer patients first began in 1967 at Wadley Institutes of Molecular Medicine (32). These researchers observed a complete remission of acute lymphatic leukemia and a significant regression in lymphosarcoma in several patients treated under clinical conditions. Continued treatment of cancer patients with L-asparaginase soon revealed untoward reactions primarily related to antibody formation, immuno-

suppression or the presence of traces of endotoxin associated with the purified enzyme of E. coli (3, 38, 42, 43, 50). As a consequence, interest was focused on organisms unrelated taxonomically to the Enterobacteriaceae with the hope of minimizing these side reactions. This approach has had some success in clinical studies (2, 36, 42). For example, Kay et al. (39) concluded that Erwinia asparaginase was an effective therapeutic agent when given to patients that were already sensitive to the E. coli enzyme. The Erwinia enzyme had no greater toxicity than did the E. coli L-asparaginase manufactured by the Bayer Company, Wuppertal-Elberfeld, Germany in doses of 6,000 IU/m^2 daily. The immunological cross-reactions of asparaginases from several Erwinia and Escherichia species were found to be very slight (83).

In recent clinical trials, significant remissions were obtained in more than 50% of the acute lymphocytic patients treated with 1,000 to 3,000 IU/kg of body weight per day (34, 48). Since dosages of this magnitude mean large amounts of purified enzyme and thus high cost to the patient, the pursuit of other strains of bacteria capable of producing high levels of L-asparaginase has intensified.

Effect of Environment on Enzyme Synthesis

Since L-asparaginase is extracted from whole cells, efficiency in production will be determined by cell density and the amount of enzyme each cell produces. The effects

of culture medium, pH, temperature, and oxygen concentration on L-asparaginase synthesis vary for the different microorganisms. A comprehensive review of this literature reveals a complex and variable set of culture conditions that effect the synthesis of L-asparaginase (6, 8, 9, 19, 25, 30, 31, 57).

In 1967, Cedar and Schwartz (16) first observed that the E. coli K-12 L-asparaginase II having antineoplastic activity was only produced under anaerobic conditions. After growing the cells at 37°C in a minimal medium and in an atmosphere containing 90% nitrogen and 10% carbon dioxide, the bacteria were harvested by centrifugation in early exponential growth. When cells were disrupted by osmotic shock, they were found to contain rather low L-asparaginase activity (0.02 IU/ml culture medium). In 1968, these same authors reported that while some enzyme was synthesized aerobically, approximately 100 times more L-asparaginase II was produced during anaerobic growth in media containing high concentrations of amino acids (17). Little L-asparaginase II was produced by cells under aerobic conditions, but when aeration was discontinued, the specific activity increased exponentially until a plateau was reached in forty minutes. This increase in enzyme synthesis was found to be dependent on de novo protein synthesis; when chloramphenicol was added after aeration was discontinued, no increase in enzyme activity was observed.

These authors also found that the addition of glucose inhibited the production of the enzyme. The greatest amounts of enzyme were obtained in media devoid of sugar provided high concentrations of amino acids were present. Interestingly, little L-asparaginase II, with antitumor activity, was formed either aerobically or anaerobically when asparagine was the sole source of nitrogen. L-Asparaginase I, having no antitumor activity, was constitutive and unaffected by the conditions of growth.

In 1968, Roberts et al. (66) reported high enzyme yields from E. coli HAP grown aerobically in a corn steep medium. Escherichia coli HAP produced 3.1 IU/ml or 0.95 IU/mg dry weight when grown under aerobic conditions. The authors also found that of the amino acids present in corn steep medium, only glutamic acid and methionine were essential for maximal L-asparaginase production. The addition of glucose to such media depressed enzyme synthesis.

The production of L-asparaginase by the submerged growth of Serratia marcescens described in 1969 by Heinemann and Howard (30), resulted in a maximal yield of 4.0 IU/ml in a medium containing 4% autolyzed yeast extract (initial pH 5.0) incubated at 26°C with limited aeration. These authors also proposed that the depressive effect of glucose on L-asparaginase activity was due to the production of fermentation acids during growth at low oxygen levels.

The introduction of membrane-type, oxygen sensitive

electrodes for conveniently monitoring the dissolved oxygen levels in liquid media enabled Heinemann et al. (31) in 1970 to investigate the effect of oxygen on the synthesis of L-asparaginase in Serratia marcescens. Peak yields of L-asparaginase were obtained in unbaffled flasks (3.0-3.8 IU/ml) and in the chemostat (2.7 IU/ml) when the level of dissolved oxygen in the culture media reached zero. A low rate of oxygen transfer from medium to growing cells was accomplished by limited aeration (1.5 vol. air/vol. medium/per hour). Yields of L-asparaginase were approximately 60% less under conditions of high oxygen transfer than at a low oxygen transfer rate.

Boeck et al. (8) studied the culture and nutritional requirements for maximum synthesis of L-asparaginase in E. coli B. Interestingly, both aerobic and anaerobic conditions appeared to affect enzyme synthesis. High L-asparaginase levels were obtained only when the combination of these two conditions were utilized. The cells were allowed to grow aerobically until the mid-to-late exponential phase and then were permitted to become anaerobic by static incubation in the presence of glucose, i.e., a bipartite procedure. Under these conditions, Escherichia coli B produced 2.6 IU of L-asparaginase/ml of fermentation broth when glucose was present. Enzyme synthesis was not induced by terminating the aeration in the absence of glucose. Production of L-asparaginase was found to be

proportional to the glucose concentration between 0.1 and 0.8 mg/ml of the growth medium. The length of static incubation was also found to be critical. Forty minutes after cessation of aeration, L-asparaginase activity peaked at 2.6 IU/ml, then slowly declined. The requirement of glucose for maximal enzyme synthesis is something of a paradox since all other studies show that glucose or any other easily fermentable carbohydrate must be absent for maximal yields. The increase in enzyme synthesis by static incubation and by the presence of glucose were easily shown when the bacteria were grown in a modified trypticase soy broth. This same induction could not be demonstrated with the corn steep medium; no explanation for this difference has appeared in the literature. Boeck and Ho (9) published additional research in 1973 using the same organism. While maximal growth was obtained aerobically, L-asparaginase synthesis was repressed. Only when the bipartite process, as previously described, was utilized did maximum synthesis proceed. Glucose, galactose or pyruvate independently supported biosynthesis of 1.5-2.6 IU of antitumor L-asparaginase/ml during static incubation of aerobically grown E. coli B. Low levels of chloramphenicol or puromycin reduced enzyme synthesis by as much as 95%. These authors suggested that pyruvate was the key compound responsible for inducing L-asparaginase synthesis.

Svobodova and Strbanova-Necinova (75) studied the in-

duction of L-asparaginase synthesis in a newly-isolated strain of E. coli. An antitumor L-asparaginase was synthesized only after depletion of oxygen from the medium or under aerobic conditions in the presence of lactate.

In 1972, Liu and Zajic (45) investigated the nutritional conditions required for optimal L-asparaginase production by Erwinia aroideae. Maximal enzyme activity was obtained when 1% lactose and 1.5% yeast extract were supplied as carbon and nitrogen sources, respectively. L-Asparaginase activity was decreased when Erwinia aroideae was grown in this medium supplemented with glucose. Glucose also was found to inhibit beta-galactosidase synthesis. Liu and Zajic (46) also studied the fermentation kinetics and continuous process of L-asparaginase production from Erwinia aroideae. The maximal yield of 4 IU/ml was reached just prior to stationary growth at an optimum temperature of 24^{o}C. Moderate aeration (1.0 volume of air per volume of medium per minute) was found to be optimal for enzyme production. The continuous fermentation process enabled enzyme yields of approximately 3.0 IU/ml. The optimum temperature for enzyme production in continuous process was 24^{o}C, the same as in the batch process. An increase in the temperature from 24-28^{o}C resulted in a 20% loss of enzyme yield. In 1973, Liu and Zajic (47) published an interesting paper concerning the effect of oxygen-transfer rate on production of L-asparaginase by Erwinia aroideae. Maximum L-asparagi-

nase synthesis, 4.25 IU/ml, was obtained when the fermentation medium was aerated with 0.5 volume air per volume of medium per minute and agitated at 500 rpm. Maximal enzyme yields were achieved when the bacteria were grown in a medium consisting of 1% lactose, 1.5% yeast extract and 0.1% K_2HPO_4. They found that the rate of absorption of oxygen into the culture medium was affected both by depletion of dissolved oxygen in the liquid and by the volumetric oxygen transfer coefficient. This is described as follows:

In an actively growing culture of Erwinia aroideae, aeration was stopped and nitrogen was introduced over the surface of the broth at a rate of 2 liters per minute to minimize surface oxygenation. The decrease in dissolved oxygen due to respiration was measured to obtain the rate of oxygen uptake.

$$\frac{d\,C_1}{dt} \quad \text{no aeration} \quad = \; - \, r\, x$$

Where C_1 is the actual concentration of dissolved oxygen; t is time; r is the specific oxygen-uptake rate and x is the cell mass. Before the critical oxygen concentration required for L-asparaginase synthesis was reached, aeration was resumed and the increase in dissolved oxygen was recorded.

$$\frac{d\,C_1}{dt} \quad \text{aeration} \quad = \; K_1\, a\, (C^* - C_1) \; - \; rx$$

Where K_1 is the volumetric oxygen-transfer coefficient and

C* is the concentration of dissolved oxygen that is in equilibrium with the existing partial pressure of oxygen in the air.

Rearranging:

$$C_l = -1/K_{la} \left(\frac{dCl}{dt} + r\ x \right) + C^*$$

Thus $K_l a$ can be obtained by plotting C_l versus

$$(d\ C_{l/dt}) + r\ x$$

The measurement of $K_l a$ provided a measurement of the overall performance of a fermentor during the aeration of a growing culture. The optimum level of $K_l a$ for L-asparaginase production was 0.98/min., but a higher $K_l a$ value of 1.2-1.9/min. was needed to achieve the highest growth. Although the oxygen transfer rate had an important effect on total L-asparaginase activity, it did not appreciably affect the specific activity with Erwinia aroideae. The maximum L-asparaginase activity of 4.25 IU/ml was obtained when the fermentation medium was moderately aerated with 0.5 volume air per volume of medium per minute and agitated at 500 rpm.

Chaikovska et al. (18) found that the greatest amount of enzyme was synthesized by E. coli at the beginning of the stationary phase of growth. Addition of glucose or increased aeration inhibited enzyme synthesis significantly.

Eryomenko and Sokolov (23), studying the factors influencing the growth and L-asparaginase activity of E. coli

ATCC 9637, reported maximum levels of enzyme activity to occur in a corn extract medium under the following conditions: an inoculum level of 3% by volume, pH of 7.2-7.5 at the beginning and 8.5 at the end of cultivation, and aeration at a rate of 30 mg 0_2/liter/min. L-Asparaginase activity ranged from 0.6-1.1 IU/mg protein for different batches when grown in 30 and 200 liter fermenters.

A soil pseudomonad was isolated by Foda et al. (24) which produced L-asparaginase when cultured in the presence of glutamic acid. The enzyme was produced optimally at a pH of 7.5 and was repressed significantly by complex nitrogen sources and by the amino acids glutamine and glycine. The highest yields were obtained in a medium containing L-glutamic acid and yeast extract buffered at a pH of 7.5. When carbon sources such as those of the tricarboxylic acid cycle were added, L-asparaginase levels were increased only as a function of increased cell mass. Little increase was seen in activity when measuring activity in terms of IU/optical density unit.

Zhukov and Eremko (82) selected clones of Pseudomonas fluorescens AG-69 with increased L-asparaginase and L-glutaminase activity. This strain on long term storage had lost the ability to produce these enzymes. Using continuous cultivation and growing the organism on agar media containing L-asparagine and L-glutamine, they succeeded in selecting isolates having L-asparaginase activity.

Rulpanova and Eremenko (69) found that biosynthesis of L-asparaginase in Bacillus mesentericus 43A was inhibited if the cells were transferred from a medium containing glycerol to one containing glucose. Inhibition also occurred with the addition of citrate, fumarate or malic acid. No inhibition was seen in the presence of α-ketoglutarate, oxalacetate or pyruvate. Biosynthesis of L-asparaginase in this culture seems to be regulated mainly by the mechanism of catabolite repression. These authors also studied the regulation of L-asparaginase synthesis by growing the bacteria in the presence of amino acids such as aspartate and glutamate, alone and in combination. The addition did not enhance activity; in fact, a decrease in enzyme activity was often observed.

Netrval (57) reported that 18 amino acids and 3 of 7 organic acids enhanced the specific activity of L-asparaginase EG-2 in shake cultures of E. coli.

The production of deaminases in several bacterial cultures was studied by Vinogradov (79). Among forty cultures, seventeen strains catalyzed hydroxylaminolysis of L-asparagine and L-glutamine; among these cultures, seven strains belonged to the genus Pseudomonas. El-Din and Foda (22) have recently studied the kinetics and properties of L-glutaminase and L-asparaginase activity in a strain of Pseudomonas ovalis which produced these enzymes simultaneously on induction by L-glutamine or L-as-

asparagine in the growth medium. Optimum pH for enzyme formation was found to be 7.0. Significant amounts of L-glutaminase and L-asparaginase activities (1.5 IU/ml) appeared toward the end of the exponential growth phase. These authors also showed that enzyme formation was linked to cellular growth by assaying enzyme levels in the presence of known growth inhibitors such as streptomycin sulfate and tetracycline.

Garaev and Golub (27) studied the mechanism of the effect of glucose on L-asparaginase synthesis by E. coli. These authors found that the synthesis of L-asparaginase was almost completely suppressed if glucose was added at a concentration of 0.55% to the growth medium. Lactate was found to stimulate L-asparaginase synthesis. Glucose was found to be responsible for the catabolite repression and inhibition of the components of a system involved in lactate transport. Thus, the inhibition of L-asparaginase synthesis by glucose seemed to be due, at least in part, to the fact that it prevented the assimilation of lactate by the cells.

It can be concluded that the environmental and nutritional conditions required for maximal L-asparaginase synthesis is highly variable, even among microorganisms of the same species. Certain factors, however, such as aeration rate, pH, and the presence of relatively high concentrations of amino acids and/or organic acids such as lactate, oxlace-

tate, pyruvate, etc., provide a common basis of metabolic control in the synthesis of L-asparaginase. The synthesis of L-asparaginase in E. coli is obviously a complex process and critically depends on the appropriate conditions for maximal activity. To date, no thorough attempt at elucidating the mechanism of regulation of the synthesis of the antitumor L-asparaginase has been made. The research data presented in this dissertation culminate an attempt to provide additional insights into this complex physiological system.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains: Escherichia coli A-1 (met^-F^-) and E. coli HAP (met^+) were obtained from the stock culture collection at Wadley Institutes of Molecular Medicine, Dallas, Texas. The designation Las^+ was introduced by Barnes, et al. (4, 5) to indicate L-asparaginase production; the genotype of E. coli A-1 was presented as ($met^-Las^+F^-$). Escherichia coli CGSC 4318 (thi^-leu^-Hfr) was kindly supplied by Royston Clowes, Department of Molecular Biology, University of Texas at Dallas. This designation has been amended to CGSC 4318 ($thi^-leu^-Las^-$Hfr) (4, 5). Isolated recombinants from mating E. coli CGSC 4318 with E. coli A-1 were coded using letter R (recombinant) and the sequential numerical designation of the colony from which the recombinant was isolated.

Media: Working cultures were maintained on slants of 3% nutrient agar (3g of Difco commercial powder per 100 ml of distilled water). Stock cultures were grown at 37°C in 15 X 45 mm sterile vials containing a medium of the following composition: Difco Nutrient Broth powder, 0.9g; NaCl, 0.5 g; Difco Agar, 0.75g; distilled water to 100 ml. They were stored at room temperature. Liquid media used for

growing test strains were prepared by dissolving the selected commercial powder medium to the strength indicated in distilled water and adjusting the pH to 7.0. Minimal agar was prepared by adding NH_4Cl, 0.5g; Na_2SO_4, 0.2g; K_2HPO_3, 0.3g; KH_2PO_4, 0.1g; $MgSO_4 7H_2O$, 0.1g; and glucose 0.1g per 100 ml of distilled water.

Chemicals: Amino and tricarboxylic acids were purchased from Sigma Chemical Co., St. Louis, Missouri. Aqueous stock solutions were prepared, adjusted to pH 7.0, filter sterilized (Millipore Corporation, Bedford, Mass.) and added aseptically to the respective media. Monosodium glutamate, commercial grade, was purchased from J. T. Baker Chemical Co., Phillipsburg, New Jersey. All other chemicals used were of reagent grade quality and were obtained from diverse sources.

Test Tube Cultures: Stock cultures of parental and recombinant strains of E. coli were streaked on plates of 3% nutrient agar and checked for purity. Test tube cultures (4ml of the respective medium in a 16 X 100 mm test tube) were prepared with an inoculum obtained by transferring a portion of a single colony with a straight needle. Broth cultures were grown overnight at 37°C on a New Brunswick rotary shaker using a conventional slanting rack (20° angle) and a shaking rate of 70 rpm. When determining the

enzyme levels of a single isolate grown in various test media, the following procedure was used: Three isolated colonies were picked and the cells were resuspended in 3 ml of sterile 0.85% NaCl to a density of 2-4 X 10^8. Tubes containing 4 ml of the test broth were inoculated with 0.05 ml of this suspension and incubated for 20 hours at 37°C on the rotary shaker as described above.

Fermentor Cultures: Isolated colonies from plates of 3% nutrient agar were resuspended in saline to a density of 1 X 10^9 cells/ml. Five milliliters of this suspension were inoculated into 500 ml of the appropriate medium in a Bio-flow Model C30 fermentor with pH and dissolved 0_2 monitoring capabilities (New Brunswick Scientific Co., New Brunswick, New Jersey). Fermentor temperature was maintained at 37°C, air flow at 560 cc/min., and agitator (propeller) speed at 400 rpm. The pH was continuously recorded and could be automatically controlled with 2 N HCl or 2 N NaOH. When desired, sterile 25% glucose solution was added to achieve a 0.5% concentration; foaming was controlled with Corning Antifoam B reagent.

Mating Procedure: Escherichia coli 4318 and E. coli A-1 were grown overnight at 37°C in test tubes containing 4 ml of 3% nutrient broth and incubated until mid-log phase growth (2 X 10^8 viable cells/ml) was attained. Two milliliters of each culture were mixed and incubated without

agitation at 37°C for ninety minutes. After the mating mixture was centrifuged and washed twice with 0.9% saline, 0.1 ml of each parental culture plus the mixture were spread on minimal agar plates. Plates were then incubated 4-5 days at 37°C. Control tubes of donor and recipient cells alone failed to yield colonies. Microorganisms that formed colonies on this medium were known to be genetic recombinants because they grew on the minimal agar devoid of required amino acids. Isolated colonies were subcultured at least five times on minimal agar before L-asparaginase activity was tested.

L-Asparaginase Assay: A linear reference was drawn using anhydrous $(NH_4)_2SO_4$ prepared in the following manner: $(NH_4)_2SO_4$, (94.34 mg) was dissolved in 100 ml of distilled water. This solution contained 200 µg nitrogen/ml. Appropriate dilutions were made to obtain solutions containing 2, 4, 8, 10, 20, and 40 ug nitrogen in 0.4 ml of distilled water. To each of these concentrations was added 0.1 ml of trichloroacetic acid (24%), 7 ml distilled water and 1 ml of Nessler's Reagent. The color was allowed to develop for five minutes and the absorbance read at 390 nanometers against a blank which had all of the constituents except ammonium sulfate. A linear relation between optical absorbance and, nitrogen content, to 20 ug nitrogen, was obtained. Fourteen ug of nitrogen (1 umole) corresponded to an absorb-

ance of 0.42. One International Unit of L-asparaginase activity is defined as that amount of enzyme required to release one μ mole of ammonia from L-asparagine per minute at 37°C. The release of ammonia was quantitated by either Nesslerization or by the Logsdon procedure (49). L-Asparaginase activity using Nessler's Reagent was performed as follows: 0.1 ml of a cell suspension washed 2X with 0.9% saline was added to 1.9 ml of a 0.011 M solution of L-asparagine (37°C) in 0.1 M tris-phosphate buffer at pH 8.0. The blank consisted of the same except that 0.5 ml of a 24% solution of trichloroacetic acid was added to inhibit the reaction. After incubating blank and test samples for fifteen minutes at 37°C, 0.5 ml of TCA (24%) was added to the test samples. The tubes were then centrifuged and 0.5 ml of the test sample was added to 7.5 ml distilled water and mixed. One milliliter of Nessler's Reagent was added, the sample was mixed and, after development, the resulting yellow color was read at 390 nanometers. The reading was multiplied by a factor of 7.8 to give IU/enzyme per milliliter of medium. This factor was derived as follows:

use 0.1 ml for assay	X 10
total volume of assay = 2.5 ml	
remove 0.5 ml	X 5
assay time, 15 min.	÷ 15

$$F_d = \frac{5 \times 10}{15} = 3.33 = \text{dilution factor}$$

Using Beer's Law, the following relationship exists:

$A = Ecl$, where

A = absorbance

E = molar extinction coefficient

c = moles/liter

l = Thickness of light absorbing solution

$$c = \frac{A_{390}}{Exl} \times F_d \qquad \text{therefore:} \quad c = \frac{A\ (3.3)}{0.42}$$

L-Asparaginase activity was also measured using the Technicon Auto-analyzer Model I (Technicon Corp., Terrytown, New York) following the procedure described by Logsdon (49) with the following modifications. Hypochlorite solution was prepared by adding 200 ml of 6% NaOCl ("Purex") to 800 ml of a 0.48 M solution of K_2CO_3. Alkaline phenol was prepared by adding 100 ml of liquefied phenol (88%) to one liter of 0.9 N NaOH. The substrate for enzyme assay was 0.011 M L-asparagine dissolved in a buffer solution made of 0.2 M K_2HPO_4 titrated with 0.1 M citric acid to pH 7.0. The Logsdon assay was calibrated and checked periodically by asaying L-asparaginase activity of *E. coli* A-1 and *E. coli* HAP.

Dry Weight Measurements: Millipore filters (0.45 u, 45 mm diameter) were exposed to 100° for five hours in an oven. The filters were stored under vacuum over Drierite until used. After taring the filters, three milliliters of the

test suspension were filtered under vacuum without touching the side wall of the Millipore assembly. This was followed by washing with ten milliliters of distilled water. The filter was then gently removed and placed on a drying pan contained in a 100^{o}C dry-heat oven for five hours. The filter was allowed to cool to room temperature and weighed on an analytical balance. Dry weight was determined by averaging duplicate samples.

CHAPTER IV

RESULTS

The data in Table I show the yields of L-asparaginase (per unit of cell dry weight) in four trials with four different media. Obviously, the composition of the medium and final pH affect the synthesis of L-asparaginase by E. coli A-1. To study the individual components responsible for maximum activity, a medium of 3.0% nutrient broth was used. This concentration, while providing good growth, produced cells with a rather low enzyme yield, namely, 3.0 - 4.0 IU/ml medium. To this medium, individual filter sterilized amino acids were added at different concentrations. Figure 1 shows five amino acids out of the twenty-one tested to increase enzyme yields.

Growth of E. coli A-1 in the minimal medium supplemented with 0.5% glucose was good (1×10^9 viable cells/ml in 20 hours), but no L-asparaginase activity was found (Table II). The addition of a mixture of L-amino acids to this medium produced cells with only 0.2 IU L-asparaginase/mg dry weight. Further studies indicated that the major part of the enhancement was due to glutamic acid, although glutamic acid did not support L-asparaginase synthesis in the absence of the other amino acids (Table II). The concentra-

Figure 1. Effect of L-amino acids added to 3% nutrient broth on L-asparaginase activity by Escherichia coli A-1. Cells were grown overnight in shaking test tube cultures at 37°C. The L-amino acids used were glutamine, gln; glutamate, glu; serine, ser; methionine, met; and asparagine, asn.

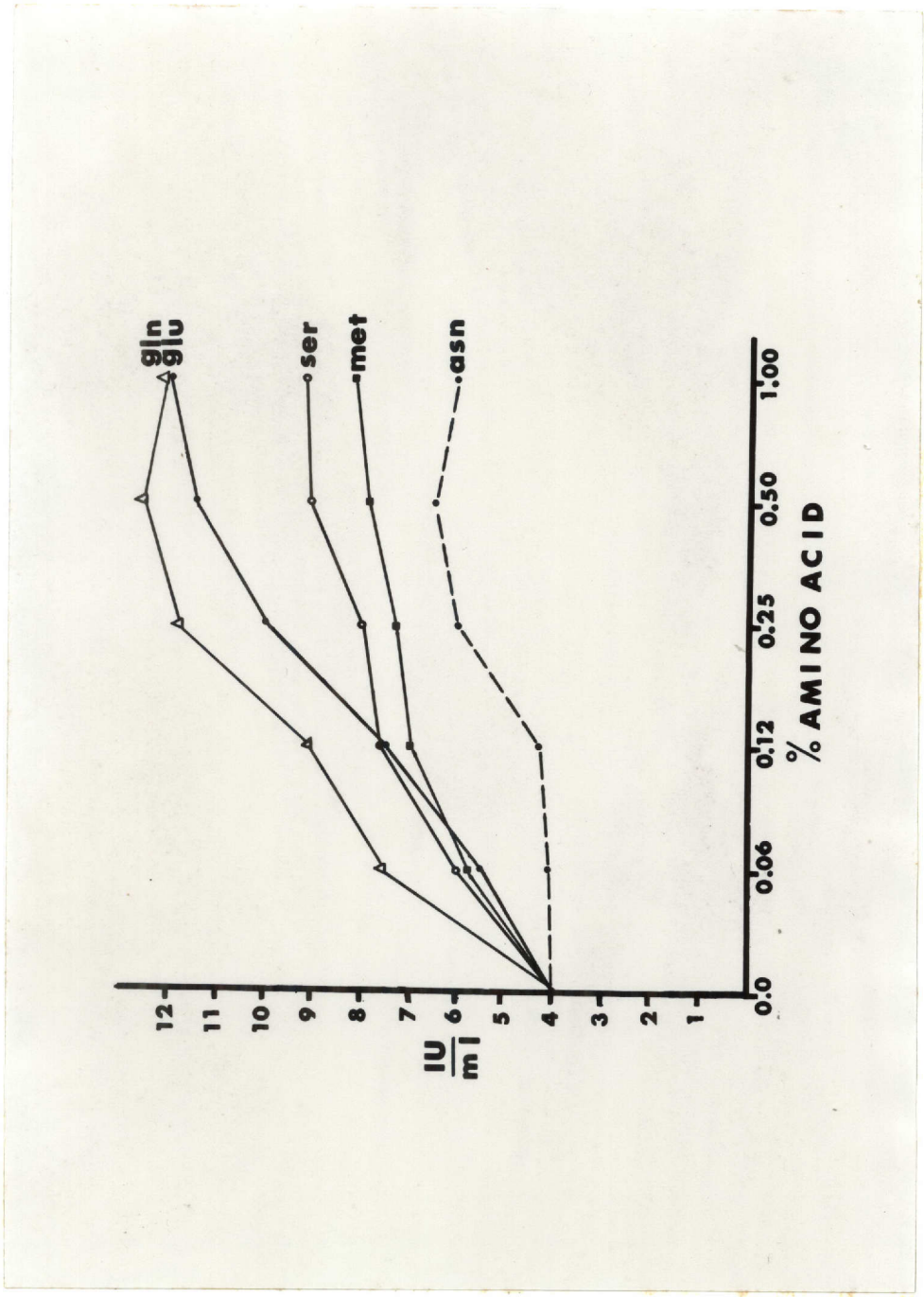

gln
glu
ser
met
asn
IU/ml
12
11
10
9
8
7
6
5
4
3
2
1
0.0
0.06
0.12
0.25
0.50
1.00
% AMINO ACID

TABLE I

YIELD OF L-ASPARAGINASE IN 20 HOUR CULTURES OF *ESCHERICHIA COLI* A-1 GROWN IN DIFFERENT MEDIA

Medium	g of powder/ 100 ml	Final pH[b]	IU/ml[c]	IU/mg Dry Cells
Brain heart Infusion	12.5	6.47	3.1	2.9
	6.25	7.47	9.5	6.6
	3.12	8.14	9.0	5.6
	1.56	8.31	0.5	0.5
Nutrient Broth	12.5	6.97	9.8	4.4
	6.25	7.98	9.0	3.9
	3.12	8.46	3.9	2.7
	1.56	8.47	0[a]	0
Peptone	12.5	6.79	6.0	2.8
	6.25	8.36	3.5	2.2
	3.12	8.42	0	0
	1.56	8.29	0	0
Yeast Extract	12.5	6.52	9.0	3.2
	6.25	7.63	9.5	3.0
	3.12	8.19	8.2	3.5
	1.56	8.63	4.5	2.6

[a] Zero indicates amounts less than 0.1 IU.

[b] Supernatant pH of 20 hour cultures.

[c] Average value of duplicate trials.

TABLE II

EFFECT OF AMINO ACIDS ADDED TO MINIMAL MEDIUM ON THE ACTIVITY OF L-ASPARAGINASE BY ESCHERICHIA COLI A-1

Additions to minimal medium	IU/ml[d]	IU/mg Dry Cells
0.5% glucose	0	0
AA[a]	0.2	0.2
1%MSG[b]	0	0
AA + 0.5% MSG	4.0	3.2
AA + 1.0% MSG	5.7	5.2
AA + 1.5% MSG	6.2	6.2
AA + 2.0% MSG	6.2	6.3
AA + 2.5% MSG	6.0	6.3
AA + 3.0% MSG	5.5	6.7
AA + 3.5% MSG	5.1	7.3
AA + 4.0% MSG	3.5	4.9

[a] The amino acid (AA) mixture contained 0.4% (wt/vol) asparagine, serine, and methionine; 0.3% lysine and aspartic acid, 0.2% phenylalanine, arginine, leucine, isoleucine, valine, proline, alanine, and threonine; 0.1% histidine and glycine; and 0.01% tyrosine and cysteine.

[b] Monosodium glutamate, commercial grade.

[c] Cells were grown overnight in shaking test tube cultures at 37°C.

[d] Average value of duplicate trials.

tion of glutamate required for maximum enzyme activity was rather high. Maximal activity was achieved in the amino acid supplemented medium containing 3.5% of monosodium glutamate. This experiment clearly indicates a synergistic effect between L-glutamic acid and exogenous amino acids for the production of maximal L-asparaginase activity by E. coli A-1.

It was found that L-glutamine, L-glutamic acid or commercial grade monosodium glutamate were equally effective in increasing enzyme levels when added to 3% nutrient broth (Table III).

The highest yields of L-asparaginase were obtained in cells recovered from the minimal media, washed, and resuspended in the induction medium (Table IV). The data in Table IV also show the combined effect of nutrients in nutrient broth and of monosodium glutamate (MSG). It should be noted that nutrient broth might contain glutamic acid in low amounts. The increase in enzyme activity seen when increasing the nutrient broth concentration alone is probably a reflection of the presence of glutamic acid. The addition of monosodium glutamate allows the use of less nutrient broth to achieve high enzyme yields (Table IV). For example, a 12.5% solution of nutrient broth supports an activity of L-asparaginase of 9.8 IU/ml (Table I). To achieve 10.6 IU/ml, it only takes 3.0% nutrient broth plus 1% monosodium glutamate (Table III).

TABLE III

EFFECT OF L-GLUTAMIC ACID, L-GLUTAMINE, AND MONOSODIUM GLUTAMATE ON L-ASPARAGINASE ACTIVITY OF ESCHERICHIA COLI A-1

Additions to 3% Nutrient Broth[a]	L-Asparaginase		L-Glutaminase (IU/ml)
	IU/ml[c]	IU/mg Dry Cells	
None	3.4	2.9	0
0.25% glu	9.1	6.0	0.2
1.0%	10.0	5.1	0.2
0.25% gln	9.6	4.8	0.2
1.0%	9.6	4.5	0.2
0.25% MSG	10.0	5.0	0.2
1.0%	10.6	4.5	0.2

[a] Abbreviations: glu, Glutamic acid; gln, glutamine; MSG, monosodium glutamate, commercial grade.

[b] Cells were grown overnight in shaking test tube cultures at 37°C.

[c] Average value of duplicate trials.

TABLE IV

L-ASPARAGINASE ACTIVITY BY ESCHERICHIA COLI A-1 GROWN IN MINIMAL MEDIA

Induction Medium[a]	Final pH	IU/ml[d]	IU/mg Dry Cells
Minimal medium	5.25	0	0
0.5% NB[b]	7.30	0	0
10% NB	8.25	0.5	0.4
2.0% NB	8.41	4.2	2.6
3.0% NB	8.59	5.5	3.4
0.5% NB + 1% MSG	8.65	0.5	0.5
1.0% NB + 1% MSG	8.50	5.3	3.3
2.0% NB + 1% MSG	8.40	19.6	8.0
3.0% NB + 1% MSG	8.51	27.5	8.9

[a] The cells were removed from the growth medium at late log phase, washed, and resuspended in an equal volume of induction medium and grown overnight.

[b] NB, nutrient broth.

[c] Cells were grown overnight in shaking test tube cultures at 37°C.

[d] Average value of duplicate trials.

It soon became obvious that L-glutamate was required for the maximal synthesis of L-asparaginase. Additional studies were performed to determine if other dehydrated media could be substituted for corn steep liquor medium commonly used in the production of L-asparaginase in large scale (1,000 liter) cultures. Table V shows that of the twelve formulations tested (courtesy of Dr. Edward Savard, Hoffman-LaRoche, Inc., New Jersey), three produced cells having relatively high enzyme activities. These required peptone and/or yeast extract in addition to 10 mg monosodium glutamate/ml. However, none supported as good a specific L-asparaginase activity as 3% nutrient broth supplemented with 1% MSG.

Based on previous physiological requirements for optimal enzyme yield, two media were developed which showed significant improvement over the commonly used corn steep liquor medium (Table VI). Corn steep liquor medium requires a pH adjustment and clarification before autoclaving; the inconsistency of the final product gave variable enzyme yields. In addition, microscopic examination of this medium revealed gross contamination by gram positive and gram negative microorganisms. It is possible that toxic molecules from these microbes remain associated with the enzyme in the final purified state. The media described in Table VI are simple to prepare when compared to corn steep liquor medium.

TABLE V

L-ASPARAGINASE ACTIVITY BY ESCHERICHIA COLI A-1 GROWN OVERNIGHT AT 37°C IN SHAKING TEST TUBE CULTURES USING VARIOUS MEDIA SUPPLEMENTED WITH 25 UG METHIONINE/ML

Medium Number	MSG (10 mg/ml)	pH[a]	Growth O. D. 600	Activity IU/ml
1	-	7.07	1.59	0.0
2	+	8.06	1.87	0.0
3	+	8.03	2.04	0.5
4	+	8.12	1.84	0.5
5	+	7.83	2.21	7.0
6	+	7.93	2.26	12.0
7	+	7.73	2.13	9.5
8	+	7.99	2.13	14.0
9	+	7.78	1.99	9.5
10	+	8.02	2.06	10.0
11	+	7.95	2.10	9.5
12	+	8.00	2.03	8.0
13 Control 3% Nutrient Broth	+	8.28	2.12	17.5

[a] pH is of supernatant after 20 hours growth

TABLE V
(CONTINUED)

COMPOSITION OF THE VARIOUS MEDIA

Stock Solutions:

Reagent	g/100 ml H_2O
K_2HPO_4	3.9
KH_2PO_4	1.9
$(NH_4)_2SO_4$	1.0
$MgSO_4$ (anhydrous)	0.1
$Na_3C_6H_5O_7.2H_2O$ (Sodium citrate)	0.7

Carbohydrates 20% w/v solution of glucose or mannitol, sterilized by filtration through a 0.45 u millipore filter.

	g/100 ml H_2O
Gibco Fresh Yeast Extract	25.0
BBL Trypticase Peptone	25.0
BBL Acidicase Peptone	25.0
Sigma L-methionine	0.5

Medium #1

Reagent	ml of stock solution
K_2HPO_4	6.0 ml
KH_2PO_4	4.0 ml

TABLE V

(CONTINUED)

Reagent	ml of stock solution
$(NH_4)_2SO_4$	10.0 ml
$NA_3C_6H_5O_7$	10.0 ml
Glucose	1.5 ml
H_2O	q.s. to 100.0 ml

Medium #2. Same as medium #1, but substitute 2 ml of stock yeast extract for water.

Medium #3. Same as medium #2, but add 4 mg L-methionine.

Medium #4.

Reagent	ml of stock solution
K_2HPO_4	6.0 ml
KH_2PO_4	4.0 ml
$(NH_4)_2SO_4$	10.0 ml
$Na_3C_6H_5O_7$	10.0 ml
Mannitol	1.5 ml
Yeast Extract	2.0 ml
H_2)	q.s. to 100.0 ml

Medium #5. Same as #4, but add 10 ml stock BBL Acidicase Peptone Solution.

Medium #6. Same as #4, but add 10 ml stock BBL Trypicase Peptone Solution.

TABLE V
(CONTINUED)

Medium #7. Same as #1, but add 10 ml stock BBL Acidicase Peptone and 2 ml of stock yeast extract solution.

Medium #8. Same as #1, but add 10 ml stock BBL Trypticase Peptone and 2 ml of stock yeast extract solution.

Medium #9.

Reagent	ml of stock solution
K_2HPO_4	6.0 ml
Glucose	1.5 ml
Yeast Extract	2.0 ml
BBL Trypticase	10.0 ml
Adjust pH to 7.2 with KH_2PO_4	3.0 ml
H_2O	q.s. to 100.0 ml

Medium #10. Same as #9, but substitute 1.5 ml of stock mannitol solution for glucose.

Medium #11. Same as #9, but substitute 10 ml stock BBL Acidicase Solution for trypticase peptone.

Medium #12. Same as #9, but substitute 10 ml stock BBL Acidicase Solution for trypticase peptone and 1.5 ml of stock mannitol solution for glucose.

TABLE VI

MEDIA FOR USE IN
LARGE SCALE FERMENTATION

Medium No. 1

	g/l
K_2HPO_4	2.3
NaH_2PO_4	0.76
NH_4SO_4	1.0
$Na_3C_6H_5O_7$	0.68
Yeast Extract (BBL)	2.5
Peptone (Difco)	5.0
MSG	10.0

No pH adjustment required
(pH = 7.1)

Medium No. 2

* N-Z Amine HD	10.0
MSG	10.0

No pH adjustment required
(pH = 6.9)

* Humko-Sheffield, New Jersey

Fermentation Studies

A series of experiments with the Bioflo fermentor were used to confirm the effect of MSG on L-asparaginase activity in mass cultures of E. coli A-1. These studies showed that higher L-asparaginase activity was obtained when the pH of the culture was maintained at 7.5 and that glucose interferred with L-asparaginase production, especially if MSG were present in the medium. A pH of 7-8 was found to support maximal L-asparaginase activity. If glucose were added to 3% nutrient broth containing 1% MSG, L-asparaginase synthesis was much lower than that obtained with only MSG added. These data show that glucose reverses the enhanced synthesis of L-asparaginase by glutamate, even if the pH were controlled at an optimum of 7.5 (Table VII).

By adding 0.5% glucose to a 3% nutrient broth medium, it was discovered that growth of the microorganism could be separated from the synthesis of L-asparaginase. That is, the presence of 0.5% glucose in the medium inhibited the synthesis of L-asparaginase so that cultures could be grown to maximal densities without producing the enzyme. Then by a manipulation of medium pH enzyme synthesis could be induced in pregrown cultures (Figure 2). This conveniently allowed the study of the rate of enzyme synthesis under varied oxygen conditions as shown in Figure 2. Higher yields

TABLE VII

EFFECT OF GLUCOSE AND CULTURE pH
ON L-ASPARAGINASE ACTIVITY IN
FERMENTOR CULTURES OF *ESCHERICHIA COLI* A-1[a]

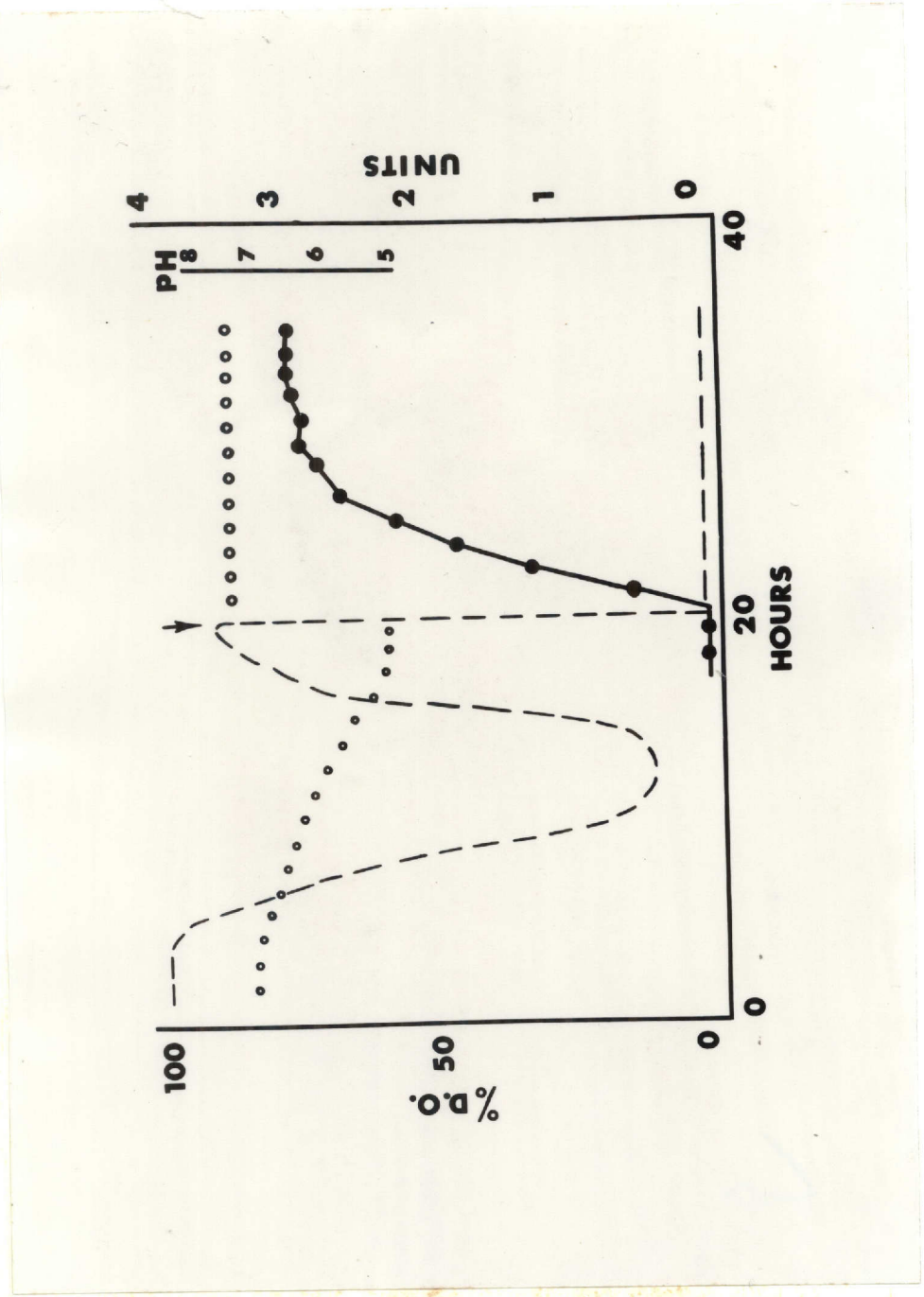
Additions to 3% nutrient broth	pH Controlled	Culture pH	IU/ml[c]	IU/mg Dry Cells
None	+	7.5	5.0	2.7
None	+	5.5	0	0
0.5% glucose	+	7.5	6.8	2.9
0.5% glucose	-	5.5	0	0
1% MSG	+	7.5	19.8	8.8
1% MSG	+	5.5	1.0	0
1% MSG + 0.5% glucose	+	7.5	7.0	2.9
1% MSG + 0.5% glucose	-	5.5	0	0

[a] Cultures were grown overnight at 37°C, 560 cc air/minute and propellor set at 400 rpm.

[b] The pH was established and maintained as indicated.

[c] Average value of duplicate trials.

Figure 2. Kinetics of L-asparaginase synthesis by E. coli A-1 in the Bioflo Fermentor. Grown medium is 3% nutrient broth plus 0.5% glucose. The broken line represents percent dissolved oxygen, the open circles represent pH, and the solid line L-asparaginase synthesis in international units per milligram cell dry weight. The arrow signifies a pH shift from 5.5 to 7.5. Medium pH was adjusted (arrow) and controlled automatically thereafter.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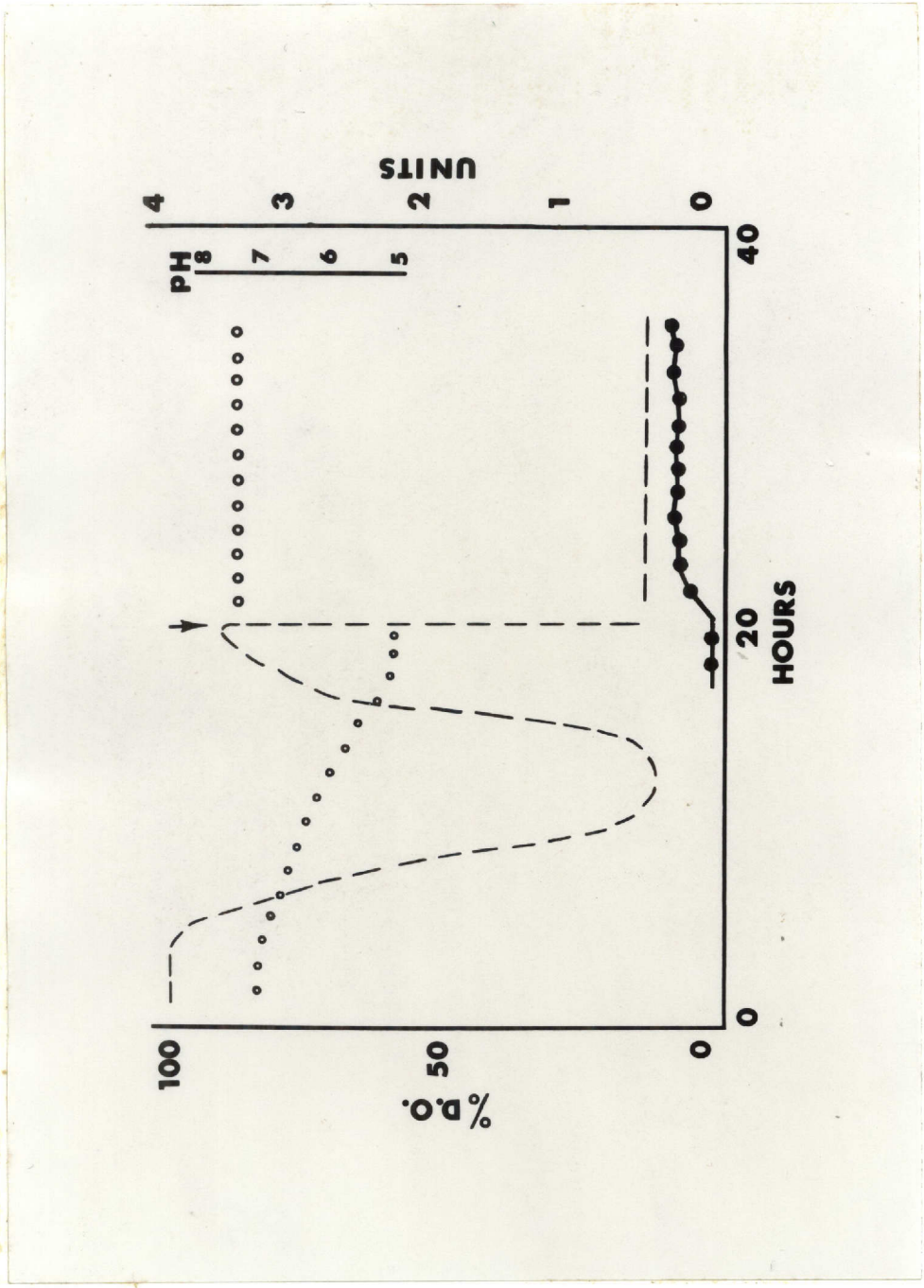
of L-asparaginase activity were found in cultures of E. coli A-1 grown in 3% nutrient broth plus 0.5% glucose in which there was no discernible dissolved oxygen than in those with measurable dissolved oxygen. After overnight growth, pH of the culture was adjusted to 7.5 and maintained by automatic control to attain the "critical level" of dissolved oxygen. Adjusting the pH to 7.5 within 15 minutes brought the level of dissolved oxygen to zero without further manipulation, although agitator velocity was maintained at 400 rpm and air flow at 560 cc/min. The effect of approximately 0.8 mg of 0_2 per liter of medium (10% dissolved oxygen) is seen in Figure 3. In essence, the rate of L-asparaginase synthesis could be controlled by manipulation of the culture pH and, as a consequence, the dissolved oxygen level of the medium. The effects of pH and dissolved oxygen appear to be interdependent in that L-asparaginase activity was negligible at a pH of 5.2, diminished when the pH was lower than 7.5, or when the dissolved oxygen was greater than zero.

If the flow of air was stopped and the culture allowed to become anaerobic, L-asparaginase synthesis was markedly lower than if aeration was continued. Figure 2 shows that under optimal environmental conditions, i.e., dissolved oxygen at 0% saturation and pH controlled at 7.5, enzyme synthesis achieved a level of 3 IU L-asparaginase/mg dry

Figure 3. Effect of dissolved oxygen (10% of saturation) on L-asparaginase synthesis by *E. coli* A-1. The arrow indicates a pH shift from 5.5 to 7.5 and an increase in propellor RPM to achieve a 10% dissolved oxygen level. Symbols are the same as in Figure 2.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cells. Figure 4 shows that under anaerobiosis, i.e., no air being supplied to the fermentor, enzyme synthesis was much lower, achieving 0.2 IU L-asparaginase/mg cell dry weight. The synthesis of L-asparaginase in E. coli A-1 depends on proper culture pH and on oxygen uptake, but is inhibited by excess oxygen dissolved in the medium. This critical relationship has not been previously reported.

It is, apparently, the de novo synthesis of L-asparaginase that is involved, since no L-asparaginase activity is observed, even under optimal conditions, upon adding chloramphenicol (2 μg/ml) to the culture (Figure 5). This point was well established in previous reports (9, 75). This implies that L-asparaginase is formed de novo when the pH is brought to 7.5 concomitant with the disappearance of dissolved oxygen. On the other hand, stimulation of L-asparaginase activity (i.e., synthesis) does not require extensive cellular reproduction. Cells of E. coli A-1 grown in minimal media were harvested, washed, and transferred to the 3% nutrient broth 1% MSG medium and permitted to attain the critical rate of oxygen transfer in the new medium. The synthesis of L-asparaginase started in 8 hours, although cell numbers did not increase more than two fold during that time (Figure 6). In experiments of this type, L-asparaginase synthesis could be initiated simply by adjusting the pH to 7.5 and allowing the dissolved oxygen to reach the critical transfer rate.

Figure 4. Effect of anaerobiosis on L-asparaginase synthesis by E. coli A-1. The arrow indicates a pH shift from 5.5 to 7.5 and turning off the air supply to the fermentor. Symbols are as in Figure 2.

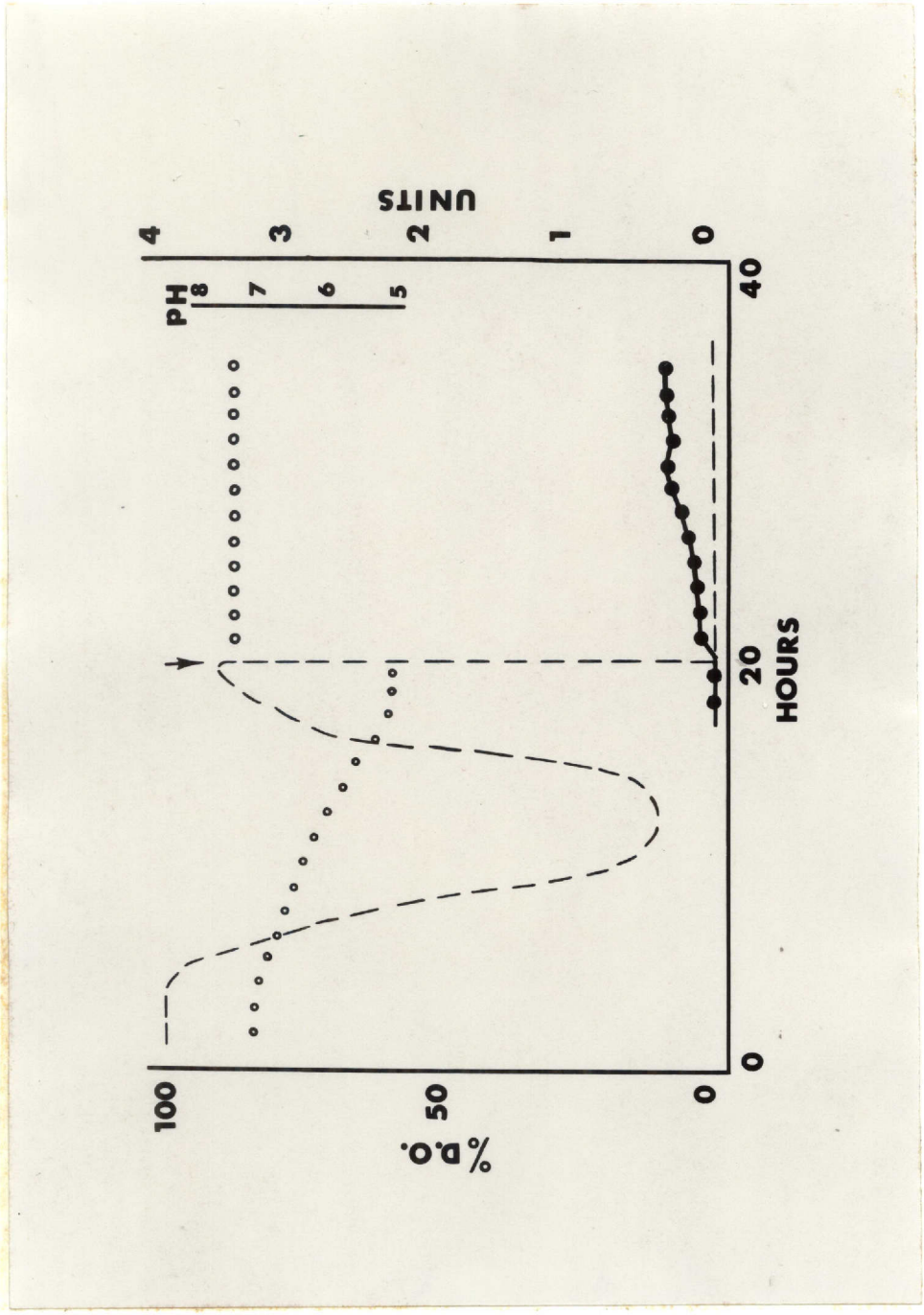
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Figure 5. Inhibition of L-asparaginase synthesis by the addition of 2 ug/ml chloramphenicol (CM) at the time of pH shift from 5.5 to 7.5. Symbols are as in Figure 2.

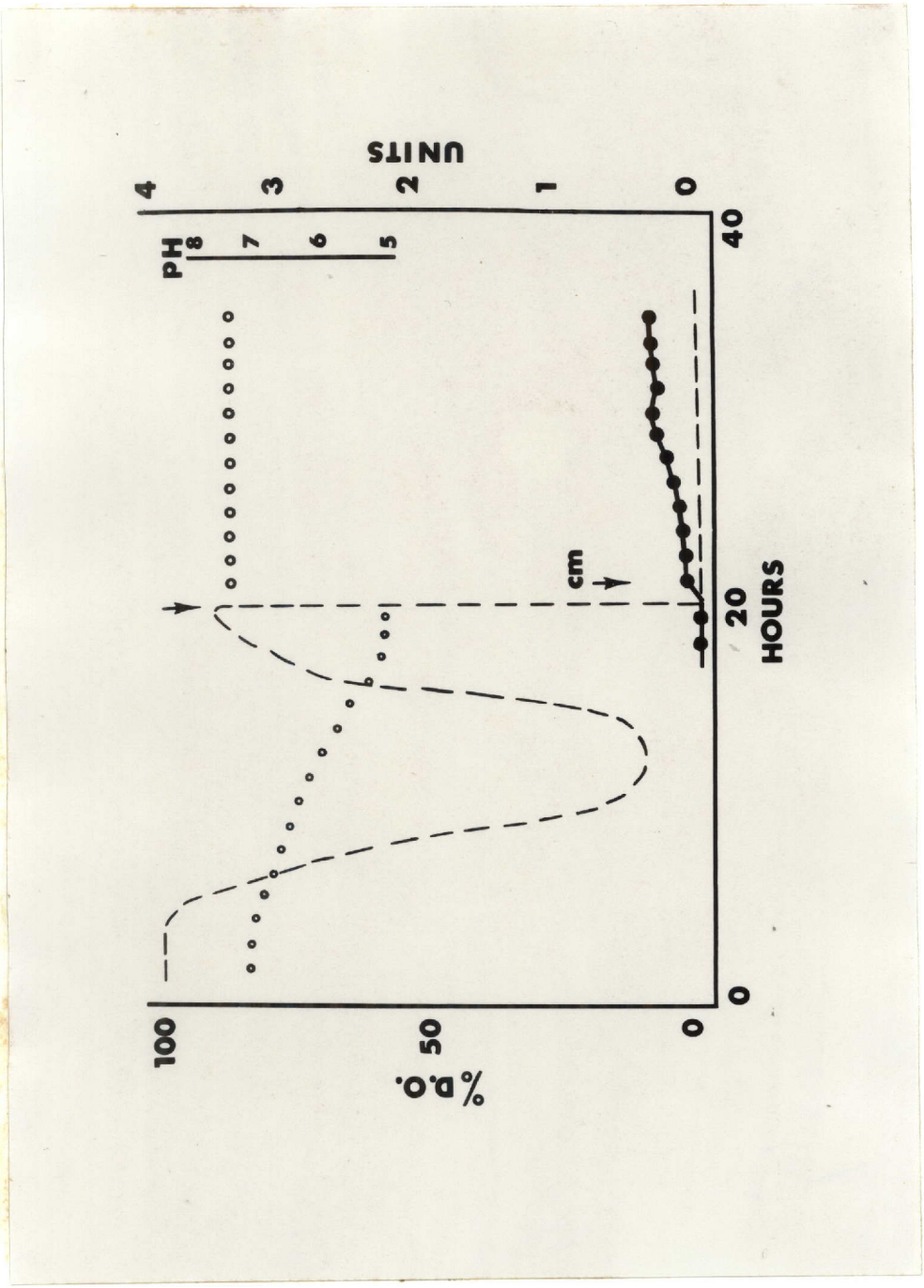
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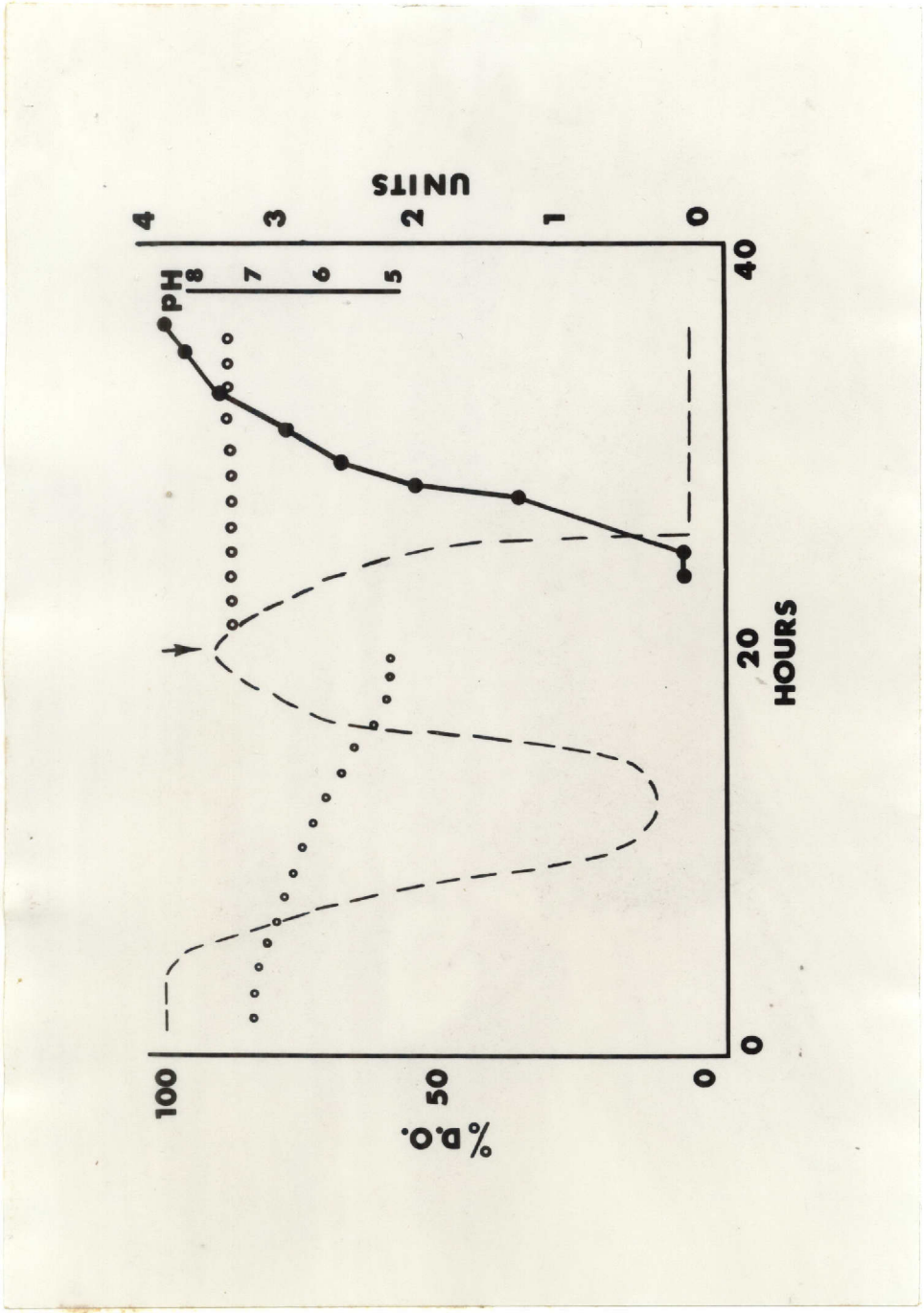
Figure 6. Synthesis of L-asparaginase by Escherichia coli A-1 pregrown in minimal media and transferred (arrow) to 3% Nutrient Broth plus 1% MSG. Symbols are as in Figure 2.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Mating Study

Escherichia coli A-1 was recognized as an F^- strain by mating experiments with known F^- and Hfr E. coli strains. This organism was able to receive donor markers for methionine with a recombination frequency of about 0.05%. Using this nutritional marker as proof of recombination, we obtained 350 isolates after primary selection of minimal agar. Approximately 95 were subcultured five times on minimal agar and established as stock cultures. The level of L-asparaginase produced by these isolates fell into three categories. Table VIII shows enzyme activities of the parental strains and three recombinants representing three categories of enzyme production. Escherichia coli HAP is the parental strain from which E. coli A-1 was derived (4) and is included only for comparison. Recombinants (R), like the parental strain E. coli A-1, produced L-asparaginase, but little L-glutaminase in the presence of MSG (Table VIII). A comparison of L-asparaginase activity in International Units per milliliter shows that, in the test tube culture system, the isolate designated R-17 produced very low levels of enzyme compared with E. coli A-1. On the other hand, if cell growth is taken into consideration (International Units per milligram of dry cells), it becomes evident that R-17 cells produce almost as much enzyme as the parental recipient E. coli A-1. Although E. coli R-26 grew relatively

TABLE VIII

L-ASPARAGINASE ACTIVITY IN TEST TUBE CULTURES OF PARENTAL AND RECOMBINANT STRAINS GROWN IN 3% NUTRIENT BROTH PLUS 1% MSG IN SHAKING TEST TUBES OVERNIGHT AT 37°C

E. coli strain	Genotype	L-Asparaginase[b] (IU/ml of culture fluid)	Cell Dry Wt (mg/ml)	L-Asparaginase (IU/mg of dry cells)	L-Glutaminase (IU/ml)
HAP	Prototrophic	2.5	2.60	1.0	0.0
A-1	$Met^{-}Las^{+}F^{-}$	10.8	2.20	4.9	0.2
4318	*thi*$^{-}$ *leu*$^{-}$$Las^{-}Hfr$	0.0	1.60	0.0	0.0
R-26 (1)[a]	Prototrophic	0.2	1.80	0.1	0.0
R-17 (2)	Prototrophic	3.2	0.76	4.2	0.0
R-69 (3)	Prototrophic	20.3	2.53	8.1	0.3

[a] (1) Includes all recombinants that produce 1 IU/ml; (2) includes those that produce 1 but 10 IU/ml; (3) includes those that produce 10 IU/ml.

[b] Enzyme activities represent average values of duplicate trials of a single recombinant chosen as representative of each class.

well in the test medium when compared to E. coli A-1, its L-asparaginase activity was very low, having a specific activity of only 0.1 IU/mg dry cells. L-Asparaginase activity of E. coli R-69 was approximately 100% higher than that of E. coli A-1, whereas the amount of growth of R-69 was only 13% greater.

To confirm the results from these studies, a series of experiments with the Bioflo fermentor was carried out. Previous studies with E. coli A-1 had shown that maximum enzyme levels are achieved in a three percent nutrient broth plus one percent MSG medium when the pH is maintained at 7.5 and the dissolved oxygen level remains at 0% saturation while aeration continued. L-Asparaginase activity of the recombinant strains was generally found to be greater than that obtained in test tube cultures, but they maintained the same relationship to each other (Table IX). For example, in test tube cultures, L-asparaginase activity of R-69 was >A-1, >R-17, >R-26. This same order holds true for the fermentor studies even though the level of enzyme produced by each strain was increased. Some of the increase in L-asparaginase activity of E. coli R-69 can be explained on the basis of better growth in the fermentor. However, L-asparaginase activity of R-69 per unit of cell dry weight was improved by 10% over that of E. coli A-1 and the 54% increase in International Units per milliliter

when compared with E. coli A-1 justifies the use of this strain for large-scale production. The enhanced activity of R-69 versus A-1 was found to be maximal using a 3% nutrient broth plus 1% MSG. Enzyme assays consistently showed that R-69 produced greater levels of enzyme than A-1, however, this level would vary depending on medium composition. L-Asparaginase levels for E. coli R-17 when grown under fermentation conditions was not significantly greater than the levels achieved in test tube studies, although its growth in the fermentor were 58% better. A similar comparison of R-69 shows L-asparaginase activity improved approximately 50% and growth only increased 17% (Table IX).

Additional characterization of the recombinant strains in the fermentor is compiled in Figure 7. Fermentor studies with the parental recipient, E. coli A-1, have shown that maximal enzyme synthesis is obtained in cells after 20 hours when induced by adjusting the pH to 7.5 and allowing the dissolved oxygen level to drop to zero (Figure 2). In the fermentor, E. coli R-69 produced more L-asparaginase than E. coli A-1, while strains R-17 and R-26 produced much less of the enzyme under the same conditions.

Because E. coli R-69 produced higher levels of L-asparaginase than E. coli A-1, the organism currently used for commercial production, it was selected for further studies.

Figure 7. Synthesis of L-asparaginase by Escherichia coli A-1, HAP and three prototrophic recombinants. Symbols are as in Figure 2.

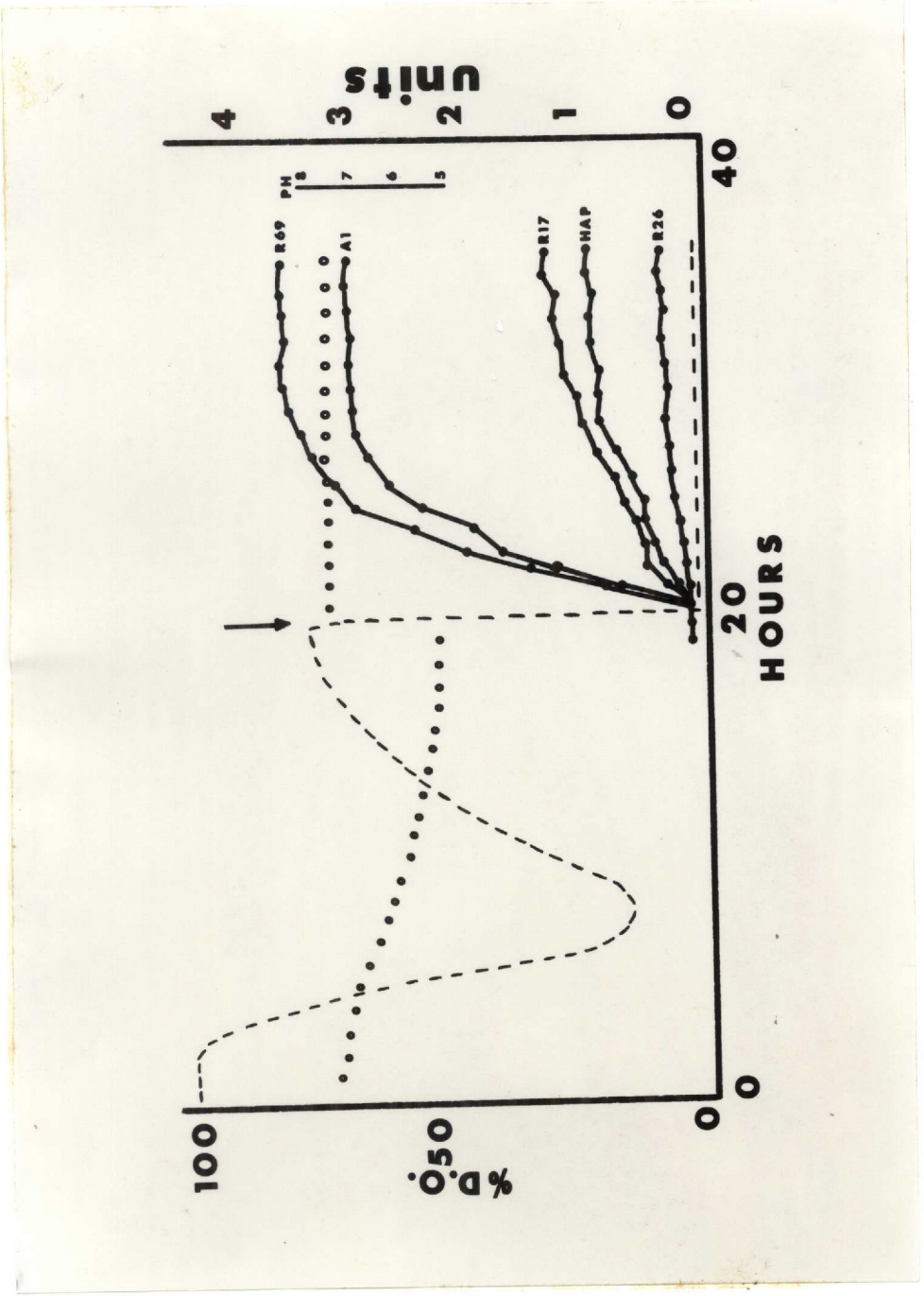
units
4
3
2
1
0
PH
8
7
6
5
R69
A1
R17
HAP
R26
40
20
HOURS
0
100
%D.O.
50
0

TABLE IX

CHARACTERISTICS OF PARENTAL AND RECOMBINANT STRAINS GROWN IN 3% NUTRIENT BROTH PLUS 1% MSG IN THE BIOFLO FERMENTOR AT A CONTROLLED pH OF 7.5

E. coli strain	Genotype	L-Asparaginase[b] (IU/ml of culture fluid)	Cell Dry Wt (mg/ml)	L-Asparaginase (IU/mg of dry cells)	L-Glutaminase (IU/ml)
A-1	$Met^{-}Las^{+}F^{-}$	19.8	2.30	8.7	0.3
4318	*thi*$^{-}$ *leu*$^{-}$$Las^{-}$Hfr	0.0	1.89	0.0	0.0
R-26 (1)[a]	Prototrophic	0.3	2.10	0.1	0.0
R-17 (2)	Prototrophic	4.1	1.20	3.3	0.0
R-69 (3)	Prototrophic	30.5	3.10	9.8	0.4
HAP	Prototrophic	3.2	3.50	0.9	0.0

[a] (1) Includes recombinants that produce 1 IU/ml; (2) includes those that produce 1 but 10 IU/ml; (3) includes those that produce 10 IU/ml.

[b] Enzyme activities represent average values of duplicate trials of a single recombinant chosen as representative of each class.

L-Asparaginase synthesis in this recombinant was inhibited by dissolved oxygen levels greater than zero in the same manner as seen with E. coli A-1. The inhibition of enzyme synthesis by excess oxygen dissolved in the medium was found to be reversible (Figure 8). Raising the propellor speed (600 rpm) to increase the dissolved oxygen level in the medium greatly inhibited L-asparaginase synthesis during the four-hour pulse when dissolved oxygen was elevated to the 50% saturation level. When the propellor was reduced to the normal operating speed of 400 rpm, the dissolved oxygen rapidly dropped to 0% and enzyme synthesis resumed. The addition of 1% glucose at the time of enzyme induction produced a transient repression of L-asparaginase synthesis (Figure 9).

Recent studies by Netrval (56, 57) have shown that many of the tricarboxylic acid cycle compounds enhance L-asparaginase acitivity in E. coli. Table X shows that, in every case, L-asparaginase levels were higher in R-69 than in E. coli A-1, and that monosodium glutamate and oxalacetate increased enzyme yields more than six other organic acids. While strain A-1 did not respond, by increased L-asparaginase acitivity, to any of the other six acids, strain R-69 responded well to four of them (malate, succinate, α-ketoglutarate and fumarate). The significance of this is not known.

Figure 8. Reversible inhibition of L-asparaginase synthesis in E. coli R-69 by dissolved oxygen (50% saturation). At 24 hours the propellor RPM was increased to bring the dissolved oxygen level to 50%. After 5 hours, the propellor RPM was reduced to its normal speed. Symbols are as in Figure 2.

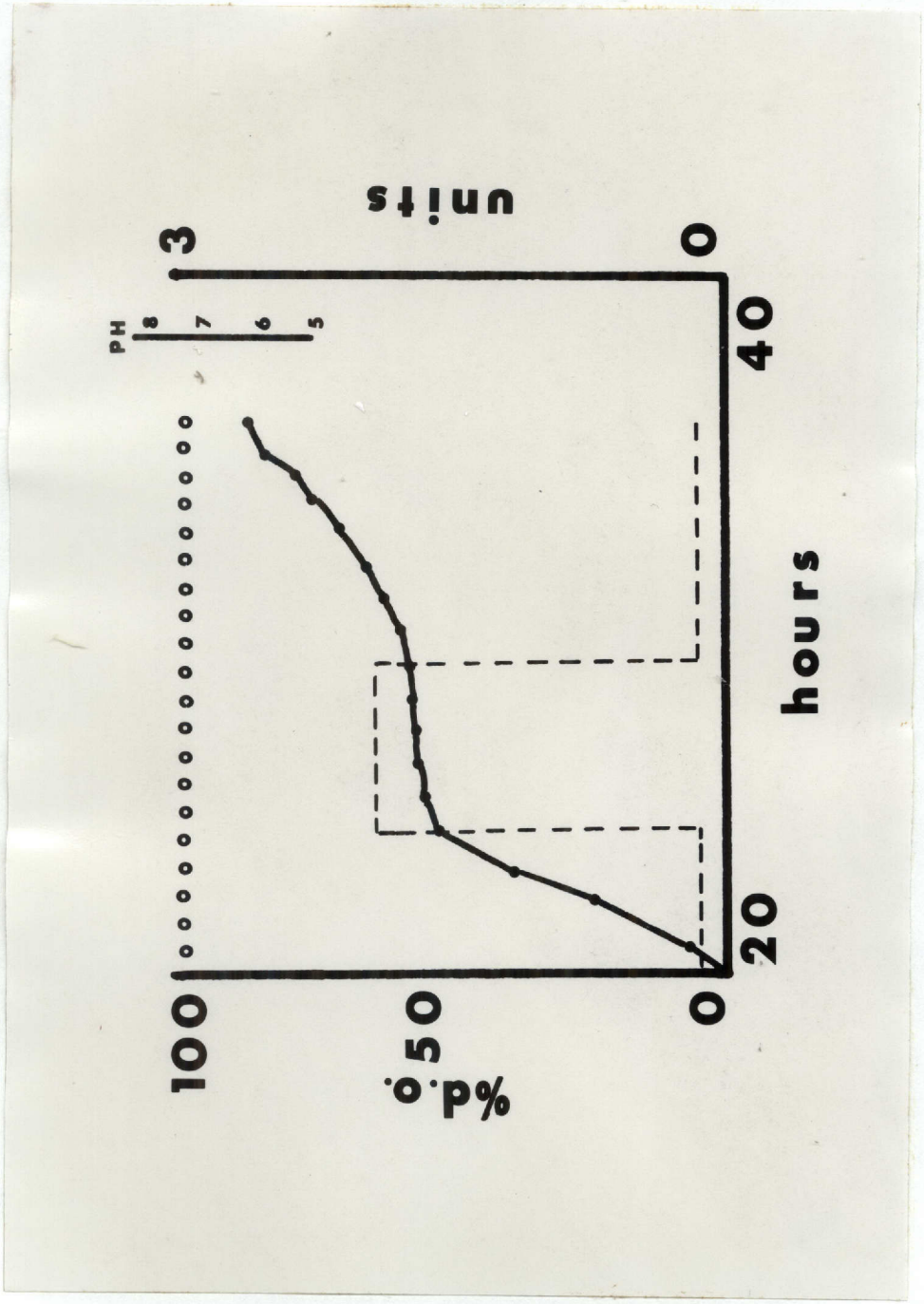
units
3
0
PH
8
7
6
5
40
hours
20
0
100
d.50
%

Figure 9. Transient inhibition of L-asparaginase synthesis in E. coli R-69 by the addition of 1% glucose at 24 hours. Symbols are as in Figure 2.

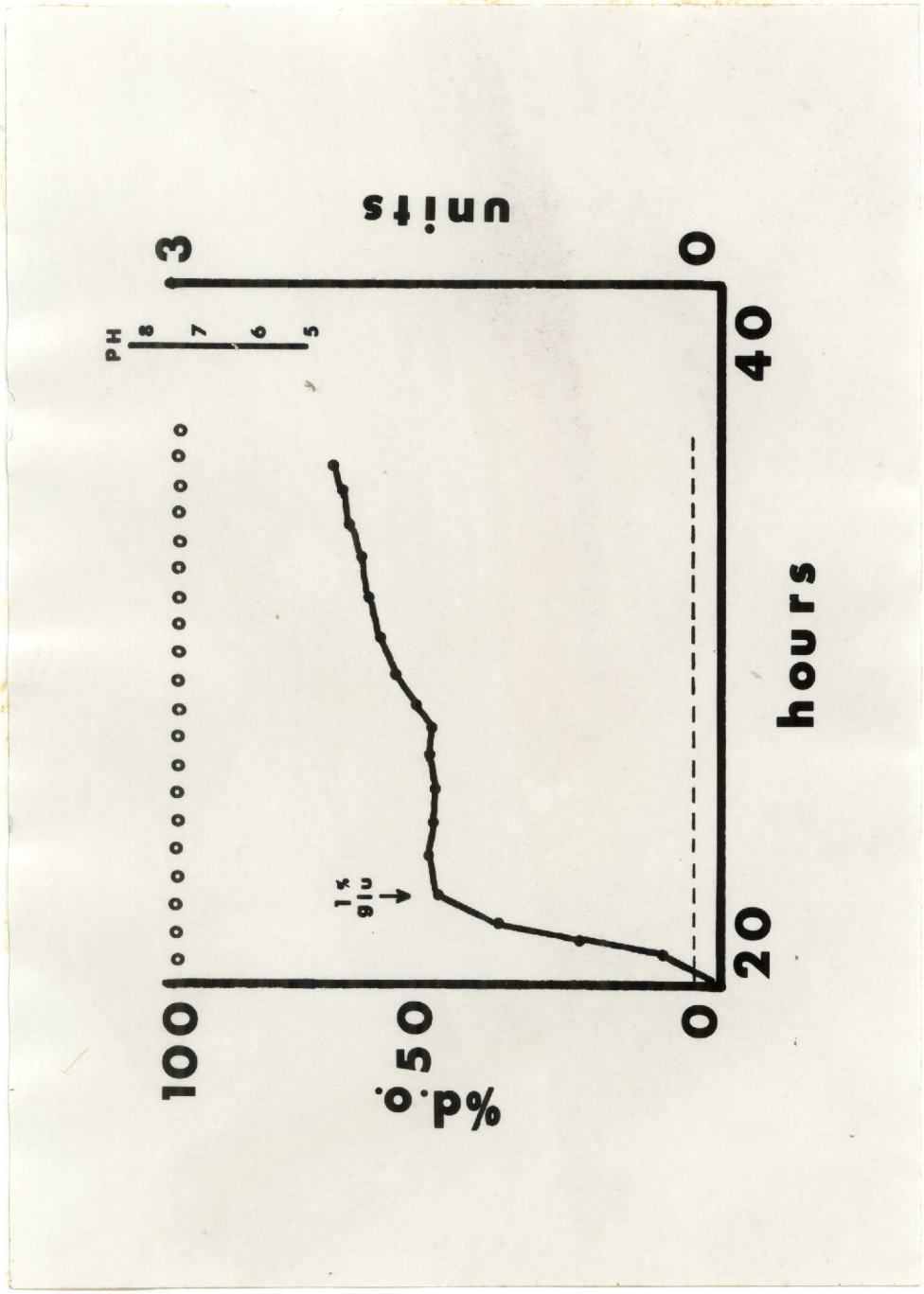
units
3
0
PH
8
7
6
5
40
hours
20
0
1% glu
100
50
0
%d.o.p

TABLE X

EFFECT OF VARIOUS TRICARBOXYLIC ACID CYCLE INTERMEDIATES ON THE ACTIVITY OF L-ASPARAGINASE BY *ESCHERICHIA COLI* A-1 AND *ESCHERICHIA COLI* R-69 IN SHAKING TEST TUBE CULTURES

Addition (0.5%) to 3% nutrient broth	L-Asparaginase Activity[a] (IU/mg dry cells)	
	E. coli A-1	*E. coli* R-69
None	2.7	2.9
MSG	4.8	7.7
Oxalacetate	4.6	6.5
Malate	3.2	5.0
Succinate	3.1	5.0
α-Ketoglutarate	2.9	4.6
Fumarate	2.8	4.5
Citrate	2.7	3.3
Isocitrate	2.7	3.2

[a] Average value of duplicate trials

CHAPTER V

DISCUSSION

Culture requirements for maximal synthesis of L-asparaginase vary from one microorganism to the other. Indeed, the rate of synthesis varies greatly in the same organism with environmental conditions. In this report, we have shown that L-asparaginase synthesis in cells of E. coli A-1 depend upon a complex of interactions, namely, nutrients, pH, oxygen transfer rate, and the presence of specific compounds such as glutamic acid, as well as genetic factors. These controlling factors are interdependent; thus, a change in one, greatly affects another. For example, the nutrient composition and concentration affects the rate of synthesis of L-asparaginase and that of other proteins as well so that the rate of growth of the organism varies also. On the other hand, if the pH is not precisely controlled, the organism may grow luxuriantly, but it may not synthesize L-asparaginase. It appears that this enzyme is simply one of many proteins directed by genes which are activated or repressed according to the culture conditions.

The effect of monosodium glutamate on synthesis is perplexing. The natural substrate for L-asparaginase is L-asparagine, but the maximal enhancement of L-asparaginase

synthesis by E. coli A-1, and other closely related strains, is obtained by adding glutamine or glutamate to the medium. Imada et al (37) showed that many organisms which produce L-asparaginase also produce L-glutaminase: in another study (17), it was shown that L-asparagine induced L-asparaginase only under anaerobic conditions. In our experiments, glutamate or glutamine probably do not function as classical enzyme inducers. Even L-asparagine, the natural substrate, does not support as much L-asparaginase activity as certain other amino acids (Figure 1): in fact, other amino acids are required for its synthesis (Table II). We have not ascertained the mechanism of action of synthesis of this unusual enzyme. It is well known, however, that the amide group of L-glutamine serves as the immediate source of nitrogen atoms in a wide variety of compounds including the purines and pyrimidines and the amino acids, histidine, asparagine, glutamic acid, tryptophan, and arginine. Also, the coenzymes, folic acid and nicotinamide nucleotides are derived from this nitrogen source. The regulation of glutamine in bacterial metabolism is under the control of several enzymes which are susceptible to repression and feedback inhibition by end products of glutamine metabolism. In addition, a cascade system consisting of several metabolite-regulated enzymes and a small regulatory protein modulate the adenylylation and deadenylylation of glutamine synthetase (28).

Based on the increase seen in enzyme activity when compounds of the tricarboxylic acid cycle are added, as shown in Table X, it is proposed that oxalacetate is closer to the true inducer molecule than monosodium glutamate, since it is chemically simpler and induces comparable levels of enzyme activity. However, the nature of induction by this compound is not known.

Netrval (56, 57) has speculated about a mechanism in which L-asparagine was the actual agent stimulating or inducing the synthesis of L-asparaginase, whereas the other substances were presumed to be transformed metabolically into L-asparagine. Netrval also found that most of the amino acids and many dicarboxylic acids have some stimulatory effect and, in view of this, the explanation offered by Netrval is no longer satisfactory. Using several amino acids and lactate, it was shown that the stimulatory effects of two different amino acids, or of one amino acid and lactate were usually cumulative. From these findings the author proposed that the mechanism of the stimulatory effect was nonspecific. Netrval (56, 57) also stated that there appeared to be a definite connection between the Krebs cycle and the regulation of L-asparaginase synthesis.

The cumulative effects of groups of amino acids on the synthesis of L-asparaginase was first described by Cedar and Schwartz (16, 17). Interestingly, supplementation of the casein hydrolysate medium with L-asparagine or L-glutamine,

alone or in combination did not stimulate the production of the enzyme. The formation of L-asparaginase was inhibited by the addition of fermentable sugar, particularly glucose: xylose, maltose, and galactose inhibit enzyme production to a lesser extent (16). Cedar and Schwartz (17) speculated about a system of control involving an inducer derived from the metabolism of amino acids during anaerobic growth. They also suggested that there could be a metabolite which acts as a suppressor of L-asparaginase synthesis during growth of the organism, and that the utilization of amino acids during anaerobic conditions resulted in a decrease in the concentration of this repressor. Neither the substrate, L-asparagine, nor its products, L-aspartate and ammonia, increase enzyme activity (16, 17). One potential problem with the data of Cedar and Schwartz was that the amino acid studies were performed in a minimal medium containing one percent glucose. Cedar and Schwartz clearly showed that glucose inhibited enzyme synthesis yet they used it as a carbon source in these experiments. This could be a reason why glutamine failed to stimulate enzyme yield, even in casein hydrolysate-supplemented media.

Glutamine occupies a central position in bacterial metabolism. Control of glutamine synthesis is necessary to the control of amino acid synthesis and to the control of

protein synthesis and growth (28). Under conditions required for high L-asparaginase synthesis by E. coli A-1, it would seem reasonable that glutamine synthetase levels would be quite low since it is known that the enzyme is repressed in high concentrations of ammonia (73). Boris and Magasanik (65) have recently suggested that L-asparaginase synthesis in Klebsiella aerogenes is possibly under the control of glutamine synthetase. These authors found that mutants lacking glutamine synthetase also failed to produce L-asparaginase. Conversely, strains which produced high constitutive levels of glutamine synthetase contained high levels of L-asparaginase activity. The formation of L-asparaginase appeared to be regulated in parallel with that of other enzymes capable of supplying the cell with ammonia or glutamate.

The concomitant requirement of a controlled pH and no dissolved oxygen for maximal L-asparaginase synthesis describes how interdependent these factors are. That is, the pH of the medium will affect the rate of bacterial division, and consequently, the rate at which oxygen is used by the culture biomass. Dissolved oxygen levels obviously affect the rate of bacterial metabolism, hence, growth and ultimately the medium pH. For optimum levels of L-asparaginase activity, the pH has to be between 7 and 8 and a dissolved oxygen level neither above nor below the critical oxygen level described. The enzyme induction by pH change

at 20 hours shown in Figure 2 represents a novel approach at studying enzyme synthesis after establishing maximal growth of the culture.

It should be mentioned at this time that data derived from the fermentor studies show that the addition of glucose can cause both a transient and permanent repression of enzyme synthesis. A transient repression occurs if 1% glucose is added during the period of active synthesis of L-asparaginase. Enzyme synthesis resumes, however, after approximately 5 hours (Figure 9). The increase in enzyme yield obtained by adding 1% MSG could not be observed in these studies due to the presence of glucose: therefore, glutamate was not added (Table VII). It was found, however, that the "glutamate effect" could be observed if the glucose-grown cells were washed and resuspended in fresh MSG-supplemented media containing no glucose (Figure 6). This suggested that some kind of repressor resulting from glucose metabolism was present and that once removed, enzyme synthesis commenced. These results are in general agreement with the literature since glucose is known to inhibit or repress many degradative enzymes (73). For induction studies, the presence of glucose (0.5%) was necessary to achieve a certain amount of growth without producing L-asparaginase (Figure 2). This condition was met by a drop in pH from 7.0 to 5.5 during growth. This drop in pH was accompanied by a rise in the dissolved

oxygen level which by itself would inhibit the synthesis of the enzyme. When the pH was artificially raised to 7.5 at 20 hours, the dissolved oxygen was depleted in a matter of 15 minutes by the metabolic activity of the bacteria enhanced at pH 7.5. L-Asparaginase synthesis then began, reaching maximal levels in approximately 8 hours.

The question could be asked concerning the induction of L-asparaginase by this method; is it a specific induction or only of a general and correlary nature? That is, does the pH change and consequent induction of L-asparaginase represent a specific response by that enzyme alone or does this pH change cause a general induction of other enzymes such as glutamine synthetase and glutamate dehydrogenase which can also play a role in nitrogen metabolism? One point of view could be that these conditions represent a general induction phenomonon and are solely designated to provide the cells with conditions for transporting the various amino acids required for protein synthesis. These amino acids are then used in part for L-asparaginase synthesis rather than being primarily used for cell wall, cell membrane, DNA or RNA synthesis. In other words, the biosynthetic machinery is ready to channel more of the amino acid building blocks into proteins other than those needed for growth.

The requirement of a specific aeration rate for optimal enzyme synthesis also raises a question as to what mechanism

of control is exerted when dissolved oxygen levels either exceed or fall below this level. It is well known that the adenylate charge of the bacterial cell expresses the overall energy state of the organism in question (73), and that adenosine triphosphate (ATP) is involved in the control as a feedback inhibitor of isocitrate dehydrogenase, an allosteric enzyme. It seems reasonable that under conditions of oxygen saturation, high ATP levels would block the TCA cycle via isocitrate dehydrogenase, and hence, block the metabolic breakdown of glutamate required for the anaplerotic process of L-asparaginase synthesis. Dissolved oxygen levels below that of the optimum would perhaps allow the accumulation of NADH which is also known to act as a feedback inhibitor of isocitrate dehydrogenase. Since the activity of this enzyme is obviously controlled to some degree by the adenylate charge, it could be indirectly involved in the regulation of L-asparaginase synthesis. The relationship between oxygen tension and the synthesis of a specific enzyme must be complex and is certainly beyond our understanding at this time.

In contrast to the many reports on the physiological conditions that affect the kinetics of L-asparaginase synthesis, little work has been reported on the genetics of E. coli or any other bacteria with respect to synthesis of this enzyme. The isolation of strains that produce large amounts of enzyme is made difficult by the lack of suitable

media and adequate techniques for selection. In view of this, it was assumed that a genetic procedure could be designed to yield strains of E. coli that would produce large amounts of L-asparaginase over and above that of the parental strain E. coli A-1.

The non-isogenic mating experiment described in the RESULTS section of this report resulted in the isolation of recombinant progeny with varying capacities to synthesize the enzyme. Approximately 10% produced greater levels of L-asparaginase than the parental strain E. coli A-1. The remainder produced lower levels, but are considered to be recombinants since they are prototrophic organisms obtained by mating auxotrophic parental strains, and, also, because the amounts of L-asparaginase are different from those produced by either parent strain. The isolation of recombinants capable of producing 30 IU/ml from the parental strain, E. coli A-1, which produced 20 IU/ml, and CGSC 4318, which is a nonproducer, raises questions regarding the genetics of L-asparaginase synthesis in E. coli. Are these recombinants stable partial diploids and the increase in enzyme activity simply an expression of more than one gene? Or have there been recombinational events which have deleted specific repressors? These recombinants also differed from the parental strains in growth characteristics. Culture studies showed that variations in total cell yield were independent of L-asparaginase synthesis. It is ob-

vious that E. coli possesses a complex physiological mechanism for the production of L-asparaginase, but the non-isogenic mating study presented indicates that the genetic mechanism involved also may be very complicated. Non-isogenic crosses could be exploited as a means of producing large amounts of proteins or enzymes by bacteria which, in the past, have been difficult to produce by conventional methods.

On a more practical note, however, the work reported here has yielded a new prototrophic recombinant, E. coli R-69, which is now being used in the large-scale (1,000 liter) production of L-asparaginase. This organism routinely produces 30 IU/ml of culture fluid, an increase of 10 IU/ml over that produced by E. coli A-1. The total yield from E. coli R-69 was improved by some 10^7 IU of L-asparaginase per run in the 1,000-liter fermentor as compared to E. coli A-1.

It should also be noted that the yield of L-asparaginase using a given culture medium should be viewed in terms of the amount of cell mass devoted to L-asparaginase activity. The best results in our test tube studies (27 IU/ml) were obtained by using cells first grown in a minimal medium and transferred to 3% nutrient broth containing 1% monosodium glutamate (Table IV). Cells grown in a minimal medium containing 3.5% MSG and an amino acid pool produced only 5.0 IU/ml (Table II). The yield of enzyme

was, however, 8.9 (Table IV) and 7.3 IU per mg of dry cells (Table II), respectively. Thus, approximately the same amount of enzyme was produced in both cases. However, if measured in terms of International Units per milliliter, it appears as if the pregrown cells produced five times as much as those grown in minimal media. In view of this finding, it is suggested that comparisons of enzyme yields be made in terms of International Units per milligram cell dry weight or cell protein.

Corn steep liquor is a well known and widely used fermentation medium and has been used for some time in the large-scale production of L-asparaginase at Wadley Institutes of Molecular Medicine, Dallas, Texas, and by other commercial producers as well (66, 67). Although inexpensive, this medium is undesirable due to the time consuming labor required to resuspend, cook, and separate the soluble fraction from insoluble particulates. The medium requires adjustment of the pH to 7.0 before autoclaving and variability in the composition of the final product was found to give inconsistent enzyme yields from one fermentation run to the next. In addition, microscopic examination of this medium showed gross contamination by microorganisms. Although autoclaving kills such contaminating bacteria, it would not be desirable to have them present in the fermentation medium.

Part of the work reported here was devoted to develop-

ing a fermentation medium that would be economical, simple to prepare, free of large numbers of contaminating organisms, and capable of supporting the growth of E. coli and the production of large amounts of L-asparaginase. Based on the physiological studies described, this was accomplished. Two media were developed, one of which (Table VI) was found to be most economical, easiest to prepare, and more consistent in supporting good yields of L-asparaginase.

In summary, a detailed view of the literature presents confusing, and even contradictory, statements concerning the regulation of L-asparaginase synthesis in E. coli. While some authors claim that high yields are achieved under aerobic conditions, others state that anaerobic conditions are required. Another has even used a combination of the two conditions to achive optimum levels of enzyme. The data, while confusing, may indeed be a reflection of nothing more than variation between strains of the organisms used and the conditions under which the studies were conducted. With the recent development of dissolved oxygen membranes, some of these contradictions may be resolved. It appears that an optimal level of dissolved oxygen is required for maximal enzyme yields, and that too much or too little is inhibitory for some microorganisms. There seems to be a general agreement that high levels of amino acids and/or tricarboxylic acid cycle intermediate compounds are necessary for optimum activity. Also, the presence of

fermentable carbohydrates tend to reduce activity, even if the pH is controlled. The optimum pH lies between 7.0 and 8.0, and loss of activity results if this range is not controlled.

The literature contains no information regarding the bacterial genetics of this particular enzyme, probably because of several inherent problems: little enzyme is produced on agar media: no selection or quick assay procedure is currently available for distinguishing a high from a low producer: and maximal yields are only obtained in test tube or fermentation cultures. The mating study presented allows the isolation of recombinants having levels of enzyme activity above and below that of the parental strain, E. coli A-1. It seems reasonable to assume that an E. coli A-1 Hfr could be produced and that it could be mated with isogenic F^- recipients of the same strain, i.e., E. coli A-1. By this method, it would be possible to map the position of the L-asparaginase gene(s). The data presented in this study show that L-asparaginase synthesis in E. coli is the result of a very complex sequence of interdependent events. The specific nature of these events is not understood at this time, but some general regulatory mechanisms have been observed and reported.

Additional data that comprise part of this dissertation are included in the two reports attached. These were published in Applied and Environmental Microbiology and

BIBLIOGRAPHY

1. Adamson, R. H., and S. Fabro. 1968. Antitumor activity and other biological properties of L-asparaginase. Cancer Chemotherapy Rep. 52:617-626.

2. Ashworth, L. A., A. P. MacLennan. 1973. Immunological tolerance to Erwinia carotovora L-asparaginase. Int. Arch. Allergy Appl. Immunol. 45:915-929.

3. Ashworth, L. A., A. P. MacLennan. 1974. Comparison of the L-asparaginases from Escherichia coli and Erwinia carotovora as immuno suppressants. Cancer Res. 34:1353-1359.

4. Barnes, W. R., G. L. Dorn, and G. R. Vela. 1977. Effect of culture conditions on the synthesis of L-asparaginase by E. coli A-1. Appl. and Env. Microbiol. 33:257-261.

5. Barnes, W. R., G. R. Vela, and G. L. Dorn. 1978. Physiology of L-asparaginase synthesis in recombinants of Escherichia coli A-1. Appl. and Env. Microbiol. 35:766-770.

6. Bascomb, S., G. T. Banks, M. T. Skarstedt, A. Fleming, and K. A. Bettleheim. 1975. The properties and large-scale production of L-asparaginase from Citobacter. J. Gen. Microbiol. 91:1-16.

7. Bilimoria, M. H. 1969. Conditions for the production of L-asparaginase 2 by coliform bacteria. Appl. Microbiol. 18:1025-1030.

8. Boeck, L. D., R. W. Squirer, M. W. Wilson, and P. P. K. Ho. 1970. Effect of glucose and low oxygen tension on L-asparaginase production by a strain of Escherichia coli. Appl. Microbiol. 20:964-969.

9. Boeck, L. D., and P. P. K. Ho. 1973. L-asparaginase production during static incubation of aerobically grown Escherichia coli. B. Can. J. Microbiol. 19:1251-1257.

10. Boyse, E. A., L. J. Old, H. A. Campbell, and L. T. Mashburn. 1967. Suppression of murine leukemia by L-asparaginase. J. Exp. Med. 125:17-31.

11. Broome, J. D. 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effect. Nature. 191:1114-1115.

12. Broome, J. D. 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its anti-lymphoma effect. J. Exp. Med. 118:99-120.

13. Broome, J. D. 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its anti-lymphoma effect. J. Exp. Med. 118:121-148.

14. Broome, J. D. 1968. Studies on the mechanism of tumor inhibition by L-asparaginase. J. Exp. Med. 127:1055-1072.

15. Campbell, H. A., L. T. Mashburn, E. A. Boyse, and L. J. Old. 1967. Two L-asparaginase from E. coli. J. Biochem. 6:721-725.

16. Cedar, H., and I. H. Schwartz. 1967. Localization of the two L-asparaginases in anaerobically grown Escherichia coli. J. of Biol. Chem. 242:3753-3758.

17. Cedar, H., and J. H. Schwartz. 1968. Production of L-asparaginase II by E. coli. J. Bacteriol. 96:2043-2048.

18. Chaikorska, S. M., R. A. Makarova, A. N. Meshkov, and M. M. Levitov. 1974. Effect of cultivation conditions on the biosynthesis of L-asparaginase by E. coli strains. All-Union Research Institute of Antibiotics. (Russian). Mikrobiologia. 1:369-374.

19. Davidson, L., M. Burkom, A. Sam, L. Chang, and B. Kitto. 1977. L-Asparaginase from Citrobacter freundii. Biochimica Biophysica Acta. 480:282-294.

20. Dolowny, W. D., L. M. Elrod, R. N. Ammeraal, and R. Schrek. 1967. Toxicity of L-asparaginase to resistant and susceptible lymphoma cells in vitro. Proc. Soc. Exp. Biol. (N. Y.) 125:598-601.

21. Ehrman, M., H. Cedar, and J. H. Schwartz. 1971. L-Asparaginase II of Escherichia coli. J. Biol. Chem. 246:88-94.

22. El-Din, S. M., and S. Foda. 1976. Kinetics and properties of L-glutaminase and L-asparaginase activities of Pseudomonas ovalis. Zentbahl. Bakteriol. Naturwiss. 131:399-496.

23. Eryomenko, V. V., and N. N. Sokolov. 1974. Cultivation conditions of E. coli ATCC 9637 with a high asparaginase activity. Paik. Biokhimia. Mikrobiologia. 10:53-59.

24. Foda, M. A., E. Z. Khafogy, and M. B. Samio. 1974. Production of L-asparaginase by Pseudomonas ovalis. Zentealbl. Bakteriol. Naturwiss. 129:525-532.

25. Gaffar, S., and Y. I. Shethra. 1975. Partial purification and antitumor activity of L-asparaginase from Azotobacter vinelandii. Curr. Sci. 44:727-729.

26. Gaffar, S., and Y. I. Shethra. 1977. Purification and some biological properties of L-asparaginase from Azotobacter vinelandii. App. Env. Micro. 33:508-514.

27. Garaev, M. M., and E. I. Golub. 1977. Mechanism of action of glucose on L-asparaginase synthesis by Escherichia coli bacteria. Mikrobiologia. 46:433-439.

28. Ginsberg, A., and E. R. Stadtman. 1973. Regulation of glutamine synthesis in Escherichia coli. In S. Prusiner (ed.), the enzymes of glutamine metabolism. Academic Press, Inc., New York.

29. Haley, E. E., G. A. Fischer, and A. D. Welch. 1961. The requirement of L-asparaginase of mouse leukemia cells in L5178 in culture. Cancer Res. 21:532-537.

30. Heinemann, B., A. J. Howard. 1969. Production of tumor-inhibitory L-asparaginase by submerged growth of Serratia marcescens. Appl. Microbiol. 18:550-554.

31. Heinemann, B., A. J. Howard, and H. J. Palocz. 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by Serratia marcescens. App. Micro. 19:800-804.

32. Hill, J. M., J. Roberts, E. Loeb, A. Khan, A. MacLellan, and R. W. Hill. 1967. L-asparaginase therapy for leukemia and other malignant neoplasms. J. Am. Med. Assoc. 202:882-888.

33. Hill, J. M., E. Loeb, A. MacLellan, A. Khan, and N. O. Hill. 1970. L-asparaginase therapy: Clinical results in acute leukemia and other malignant diseases. International Symposium on L-asparaginase. Paris. 273-280.

34. Hill, J. M., E. Loeb, A. MacLellan, N. Hill, A. Khan, and A. Albaycak. 1972. Treatment of acute leukemia. Wadley Medical Bulletin. 2:63-76.

35. Holmquist, N. D. 1963. Effect of normal sera of several related rodents on 6C3HED lymphoma in vivo. Proc. Soc. Exp. Biol. (N.Y.) 113:444-445.

36. Igarasi, S., A. Imada, K. Nalsahama, T. Matsummota, K. Otsu. 1974. New antilymphoma L-asparaginase from Fusarium species. Experentia. 30:814-816.

37. Imada, A., S. Igarasi, K. Nakahoma, and M. Isono. 1973. Asparaginase and glutaminase activities of microorganisms. J. Gen. Microbiol. 76:85-99.

38. Ito, T., T. Ise, T. Sawabe, W. Nakayama, and H. Okure. 1973. Studies on antibody formation by L-asparaginase. Jap. J. Clin. Hemotol. 14:604-610.

39. Khan, A., J. M. Hill. 1970. Suppression of skin hypersensitivity and antibody formation by L-asparaginase. J. Immunol. 104:679-683.

40. Kidd, J. G. 1953. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum or rabbit serum. J. Exp. Med. 98:565-582.

41. Kidd, J. G. 1953. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. II. Studies on the nature of the active serum constituent. J. Exp. Med. 98:583-606.

42. King, O. Y., J. R. Wilbin, D. M. Munford, W. W. Sutow. 1974. Therapy with Erwinia L-asparaginase in children with acute leukemia after anaphylaxia to E. coli L-asparaginase. Cancer. 33:611-614.

43. Lambert, G., J. P. Horvey, and Y. Delmar. 1971. Research on anti-asparaginase antibodies in leukemia subjects treated with this enzyme. Ann. Inst. Pasteur. 22:305-312.

44. Liu, Y. P., and R. J. Speer. 1976. The carboxyl-and-amino-terminal amino acids and other properties of L-asparaginase from Escherichia coli. J. Formosan Med. Assoc. 75:168-174.

45. Liu, F. S., and J. E. Zajic. 1972. L-asparaginase synthesis by Erwinia aroideae. Appl. Micro. 23:667-668.

46. Liu, F. S., and J. E. Zajic. 1973. Fermentation kinetics and continuous process of L-asparaginase production. Appl. Micro. 25:92-96.

47. Liu, F. S., and J. E. Zajic. 1973. Effect of oxygen-transfer rate on production of L-asparaginase by Erwinia aroideae. Can. J. of Microbiol. 19:1153-1158.

48. Loeb, E., J. M. Hill, A. MacLellan, N. Hill, A. Khan, and R. Speer. 1972. Five year experience with L-asparaginase in the treatment of acute leukemia. Wadley Medical Bulletin. 2:23-32.

49. Logsdon, E. E. 1960. A method for the determination of ammonia in biological materials on the autoanalyzer. Ann. N. Y. Aca. Sci. 87:801-807.

50. Loos, M., S. Vadlamuhi, M. Meltzer, S. Shifrin, T. Borsos, and A. Goldin. 1972. Detection of endotoxin in commercial L-asparaginase preparations by complement fixation and separation by chromatography. Cancer Res. 32:2292-2296.

51. Manning, G. B., and L. L. Campbell. 1957. The asparagine deamidase of Bacillus coagulans and Bacillus stereothermophilus. Can. J. Microbiol. 3:1001-1005.

52. Mashburn, L. T., and J. C. Wriston. 1963. Tumor inhibiting effect of L-asparaginase from E. coli. Arch. Biochem. 105:450-452.

53. Mashburn, L. T., and J. C. Wriston. 1963. Tumor inhibitory effect of L-asparaginase. Biochem. Biophys. Res. Comm. 12:50-53.

54. Mashburn, L. T., L. M. Landin, and C. S. Gordon. 1970. The relation of asparagine, asparaginase and asparagine synthesis to lymphosarcoma in the mouse. International Symposium on L-asparaginase. Paris. 13-24.

55. Mikucki, J., and J. Szarapinska-Kwaszewska. 1979. Production of Staphylococal L-asparaginase in a chemically defined medium. FEMS. Letters. 5:147-150.

56. Netrval, J. 1973. Effect of Organic and amino acids on L-asparaginase production by Escherichia coli. Arch. Mikrobiol. 92:345-352.

57. Netrval, J. 1977. Stimulation of L-asparaginase production in Escherichia coli by organic and amino acids. Folia Microbiol. 22:106-116.

58. Old, L. J., E. A. Boyse, H. A. Campbell, R. S. Brodney, J. Fidler, and J. D. Teller. 1967. Treatment of lymphosarcoma in the dog with L-asparaginase. Cancer. 20:1066-1070.

59. Old. L. G., H. F. Oettgen, E. A. Boyse, et al. 1967. Inhibition of leukemia in man by L-asparaginase. Cancer Res. 27:2619-2631.

60. Old, L. J., E. A. Boyse, H. A. Campbell, and G. M. Davis. 1963. Leukemia inhibiting properties and L-asparaginase activity of sera from certain South American rodents. Nature. 198:801-806.

61. Ott, J. L. 1960. Asparaginase from Mycobacteria. J. Bacteriol. 80:355-357.

62. Peterson, R. E., and A. Ciegler. 1969. L-asparaginase production by Erwinia aroideae. Appl. Micro. 18:64-67.

63. Prager, M. D., and N. Bachynsky. 1968. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem. Biophys. Res. Comm. 31:43-47.

64. Ramadan, M. E., R. E. Ashmar, and D. M. Greenberg. 1964. Purification and properties of glutaminase and asparaginase from a Pseudomonad. Biochem. and Biophys. 108:143-146.

65. Resnick, A. D., and Boris Magasanik. 1976. L-asparaginase of Klebsiella aerogenes. J. Biol. Chem. 251:2722-2728.

66. Roberts, J., G. Burson, and J. M. Hill. 1968. New procedure for purification of L-asparaginase with high yield from Escherichia coli. J. Bacteriol. 95:2117-2123.

67. Roberts, J., G. Burson, J. M. Hill, and R. J. Speer. 1968. Purification and properties of E. coli L-asparaginase. Div. of Biol. Chem. of the SCS 156th meeting. Abstract #152. Atlantic City, N. J.

68. Roberts, J. 1976. Purification and properties of a highly potent antitumor glutaminase - asparaginase from Pseudomonas-7A. J. of Biol. Chem. 251:2119-2123.

69. Robinson, R. S., B. Beck. 1969. L-asparaginase synthesis by Escherichia coli B. Biotech. Bioeng. 11:1211-1225.

70. Rowley, B., and J. C. Wriston. 1967. Activity of Serratia marsescens L-asparaginase. Biochem. Biophys. Res. Comm. 28:16-19.

71. Rulpanova, E. S., and V. V. Eremko. 1974. Regulation of L-asparaginase formation by amino acids in Bacillus mesentericus. Mikrobiologia. 45:259-262.

72. Sobin, L. H., and J. G. Kidd. 1965. A metabolic difference between two lines of Lymphoma 6C3HED cells in relation to asparagine. Proc. Soc. Exp. Biol. Med. 119:325-328.

73. Stadtman, E. R. 1970. Mechanisms of enzyme regulation in metabolism. In. P. Boyer (ed.), The enzymes, Academic Press, Inc., New York.

74. Sugira, K. 1968. Effect of L-asparaginase on transplantable and spontaneous tumors from mice and rats. Cancer Chem. Report. Part 1. 53:189-194.

75. Svobodova, O., Strbanova-Necinova. 1973. Induction of L-asparaginase synthesis in Escherichia coli. Biochim. Biophys. Acta. 321:643-649.

76. Tatsumi, N. 1970. Result of the treatment with L-asparaginase in malignant diseases. International Symposium of L-asparaginase. Paris. P. 323-324.

77. Tsuji, Y. 1957. Amidase action of bacteria. Chem. abst. 53:10350-10352 (1959).

78. Ubuka, T., and A. Meister. 1970. Incorporation of ^{15}N from L-asparagine-^{15}N-Amide and L-glutamine ^{15}N-Amide into the nucleic acid and protein of mouse leukemia cells. International Symposium on L-asparaginase. Paris. P. 73-78.

79. Vinogradov, B. D., A. G. Chigaleichik, D. A. Pirieva, V. M. Ushakow, and S. S. Rylkin. 1976. Formation of L-asparagine and L-glutamine deamidases by bacterial cultures. 45:23-27.

80. Wade, H. E., R. Elsworth, D. Herbert, J. Kippu, and K. Sargent. 1968. A new L-asparaginase with antitumor activity. Lancet. 2:776-777.

81. Yellin, T. O., and J. C. Wriston, Jr. 1966. Antagonism of purified asparaginase from guinea pig serum toward lymphoma. Science. 151:998-999.

82. Zhukov, A. V., V. V. Eremko. 1974. Continuous cultivation used to increase asparaginase and glutaminase activities in Pseudomonas fluorescens AG-69. Pakladnaia Biokhmia. 10:709-712.

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Effect of Culture Conditions on Synthesis of L-Asparaginase by *Escherichia coli* A-1

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The nutritional requirements and culture conditions affecting biosynthesis of L-asparaginase in a mutant of *Escherichia coli* HAP designated strain A-1 were studied. Asparaginase activity was increased by the addition of L-glutamic acid, L-glutamine, or commercial-grade monosodium glutamate. The rate of enzyme synthesis was dependent on the interaction between the pH of the culture and the amount of oxygen dissolved in the medium. A critical oxygen transfer rate essential for asparaginase formation was identified, and a fermentation procedure is described in which enzyme synthesis is controlled by aeration rate. Enhancement of L-asparaginase activity by monosodium glutamate was inhibited by the presence of glucose, culture pH, chloramphenicol, and oxygen dissolved in the fermentation medium.

L-Asparaginase therapy, alone or in combination with other drugs, is finding increased success in the management of acute lymphocytic leukemias. In recent clinical trials, significant remissions were obtained in more than 50% of patients treated with 3,000 to 9,000 IU/kg per day (6, 10). Dosages of this magnitude require large amounts of purified enzyme, and increasing recognition of successful therapy will generate increased demand.

Recent reports (1, 5, 9) show that most organisms studied yield only 1.3 to 4.0 IU of L-asparaginase per ml of culture medium. In general, the successful production of fermentation products depends on yield per unit volume of culture fluid. Since asparaginase is extracted from whole cells, efficiency in production will be determined by cell density and the amount of enzyme each cell produces. In this report, we emphasize the amount of enzyme produced per unit of cell dry weight because it describes the yield of enzyme per cell better than the commonly used designation of enzyme per milliliter of culture fluid. The organism described here, *Escherichia coli* A-1, produces high levels of enzyme in comparison with other organisms reported in the literature (1-5, 9, 12).

The effects of culture medium, culture pH, and oxygen transfer rate on asparaginase synthesis have been shown to vary for different organisms (1-3, 5, 8, 13), and a comprehensive view of this literature reveals a complex and variable set of culture parameters that affect asparaginase in different ways. For example, *Serratia marcescens* produces greater quantities of L-asparaginase with limited aeration than it does anaerobically (5), whereas either of these conditions has little effect on *Erwinia aroideae* (8). *E. coli* A-1 shows a response to dissolved oxygen unlike that reported for other bacteria or other strains of *E. coli*. The data presented prove that a precise amount of dissolved oxygen is essential for maximal asparaginase synthesis; we assume that this critical level of oxygen is a consideration of major importance not fully recognized in the past.

MATERIALS AND METHODS

Organism. *E. coli* A-1 (Met^-) was obtained from the stock culture collection at Wadley Institutes of Molecular Medicine. It is a mutant produced by ultraviolet irradiation of *E. coli* HAP (12). Stock cultures were maintained on slants of 3% nutrient agar (30 g of dehydrated Difco nutrient broth powder and 15 g of agar dissolved in 1 liter of distilled water) in the refrigerator. Working cultures were obtained by streaking out stock cultures and picking isolated colonies from plates showing no contamination.

Test media. Ten different media were tested, but only the four that gave the highest yields of asparaginase are described here. They were prepared by dissolving the commercial powder to the strength indicated in distilled water and adjusting the pH to 7.0.

Minimal medium. *E. coli* A-1 was grown in a medium consisting of: NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; K_2HPO_4, 3 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; glucose, 10 g; methionine, 25 mg; and distilled water, 1 liter.

Test tube cultures. Stock cultures of *E. coli* A-1

were streaked on plates of 3% nutrient agar and checked for purity. Three isolated colonies were picked, and the cells were suspended in 3 ml of sterile 0.85% NaCl to a density of 2×10^8 to 4×10^8 cells/ml. Tubes containing 4 ml of test medium were inoculated with 0.05 ml of this suspension and incubated for 20 h at 37°C in a rotary shaker-incubator.

Fermentor cultures. Isolated colonies from plates of 3% nutrient agar were suspended in saline to a density of 1×10^9 to 2×10^9 cells/ml. Five milliliters of suspension was used to inoculate 500 ml of the appropriate medium in a Bioflow model C-30 fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). Fermentor temperature was maintained at 37°C, air flow at 560 ml/min, and agitator (propeller) speed at 400 rpm. The pH was continually monitored and automatically adjusted, when desired, with 2 N NaOH or 2 N HCl. When used, sterile 25% glucose solution was added to the desired concentration. Foaming was automatically controlled with Corning Antifoam reagent.

L-Asparaginase assay. Cells were removed from 4 ml of the growth medium by centrifugation and resuspended in an equal volume of distilled water. Asparaginase activity was measured using a Technicon autoanalyzer model I (Technicon Corp., Terrytown, N.Y.) according to the procedure for ammonia determination described by Logsdon (11), but with the following modifications. Hypochlorite solution was prepared by adding 200 ml of 6% NaOCl (Purex) to 800 ml of deionized water containing 54 g of K_2CO_3. Alkaline phenol was prepared by adding 100 ml of liquefied phenol to 1 liter of 0.9 N NaOH. The substrate for enzyme assay was L-asparagine dissolved in a buffer solution made of 0.2 M K_2HPO_4 titrated with 0.1 M citric acid to pH 7.0. Assays are reported in international units; 1 U is the amount of enzyme that releases 1 μmol of ammonia per min at 37°C. The Logsdon assay was calibrated and checked periodically by measuring ammonia evolution using the Nessler reaction (12). Samples from the fermentor cultures could be held in ice for periods of less than 12 h before assay without change in asparaginase activity.

RESULTS

Test tube studies. *E. coli* A-1 was grown for 20 h at 37°C in test tubes containing 4 ml of medium and assayed for asparaginase activity. The data in Table 1 show the four media that gave the highest yield of asparaginase per unit of cell dry weight.

Growth of *E. coli* A-1 in minimal medium containing 0.5% glucose was adequate (1×10^9 cells/ml in 20 h), but no asparaginase activity was found. Addition of a mixture of L-amino acids to this medium produced cells with ample asparaginase activity. Further studies indicated that the major part of the enhancement was due to glutamic acid, although glutamic acid had no effect in the absence of the other amino acids. In the absence of glutamic acid there was very little asparaginase synthesis, and no other amino acid tested gave the incremental increase in enzyme activity seen with glutamic acid (Table 2).

The experiment described in Table 3 was performed in view of the peculiar induction of L-asparaginase activity by L-glutamic acid. Similar studies indicated that L-aspartic acid and L-asparagine do not enhance L-asparaginase activity, and a separate series of experiments has shown that L-glutaminase activity is not signif-

TABLE 1. *Yield of L-asparaginase in test tube cultures of Escherichia coli A-1 grown in different media*

Medium	g of powder/100 ml	Final pH	IU/ml	IU/mg of cell dry wt
Brain heart infusion	12.5	6.47	3.1	2.9
	6.25	7.47	9.5	6.6
	3.12	8.14	9.0	5.6
	1.56	8.31	0.5	0.5
Nutrient broth	12.5	6.97	9.8	4.4
	6.25	7.98	9.0	3.9
	3.12	8.46	3.9	2.7
	1.56	8.47	0[a]	0
Peptone	12.5	6.79	6.0	2.8
	6.25	8.36	3.5	2.2
	3.12	8.42	0	0
	1.56	8.29	0	0
Yeast extract	12.5	6.52	9.0	3.2
	6.25	7.63	9.5	3.0
	3.12	8.19	8.2	3.5
	1.56	8.63	4.5	2.6

[a] Zero indicates amounts less than 0.1 IU.

TABLE 2. *Effect of amino acids added to minimal medium on the synthesis of L-asparaginase in E. coli A-1*

Additions to minimal medium	IU/ml	IU/mg of cell dry wt
0.5% glucose	0	0
AA[a]	0.2	0.2
1% MSG[b]	0	0
AA + 0.5% MSG	4.0	3.2
AA + 1.0% MSG	5.7	5.2
AA + 1.5% MSG	6.2	6.2
AA + 2.0% MSG	6.2	6.3
AA + 2.5% MSG	6.0	6.3
AA + 3.0% MSG	5.5	6.7
AA + 3.5% MSG	5.1	7.3
AA + 4.0% MSG	3.5	4.9

[a] The amino acid (AA) mixture contained 0.4% (wt/vol) asparagine, serine, and methionine; 0.3% lysine and aspartic acid, 0.2% phenylalanine, arginine, leucine, isoleucine, valine, proline, alanine, and threonine; 0.1% histidine and glycine; and 0.01% tyrosine and cysteine.

[b] Monosodium glutamate, commercial grade.

icantly enhanced by L-glutamic acid or L-glutamine.

The highest yields of asparaginase were obtained in cells pregrown in minimal medium, washed, and resuspended in the induction medium (Table 4). The data in Table 4 also show the combined effect of nutrient broth and monosodium glutamate (MSG) under these conditions.

Fermentor studies. A series of experiments with the Bioflow fermentor was used to confirm the effect of MSG on asparaginase activity in mass cultures of *E. coli* A-1. These studies showed that higher asparaginase activity was obtained when the culture pH was maintained at 7.5 and also that glucose caused a significant reduction in asparaginase activity (Table 5). If glucose was added to 3% nutrient broth together with MSG, asparaginase synthesis was lower than that obtained with MSG only (Table 5). In the next series of experiments, higher yields of asparaginase activity were found in cultures grown in 3% nutrient broth plus 0.5% glucose in which there was no discernible dissolved oxygen. To attain this "critical level" of oxygen, the culture pH was adjusted to 7.5 and maintained by automatic control. Adjusting the pH to 7.5 brought the level of dissolved oxygen to zero without further manipulation, although agitator velocity was maintained at 400 rpm and air flow at 560 ml/min. Figure 1 shows the kinetics of asparaginase synthesis diminished. The effect of about 0.8 mg of O_2 per liter of medium (10% dissolved oxygen) is seen in Fig. 2. In essence, the rate of asparaginase production could be controlled by manipulation of the culture pH and, as a consequence, the dissolved oxygen content of the medium. The effects of pH and dissolved oxygen appeared to be interdependent in that asparaginase activity diminished when the pH was lower than 7.5 or when the dissolved oxygen was greater than zero.

TABLE 3. *Effect of L-glutamic acid, L-glutamine, and MSG on asparaginase synthesis by E. coli A-1*

Additions to 3% nutrient broth[a]	L-asparaginase		L-glutaminase (IU/ml)
	IU/ml	IU/mg of cell dry wt	
	3.4	2.9	0
0.25% glut	9.1	5.0	0.2
1.0%	10.0	5.1	0.2
	3.2	2.6	0.1
0.25% gln	9.6	4.8	0.2
1.0%	9.6	4.5	0.2
	3.2	2.6	0.1
0.25% MSG	10.0	5.0	0.2
1.0%	10.6	4.5	0.2

[a] Abbreviations: glut, Glutamic acid; gln, glutamine; MSG, monosodium glutamate, commercial grade.

TABLE 4. *Asparaginase activity in E. coli A-1 pregrown in minimal media*[a]

Induction medium	Final pH	IU/ml	IU/mg of cell dry wt
Minimal medium	5.25	0	0
0.5% NB[b]	7.30	0	0
1.0% NB	8.25	0.5	0.4
2.0% NB	8.41	4.2	2.6
3.0% NB	8.59	5.5	3.4
0.5% NB + 1% MSG	8.65	0.5	0.5
1.0% NB + 1% MSG	8.50	5.3	3.3
2.0% NB + 1% MSG	8.40	19.6	8.0
3.0% NB + 1% MSG	8.51	27.5	8.9

[a] Minimal medium with 0.5% glucose, used as growth medium. The cells were removed from the growth medium at late log phase, washed, and resuspended in an equal volume of induction medium. Growth in the induction medium was limited to approximately one cell division.

[b] NB, nutrient broth.

TABLE 5. *Effect of glucose and culture pH on L-asparaginase activity in fermentor cultures of E. coli A-1*

Additions to 3% nutrient broth	Culture pH[a]	IU/ml	IU/mg of cell dry wt
None	7.5	5.0	2.7
None	5.5	0	0
0.5% glucose	7.5	6.8	2.9
0.5% glucose	5.5	0	0
1% MSG	7.5	19.8	8.8
1% MSG	5.5	1.0	0
1% MSG + 0.5% glucose	7.5	7.0	2.9
1% MSG + 0.5% glucose	5.5	0	0

[a] The pH was established and maintained as indicated.

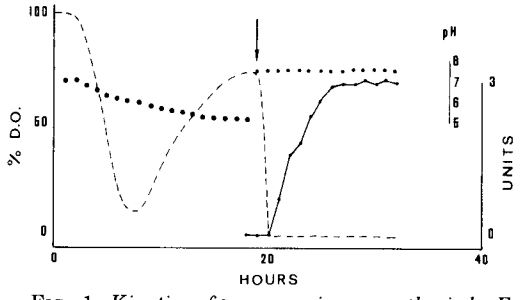

FIG. 1. *Kinetics of L-asparaginase synthesis by E. coli A-1 in the Bioflow fermentor. The broken line represents percentage of dissolved oxygen (D.O.); the solid line represents asparaginase in international units per milligram of cell dry weight; and the dots represent pH. Medium pH was adjusted (arrow) and controlled automatically thereafter.*

In contradiction to this, if the flow of air was stopped and the cultures were allowed to become anaerobic, asparaginase synthesis also ceased (Fig. 3). The synthesis of asparaginase in *E. coli* A-1 depends on proper culture pH and on oxygen uptake, but is inhibited by oxygen dissolved in the medium. This critical relationship has not been previously reported.

It is the synthesis of asparaginase that is involved, since no asparaginase activity is observed, even under optimal conditions, if chloramphenicol is added to the culture. This point is well established in previous reports (2, 13). This implies that asparaginase is formed de novo when the pH is brought to 7.5 and, consequently, dissolved oxygen to zero. To the contrary, the stimulation of asparaginase activity, i.e., synthesis, does not require extensive cellular reproduction. Cells of *E. coli* A-1 grown in minimal medium were harvested, washed, and transferred to the 3% nutrient broth-1% MSG medium and permitted to attain the critical rate of oxygen transfer in the new medium. The synthesis of asparaginase started in 8 h, although cell numbers did not increase more than twofold during that time (Fig. 4). In experiments of this type, asparaginase synthesis could be initiated at any time simply by adjusting the pH to 7.5 and allowing the dissolved oxygen to reach the critical transfer rate.

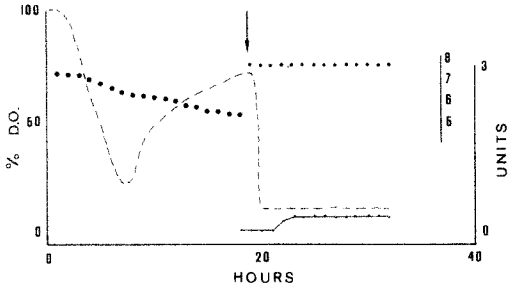

FIG. 2. *Effect of dissolved oxygen (D.O.) (10% of saturation) on asparaginase synthesis. Other details as in Fig. 1.*

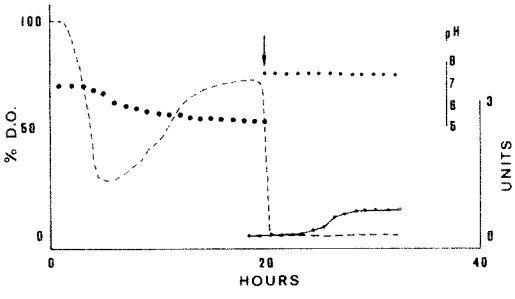

FIG. 3. *Effect of anaerobiosis on asparaginase synthesis by E. coli A-1. Symbols are as in Fig. 1. Medium pH was adjusted and air supply was turned off at the time indicated by the arrow.*

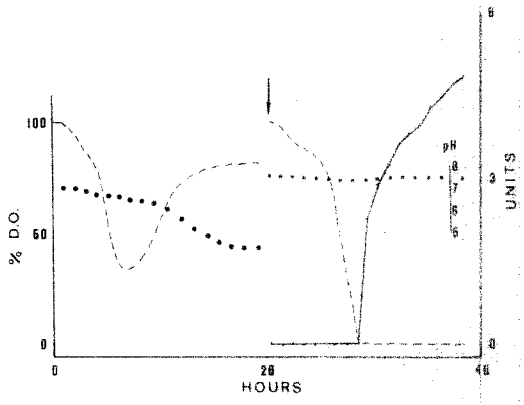

FIG. 4. *Synthesis of asparaginase by E. coli A-1 pregrown in minimal medium and transferred (arrow) to 3% nutrient broth plus 1% MSG. Symbols are as in Fig. 1.*

DISCUSSION

Nutritional requirements for maximal synthesis of L-asparaginase vary from one microorganism to the other. Indeed, the rate of synthesis varies in the same organism as a function of culture conditions. In this report, we have shown that asparaginase activity in cells of *E. coli* A-1 depends on a complex interaction of nutrients, pH, oxygen transfer rate, and the presence of specific requirements such as glutamic acid.

The effect of MSG on synthesis is perplexing. The natural substrate for L-asparaginase is L-asparagine, but the maximal enhancement of asparaginase synthesis is obtained by adding glutamine or glutamate to the medium. Imada et al. (7) showed that many organisms that produce L-asparaginase also produce L-glutaminase, and another study (13) showed that L-asparagine induced asparaginase only under anaerobic conditions. In our experiments, MSG probably does not function as a classical enzyme inducer since L-asparagine does not affect L-asparaginase activity and other amino acids are required for the induction (Table 2). We have not ascertained the mechanism of action involved in this effect. Levels of asparaginase activity of 15 to 25 IU per ml of culture have not been reported from other experiments and can be attained in ours only by the use of MSG in a suitable fermentation broth. The synthesis of L-asparaginase represents a curious biological system. In essence, asparaginase synthesis is induced by the presence of a group of amino acids but principally by glutamic acid at a specific pH and level of respiratory activity

whether cell division occurs or not.

It should also be noted that the yield of asparaginase activity using a given culture medium should be viewed in terms of the amount of cell protein devoted to asparaginase activity. The best results in our studies (27 IU/ml) were obtained by using cells pregrown in minimal medium and transferred to 3% nutrient broth plus 1% MSG. Cells grown in minimal medium plus 3.5% MSG and the other amino acids gave only 5.0 IU/ml, but the yield of enzyme was 8.9 and 7.3 IU per mg of cell dry weight, respectively. That is, approximately the same amount of effective enzyme was produced in both cases although, if measured in terms of international units per milliliter, it appears as if the pregrown cells produce five times as much as those grown on minimal medium.

In view of this finding, it is suggested that the only valid comparison of enzyme yields is in terms of international units per milligram of cell dry weight.

The effect of oxygen transfer rate shown in these experiments is essentially in agreement with the findings of Liu and Zajic (8), but *E. aroideae* does not show the critical oxygen dependence seen in *E. coli* A-1. It is obvious that a specific rate of oxygen consumption is required for asparaginase synthesis and that dissolved free oxygen in the growth medium inhibits synthesis. The relationship between oxygen tension and the synthesis of a specific enzyme must be complex and is certainly beyond our understanding at this time.

The data presented here show that asparaginase synthesis in *E. coli* A-1 is the result of a very complex sequence of culture events. The specific nature of these events is not understood, but we have identified some of them. When these findings were applied to cultures of *E. coli* A-1 in a 1,000-liter fermentor, the yields of purified L-asparaginase were increased threefold in the production laboratories of the Wadley Institute.

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LITERATURE CITED

1. **Bascomb, S., G. T. Banks, M. T. Skarstedt, A. Fleming, and K. A. Bettelheim.** 1975. The properties and large-scale production of L-asparaginase from *Citrobacter*. J. Gen. Microbiol. **91**:1-16.
2. **Boeck, L. D., and P. P. K. Ho.** 1973. L-asparaginase production during static incubation of aerobically grown *Escherichia coli* B. Can. J. Microbiol. **19**:1251-1257.
3. **Cedar, H., and J. H. Schwartz.** 1968. Production of L-asparaginase II by *Escherichia coli*. J. Bacteriol. **96**:2043-2048.
4. **Heinemann, B., and A. J. Howard.** 1969. Production of tumor-inhibitory L-asparaginase by submerged growth of *Serratia marcescens*. Appl. Microbiol. **18**:550-554.
5. **Heinemann, B., A. J. Howard, and J. H. Palocz.** 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by *Serratia marcescens*. Appl. Microbiol. **19**:800-804.
6. **Hill, J. M., E. Loeb, A. MacLellan, N. Hill, A. Khan, and A. Albayrak.** 1972. Treatment of acute leukemia. Wadley Med. Bull. **2**:63-76.
7. **Imada, A., S. Igarasi, K. Nakahama, and M. Isono.** 1973. Asparaginase and glutaminase activities of microorganisms. J. Gen. Microbiol. **76**:85-99.
8. **Liu, F. S., and J. E. Zajic.** 1973. Effect of oxygen-transfer rate on production of L-asparaginase by *Erwinia aroideae*. Can. J. Microbiol. **19**:1153-1158.
9. **Liu, F. S., and J. E. Zajic.** 1973. Fermentation kinetics and continuous process of L-asparaginase production. Appl. Microbiol. **25**:92-96.
10. **Loeb, E., J. M. Hill, A. MacLellan, N. Hill, A. Khan, and R. Speer.** 1972. Five year experience with L-asparaginase in the treatment of acute leukemias. Wadley Med. Bull. **2**:23-32.
11. **Logsdon, E. E.** 1960. A method for the determination of ammonia in biological materials on the autoanalyzer. Ann. N.Y. Acad. Sci. **87**:801-807.
12. **Roberts, J., G. Burson, and J. M. Hill.** 1968. New procedures for purification of L-asparaginase with high yield from *Escherichia coli*. J. Bacteriol. **95**:2117-2123.
13. **Svobodova, O., and S. Strbanova-Necinova.** 1973. Induction of L-asparaginase synthesis in *Escherichia coli*. Biochim. Biophys. Acta **321**:643-652.

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Physiology of L-Asparaginase Synthesis in Recombinants of *Escherichia coli* A-1

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A mating between *Escherichia coli* 4318 (*thi leu* Las$^-$ Hfr) and *E. coli* A-1 (Met$^-$ Las$^+$ F$^-$) resulted in the formation of prototrophic recombinants having L-asparaginase activities at three distinct levels. The physiology of L-asparaginase synthesis in these recombinants is described. One class of recombinants produced significantly more L-asparaginase than *E. coli* A-1. L-Asparaginase synthesis in the recombinants was inhibited by the presence of dissolved oxygen in the medium and was transiently repressed by the presence of glucose in the same manner as that observed in the parental strains. L-Asparaginase activity was increased by the addition of oxalacetate as well as other members of the tricarboxylic acid cycle.

A number of reports have appeared in recent literature describing the conditions affecting synthesis of L-asparaginase in several genera of bacteria. This literature reveals that a complex and variable set of culture conditions is required for optimum synthesis of this enzyme (2-6, 8, 9, 12-15). In a previous study (1), it was shown that the synthesis of L-asparaginase by *Escherichia coli* A-1 requires very exacting nutritional and environmental conditions. Briefly, optimum enzyme synthesis required the following: (i) an exogenous supply of amino acids such as that found in yeast extract or nutrient broth, (ii) high levels of glutamic derivatives such as L-glutamine or monosodium glutamate (MSG), (iii) moderate aeration but no dissolved oxygen in the medium, and (iv) absence of any readily oxidizable carbon source such as glucose. Under these conditions, *E. coli* A-1 produces approximately 20 IU of L-asparaginase per ml of culture fluid when grown in a small fermentor. In contrast, the majority of cultures described in the literature produce 1 to 4 IU/ml (6-8).

L-Asparaginase is a nonessential enzyme and provides the cell with no known selective advantage over organisms that do not synthesize it. For this reason, selective or indicator media capable of revealing mutants that produce large quantities of L-asparaginase have not been produced. To detect overproducers, colonies must be picked, purified, cultured, and then tested. The majority of colonies that grow after mutagenic treatment produce the same level of enzyme as untreated cells; i.e., the majority of survivors are not mutants. Furthermore, maximum enzyme levels are achieved only under the rigorous conditions described above, and little or no enzyme is produced by colonies grown on solid medium. This means that a "fermentor run" must be performed to adequately examine each clone after treatment with mutagenic agents. To avoid this step, we started with cultures known to produce L-asparaginase and attempted to increase the level of production by selective matings. The efficiency of gene transfer was determined by using auxotrophic strains and selecting for prototrophic recombinants. In this paper, we report on recombinants obtained by mating an L-asparaginase producer (F$^-$) with a nonproducer (Hfr). The purpose of this mating was to convert the F$^-$ to Hfr in preparation for matings of producer strains, but the results were more interesting from the general point of view of genetic phenomena.

(This work was submitted by W.R.B. in partial fulfillment of the requirements for the Ph.D. degree at North Texas State University, Denton, 1977.)

MATERIALS AND METHODS

Bacterial strains. *E. coli* A-1 (Met$^-$ F$^-$) and *E. coli* HAP were obtained from the stock culture collection at Wadley Institutes of Molecular Medicine. The designation Las$^+$ is suggested to indicate L-asparaginase production, and the phenotype of *E. coli* A-1 is given as Met$^-$ Las$^+$ F$^-$. *E. coli* CGSC 4318 (*thi leu* Hfr) was kindly supplied by Royston Clowes, Department of Molecular Biology, University of Texas at Dallas. We amended the designation to *E. coli* CGSC 4318 (*thi leu* Las$^-$ Hfr). Stock cultures were maintained on slants of 3% nutrient agar (3 g of commercial

nutrient agar powder per 100 ml of water) in a refrigerator. Working cultures were obtained by streaking stock cultures and picking isolated colonies.

Media. Test tube cultures of parental and recombinant strains were routinely grown in 3% nutrient broth containing 1% (wt/vol) reagent-grade monosodium glutamate (MSG). Minimal agar was prepared by adding NH_4Cl (5 g), Na_2SO_4 (2 g), K_2HPO_4 (3 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), and glucose (10 g) per liter of distilled water. Fermentor cultures were grown in 3% nutrient broth plus 1% MSG or 0.5% glucose. Tricarboxylic acid intermediates were sterilized by filtration, adjusted to pH 7.0 with 0.1 N NaOH, and added to 3% nutrient broth.

Mating procedure. *E. coli* 4318 and A-1 were grown overnight at 37°C in test tubes containing 4 ml of 3% nutrient broth in the shaker-incubator. Each culture was diluted 1:20 with sterile broth and reincubated until mid-log-phase growth (2×10^8 cells per ml) was attained. Two milliliters of each culture was mixed and allowed to incubate without agitation at 37°C for 90 min. The donor and recipient cultures and the mating mixture were centrifuged and washed twice with 0.9% saline, and then 0.1 ml of each was spread on minimal agar plates. These were incubated for 4 to 5 days at 37°C. Donor and recipient cells plated in this manner failed to yield colonies in all experiments performed. Organisms that formed colonies on this medium were known to be genetic recombinants. They were subcultured five times on minimal agar and then tested for enzyme activity.

Test tube cultures. Stock cultures of parental and recombinant strains were streaked on plates of 3% nutrient agar and checked for purity. Tube cultures (4 ml each) were prepared with inoculum obtained from isolated colonies and were incubated for 20 h at 37°C in the shaker-incubator.

Fermentor cultures. A 1,000-ml fermentor with attached pH and dissolved-oxygen controls (Bioflow model C-30, New Brunswick Scientific Co., New Brunswick, N.J.) was used to confirm test tube results and for additional studies. Fermentor temperature was maintained at 37°C, air flow was maintained at 560 cm^3/min, and agitator (propeller) speed was maintained at 400 rpm. Foaming was controlled with Corning Antifoam B reagent.

L-Asparaginase assay. Whole cells were assayed for L-asparaginase activity by nesslerization or on a Technicon Autoanalyzer (Technicon Corp., Inc., Tarrytown, N.Y.) by methods previously described (11, 14).

RESULTS

E. coli A-1 was recognized as an F^- by mating experiments with known F^- and Hfr *E. coli* strains. This organism was able to receive donor markers for *met* with a recombination frequency of about 0.05%. Using the nutritional markers as proof of recombination, we obtained 350 isolates after primary plating on minimal agar. Between 60 and 70 were subcultured five times on the minimal agar, and stock cultures were then prepared. The synthesis of L-asparaginase was measured in each of these isolates, and they were divided into three categories according to the amount of enzyme produced. Table 1 shows enzyme activities of the parental strains and three recombinants representing the three categories. *E. coli* HAP is the parental strain from which *E. coli* A-1 was derived (1) and is included only for comparison. Recombinants, like parental strains, produced L-asparaginase but not L-glutaminase in the presence of MSG (Table 1). A comparison of L-asparaginase activity in international units per milliliter shows that the isolate designated R-17 produced very low levels of enzyme compared with *E. coli* A-1. If the comparison takes cell growth into consideration (international units per milligram of cell dry weight), it becomes evident that R-17 cells produced almost as much enzyme as the parental strain. On the other hand, although *E. coli* R-26 grew relatively well in the test medium, L-asparaginase activity was very low. Again, R-69 produced levels of L-asparaginase approximately 100% higher than *E. coli* A-1, whereas the increase in growth was only 15%.

To confirm the results from these studies, a series of experiments with the Bioflow fermentor was carried out. Previous studies with *E. coli* A-1 have shown that optimum enzyme levels are

TABLE 1. *L-Asparaginase synthesis in test tube cultures of parental and recombinant strains grown in 3% nutrient broth plus 1% MSG*

E. coli strain	Phenotype or genotype	L-Asparaginase (IU/ml of culture fluid)	Cell dry wt (mg/ml)	L-Asparaginase (IU/mg of cell dry wt)	L-Glutaminase (IU/ml)	pH of supernatant
HAP	Prototrophic	2.5	2.60	1.0	0.0	7.8
A-1	Met^- Las^+ F^-	10.8	2.20	4.9	0.2	8.1
4318	*thi leu* Las^- Hfr	0.0	1.60	0.0	0.0	7.6
R-26 (1)[a]	Prototrophic	0.2	1.80	0.1	0.0	7.7
R-17 (2)[a]	Prototrophic	3.2	0.76	4.2	0.0	7.7
R-69 (3)[a]	Prototrophic	20.3	2.53	8.1	0.3	8.0

[a] (1) Includes all recombinants that produce <1 IU/ml; (2) includes those that produce >1 but <10 IU/ml; (3) includes those that produce >10 IU/ml.

achieved in 3% nutrient broth plus 1% MSG medium when the pH is maintained at 7.5 and the dissolved-oxygen level is at 0%. In our study, enzyme levels of parental and recombinant strains were generally found to be greater than those obtained in test tube cultures, but they maintained the same relationship to each other (Table 2). The increase in L-asparaginase activity of *E. coli* R-69 can be explained on the basis of better growth in the fermentor, but the amount of L-asparaginase per unit of cell dry weight remained essentially the same as that of *E. coli* A-1. Nevertheless, the 54% increase in international units per milliliter when compared with *E. coli* A-1 justifies the use of this strain for large-scale production. Although L-asparaginase levels for *E. coli* R-17 did not increase significantly, growth in the fermentor improved by 58% and, whereas growth of R-26 improved only 17%, enzyme synthesis increased by 50%.

Additional characterization of the recombinant strains is described in Fig. 1. Previous studies on the parental recipient *E. coli* A-1 have shown that maximal enzyme synthesis is attained in pregrown cells after 20 h of MSG induction by maintaining the pH at 7.5 and allowing the dissolved-oxygen level to drop to zero. *E. coli* R-69 produced L-asparaginase at a higher rate than *E. coli* A-1, whereas *E. coli* R-17 and *E. coli* R-26 produced much less enzyme under the same conditions (Fig. 1).

Because *E. coli* R-69 produced significantly higher levels of L-asparaginase than A-1, the organism currently used for commercial produc-

TABLE 2. *Characterization of parental and recombinant strains grown in 3% nutrient broth plus 1% MSG in the Bioflo fermentor*

E. coli strain	Phenotype or genotype	L-Asparaginase (IU/ml of culture fluid)	Cell dry wt (mg/ml)	L-Asparaginase (IU/mg of cell dry wt)	L-Glutaminase (IU/ml)	Supernatant pH[a]
A-1	Met^- Las^+ F^-	19.8	2.30	8.7	0.3	7.5
4318	*thi leu* Las^- Hfr	0.0	1.89	0.0	0.0	7.5
R-26 (1)[b]	Prototrophic	0.3	2.10	0.1	0.0	7.5
R-17 (2)[b]	Prototrophic	4.1	1.20	3.3	0.0	7.5
R-69 (3)[b]	Prototrophic	30.5	3.10	9.8	0.4	7.5
HAP	Prototrophic	3.2	3.50	0.9	0.0	7.5

[a] Controlled automatically with 0.1 N HCl or 0.1 N NaOH.

[b] (1) Includes recombinants that produce <1 IU/ml; (2) includes those that produce >1 but <10 IU/ml; (3) includes those that produce >10 IU/ml.

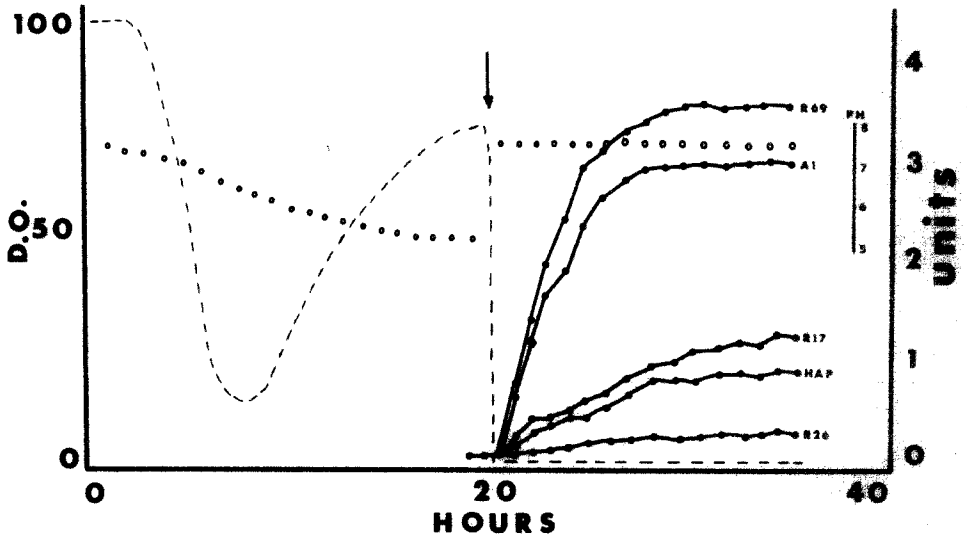

FIG. 1. *Kinetics of L-asparaginase synthesis by E. coli A-1, HAP, R-69, R-26, and R-17 in a Bioflow fermentor. Symbols: (— —) percent dissolved oxygen (D.O.), (——) international units of asparaginase per milligram of cell dry weight, and (· · · · ·) culture pH. Medium pH was adjusted (arrow) and controlled automatically thereafter. Enzyme synthesis was initiated by adjusting pH, decreasing dissolved oxygen to 0%, and adding 0.5% (wt/vol) MSG.*

tion, it was selected for further studies. L-Asparaginase synthesis for this recombinant was inhibited by dissolved-oxygen levels greater than zero in exactly the manner seen in *E. coli* A-1. As with A-1, the inhibition of enzyme synthesis by oxygen dissolved in the medium was found to be reversible (Fig. 2). The addition of glucose, once enzyme synthesis was induced, produced a transient repression (Fig. 3). Enzyme synthesis continued only after the glucose had been consumed in the same manner as occurred in A-1. Recent studies by Netrval (12, 13) have shown that many of the tricarboxylic acid cycle intermediates enhance L-asparaginase activity in *E. coli*. Table 3 shows that, in every case, L-asparaginase levels were higher in R-69 than in A-1.

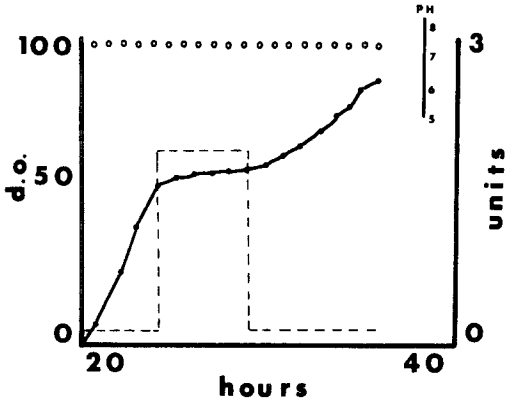

FIG. 2. *Effect of a 50% dissolved-oxygen (d.o.) pulse on the kinetics of asparaginase synthesis in E. coli R-69. Dissolved oxygen was adjusted by increasing or decreasing agitator speed. Symbols: (- - -) dissolved-oxygen pulse, (—) international units of asparaginase per milligram of cell dry weight, and (· · · ·) culture pH.*

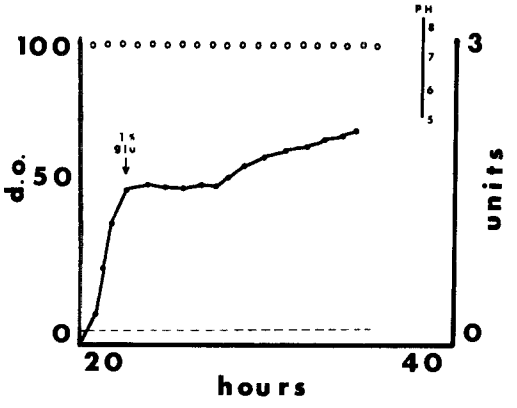

FIG. 3. *Effect of 1% glucose on the kinetics of L-asparaginase synthesis in E. coli R-69. Symbols: (- - -) percent dissolved oxygen (d.o.), (——) international units of asparaginase per milligram of cell dry weight, and (· · · ·) culture pH.*

DISCUSSION

In contrast to the many reports on the physiological conditions that affect the kinetics of L-asparaginase synthesis, little work has been reported on the genetics of *E. coli* or any other bacteria with respect to synthesis of this enzyme. The isolation of strains that produce large amounts of enzyme is made difficult by the lack of suitable media and adequate techniques for selection. Selection of mutants has also been made impossible by these deficiencies. In view of this, it was assumed that a genetic selection procedure could be designed to yield strains of *E. coli* that produce large amounts of L-asparaginase.

The mating experiment described in this report resulted in the isolation of recombinant progeny that had varying degrees of enzyme activity. Approximately 10% produced greater levels of L-asparaginase than the parental strains. The remainder produced lower levels but are considered to be recombinants since they are prototrophic organisms obtained by mating auxotrophic parental strains and also because the amounts of L-asparaginase are different from those produced by either parent strain. The isolation of recombinants capable of producing 30 IU/ml that are derived from parental strains such as A-1, which produces 20 IU/ml, and 4318, which is a nonproducer, raises many questions regarding the genetics of L-asparaginase synthesis in *E. coli*. These recombinants also differed from the parental strains in growth characteristics. Fermentor cultures showed that variations in total cell yield were independent of L-asparaginase synthesis. Examination of many recombinants showed that the two traits were transmitted randomly from 4318 to A-1. It is obvious that *E. coli* possesses a complex physiological

TABLE 3. *Effect of various tricarboxylic acid cycle intermediates on the synthesis of L-asparaginase in E. coli A-1 and E. coli R-69 in test tube cultures*

Addition (0.5%) to 3% nutrient broth	L-Asparaginase synthesis (IU/mg of cell dry wt) in:	
	E. coli A-1	*E. coli* R-69
None	2.7	2.9
MSG	4.8	7.7
Oxalacetate	4.6	6.5
Pyruvate	4.0	6.1
Acetate	4.0	6.0
Malate	3.2	5.0
Succinate	3.1	5.0
α-Ketoglutarate	2.9	4.6
Fumarate	2.8	4.5
Citrate	2.7	3.3
Isocitrate	2.7	3.2

mechanism for the production of L-asparaginase, but the brief study presented here indicates that the genetic mechanism involved may be far more complicated. Many questions, including the following, have become very pressing. (i) Does 4318 have genes that are not expressed? (ii) Does 4318 transmit a derepressor? (iii) Why do most (90%) recombinants produce less L-asparaginase than does the parent A-1? These interesting, unanswered questions will be examined later. On a more practical note, however, the work reported here has yielded a new prototrophic recombinant, R-69, which is now being used in the large-scale (1,000-liter) commercial production of L-asparaginase. This organism regularly produces 30 IU/ml of culture fluid, an increase of 10 IU/ml over production by A-1. The total yield from R-69 was improved by some 10^7 IU of L-asparaginase per run of the fermentor. The average therapeutic dose of L-asparaginase in acute leukemias ranges from 3,000 to 9,000 IU/kg of body weight (10); the use of R-69 represents an increase of some 35 to 100 daily doses per culture for a person weighing 30 kg. The time, cost, and work involved are identical for R-69 and A-1. We think that conjugation experiments may become the preferred method for work of this kind.

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LITERATURE CITED

1. **Barnes, W. R., G. L. Dorn, and G. R. Vela.** 1977. Effect of culture conditions on synthesis of L-asparaginase by *Escherichia coli* A-1. Appl. Environ. Microbiol. **33**:257–261.
2. **Boeck, L. D., and P. P. K. Ho.** 1973. L-asparaginase production during static incubation of aerobically grown *Escherichia coli* B. Can. J. Microbiol. **19**:1251–1257.
3. **Boeck, L. D., R. W. Squires, M. W. Wilson, and P. P. K. Ho.** 1970. Effect of glucose and low oxygen tension on L-asparaginase production by a strain of *Escherichia coli* B. Appl. Microbiol. **20**:964–969.
4. **Cedar, H., and J. H. Schwartz.** 1968. Production of L-asparaginase II by *Escherichia coli*. J. Bacteriol. **96**:2043–2048.
5. **Heinemann, B., and A. J. Howard.** 1969. Production of tumor-inhibitory L-asparaginase by submerged growth of *Serratia marcescens*. Appl. Microbiol. **18**:550–554.
6. **Heinemann, B., A. J. Howard, and H. J. Palocz.** 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by *Serratia marcescens*. Appl. Microbiol. **19**:800–804.
7. **Imada, A., S. Igarasi, K. Nakahama, and M. Isono.** 1973. Asparaginase and glutaminase activities of microorganisms. J. Gen. Microbiol. **76**:85–99.
8. **Liu, F. S., and J. E. Zajic.** 1973. Effect of oxygen transfer rate on production of L-asparaginase by *Erwinia aroideae*. Can. J. Microbiol. **19**:1153–1158.
9. **Liu, F. S., and J. E. Zajic.** 1973. Fermentation kinetics and continuous process of L-asparaginase production. Appl. Microbiol. **25**:92–96.
10. **Loeb, E., J. M. Hill, A. MacLellan, N. Hill, A. Kahn, and R. Speer.** 1972. Five year experience with L-asparaginase in the treatment of acute leukemias. Wadley Med. Bull. **2**:23–32.
11. **Logsdon, E. E.** 1960. A method for the determination of ammonia in biological materials on the auto-analyzer. Ann. N.Y. Acad. Sci. **87**:801–807.
12. **Netrval, J.** 1973. Effect of organic and amino acids on L-asparaginase production by *Escherichia coli*. Arch. Microbiol. **92**:345–355.
13. **Netrval, J.** 1977. Stimulation of L-asparaginase production in *Escherichia coli* by organic and amino acids. Folia Microbiol. **22**:106–116.
14. **Roberts, J., G. Burson, and J. M. Hill.** 1968. New procedures for purification of L-asparaginase with high yield from *Escherichia coli*. J. Bacteriol. **95**:2117–2123.
15. **Svobodova, O., and S. Strbanova-Necinova.** 1973. Induction of L-asparaginase synthesis in *Escherichia coli*. Biochim. Biophys. Acta **321**:643–652.