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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

# ENZYME TREATMENT OF NEOPLASIA: THE EFFECTS OF PHENYLALANINE AMMONIA-LYASE ON NORMAL AND LEUKEMIC LYMPHOCYTES

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SUBMITTED TO THE GRADUATE FACULTY

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ENZYME TREATMENT OF NEOPLASIA: THE EFFECTS OF

PHENYLALANINE AMMONIA-LYASE ON NORMAL

AND LEUKEMIC LYMPHOCYTES

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TO MY MOTHER AND MY WIFE

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TABLE OF CONTENTS


| | Page |
|---|---|
| LIST OF TABLES | vi |
| LIST OF ILLUSTRATIONS | vii |
| LIST OF ABBREVIATIONS | ix |
| Chapter | |
| I. INTRODUCTION | 1 |
| II. MATERIALS AND METHODS | 12 |
| III. RESULTS | 26 |
| IV. DISCUSSION | 62 |
| V. SUMMARY | 71 |
| BIBLIOGRAPHY | 73 |

LIST OF TABLES


| Table | | Page |
|---|---|---|
| 1. | Purification of Phenylalanine Ammonia-Lyase from Rhodotorula Glutinis | 27 |
| 2. | Stability of Different Concentrations of Phenylalanine Ammonia-Lyase at 4°C | 28 |
| 3. | Stability of Phenylalanine Ammonia-Lyase in Fischer's Media Plus 10% Horse Serum with and without L5178Y Lymphoblasts | 30 |
| 4. | Effects of Different Concentrations of Phenylalanine Ammonia-Lyase on Normal Human Lymphocytes in the Resting State | 39 |
| 5. | Effects of Different Concentrations of Phenylalanine Ammonia-Lyase on Normal Human Lymphocytes in the Dividing State | 40 |
| 6. | The Effect of Phenylalanine Ammonia-Lyase Treatment (3.35 U/15 ml) on Phenylalanine Concentration and Viable Cell Count of L5178Y Lymphoblasts in Culture | 52 |

LIST OF ILLUSTRATIONS


| Figure | | Page |
|---|---|---|
| 1. | The Action of Phenylalanine Ammonia-Lyase on the Amino Acids Phenylalanine and Tyrosine | 31 |
| 2. | A Comparison of Spectra of Commercial Preparations of Cinnamic Acid or Coumaric Acid in Alkaline Solution with that Reported by Uchiyama *et al*. | 32 |
| 3. | Absorbancy of Standard Solutions of Cinnamic Acid and Coumaric Acid | 33 |
| 4. | Rate of Formation of Cinnamic Acid and Coumaric Acid by Phenylalanine Ammonia-Lyase in Complete Fischer's Medium in the Presence and Absence of L5178Y Lymphoblasts | 35 |
| 5. | The Effect of Phenylalanine Ammonia-Lyase on DNA Synthesis in Leukocytes from a Patient with Acute Lymphoblastic Leukemia | 36 |
| 6. | The Effect of Asparaginase on DNA Synthesis in Leukocytes from a Patient with Acute Lymphoblastic Leukemia | 38 |
| 7. | The Effect of Phenylalanine Ammonia-Lyase on Cell Division of L5178Y Lymphoblasts | 42 |
| 8. | A Comparison of the Effect of Treatment with Low Concentrations of Phenylalanine Ammonia-Lyase or Asparaginase on the Growth of L5178Y Lymphoblasts | 43 |
| 9. | The Effects of Phenylalanine Ammonia-Lyase on the Growth and Viability of L5178Y Lymphoblasts | 45 |
| 10. | Effect of Treating L5178Y Lymphoblasts in Culture with Phenylalanine Ammonia-Lyase, Removing the Enzyme, and Resuspending the Cells in Fresh Media | 46 |
| 11. | The Effect of Phenylalanine Ammonia-Lyase on DNA Synthesis in L5178Y Lymphoblasts | 48 |
| 12. | The Effect of Phenylalanine Ammonia-Lyase on RNA Synthesis in L5178Y Lymphoblasts | 49 |
| 13. | The Effect of Phenylalanine Ammonia-Lyase on Protein Synthesis in L5178Y Lymphoblasts | 50 |
| 14. | Rate of Removal of L-phenylalanine-$^{14}$C(UL) from Horse Serum by Dialysis Against Earle's Balanced Salt Solution | 53 |

LIST OF ILLUSTRATIONS--Continued.


| Figure | | Page |
|---|---|---|
| 15. | Comparison of the Effects of Treatment with Phenylalanine Ammonia-Lyase with Phenylalanine Deprivation on L5178Y Lymphoblasts ..... | 55 |
| 16. | Effect of Different Concentrations of Tris-Cl Buffer on the Growth of L5178Y Lymphoblasts ..... | 56 |
| 17. | Effect of Deamination Products of L-phenylalanine and L-tyrosine on the Growth of L5178Y Lymphoblasts ..... | 58 |
| 18. | Electron Micrograph of an Untreated L5178Y Lymphoblast ..... | 59 |
| 19. | Electron Micrograph of a L5178Y Lymphoblast which had been Treated for 350 Hours with Phenylalanine Ammonia-Lyase ... | 60 |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| HCl | Hydrochloric acid |
| Tris | Tris (hydroxymethyl) amino methane HCl |
| $NaBH_4$ | Sodium borohydride |
| mM | Millimolar |
| μM | Micromolar |
| ml | Milliliter |
| μl | Microliter |
| μg | Microgram |
| pg | Picogram |
| mμ | Millimicron (1 mμ = $10^{-7}$ cm) |
| min | Minute |
| ALL | Acute lymphoblastic leukemia |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| PHA | Phytohemagglutinin |
| PAL | Phenylalanine ammonia-lyase |
| U | Units of enzyme |
| $^3H$- | Tritium labeled |
| $^{14}C$ | Carbon 14 labeled |
| UL | Uniformly labeled |
| Ci | Curie |
| μCi | microcurie |
| DPM | Disintegrations per minute |
| FHS | Fischer's medium plus 10% horse serum |

LIST OF ABBREVIATIONS—Continued.

| | |
|---|---|
| TD | Cell population doubling time |
| EBSS | Earle's balanced salt solution |
| $CO_2$ | Carbon dioxide |
| A | Absorbancy |

# ENZYME TREATMENT OF NEOPLASIA: THE EFFECTS OF PHENYLALANINE AMMONIA-LYASE ON NORMAL AND LEUKEMIC LYMPHOCYTES

## CHAPTER I

## INTRODUCTION

### Acute Lymphocytic Leukemia

The leukemias are diseases of the leukopoietic tissues. An excess of leukocytes is produced at these sites of origin, with or without a corresponding increase in leukocytes in the peripheral blood. The disease is divided into four major categories-- lymphocytic, granulocytic, monocytic, or plasmocytic-- depending on the predominant type of leukocyte present. Leukemia progresses rapidly in the acute form and more slowly in the chronic form, but it is almost inevitably fatal.

The most common form of acute leukemia affects lymphoid tissue, producing so many lymphocytes that they constitute up to 99% of the total leukocyte population usually in the immature lymphoblastic form. The disease has its highest incidence in children under five years old. In adults over 25, it is relatively rare (1). The symptoms and signs of acute leukemia include fever, anemia, lesions of the oral mucosa, hemorrhages, and enlargement of the spleen, liver, and lymph glands (2,3).

Various agents have been implicated in the etiology of leukemia. Viruses have been shown to cause certain murine leukemias (4-6), and

virus-like particles have been associated with feline (7), canine (8), bovine (9), and human (10,11) leukemias. All the naturally occurring leukemogenic viruses contain RNA and possess an RNA-dependent DNA polymerase (reverse transcriptase) (12-14). The RNA-dependent DNA polymerase would enable an oncogenic RNA virus to form a complementary DNA which could be inserted into the host DNA and possibly transmitted from generation to generation (vertical transmission). Other work has shown that reverse transcriptase is present not only in the lymphocytes from humans with acute leukemia (15) but in lymphocytes from normal donors (16). However, Todaro et al. (17) have reported that the enzyme found in normal cells can be physically separated from the enzyme of oncogenic viruses grown in the cells.

In addition to viruses, other agents have been shown to cause leukemias. Law et al. (18) used skin paintings with methylcholanthrene to induce a lymphoblastic leukemia (L1210) in a female DNA mouse. This leukemia could be maintained by subcutaneous injections of leukemic cells from this mouse into other DBA mice.

Radiation has also been implicated in the etiology of leukemia with irradiation before birth or during infancy producing acute leukemias indistinguishable from those predominating in the general population during childhood (19).

Animals with specific types of leukemias are unable to synthesize certain nonessential amino acids in adequate amounts and thus require exogenous asparagine (20) and serine (21). Consequently, asparaginase, an enzyme which deaminates asparagine to aspartic acid and ammonia, has been found effective in suppressing leukemias in mice

(22-24), lymphosarcoma in dogs (25), and acute lymphocytic leukemia, leukemic lymphosarcoma, and lymphosarcoma in humans (26,27). The process by which L-asparaginase appears to selectively starve malignant cells has been referred to as amino acid depletion therapy (27).

The malignancy most responsive to L-asparaginase treatment has been acute lymphocytic leukemia (28-32). The enzyme appears to induce remission in approximately 20-26% of the patients treated. The duration of remission varies from 30 to 175 days, with a median of 58-90 days when the patient is maintained on supportive therapy with other drugs such as methotrexate and 6-mercaptopurine. In some cases, a second brief remission of the disease can be obtained with L-asparaginase. However, resistance to this enzyme is easily acquired and cannot be overcome by increasing the dose. In mice and humans with leukemia, this resistance results from an increase in asparagine synthetase activity (33,34).

A serious side effect of asparaginase treatment is hypersensitivity (27,28,30,32,35,36). Passive hemagglutinating antibodies have been identified (37) in patients suffering from anaphylactic shock after treatment with L-asparaginase. L-asparaginase also appears to have certain immunosuppressive properties (38,39). Other effects of L-asparaginase treatment have been hypoalbuminemia, hypofibrinogemia, and hypocholesterolemia (32,40-43). Apparently, L-asparaginase produces little impairment in bone marrow production (27,28,34,35), but myelosuppression has been noted (32). Hyperglycemia and glycosuria have also been reported (40,44). Some neurological disturbances, including lethargy, somnolence, and confusion, have been observed after L-asparaginase treatment (28,35,40).

Several studies (45,46) showing the distribution and clearance of L-asparaginase indicate that the enzyme is most concentrated in the lymph, bile, and cerebrospinal fluid within 2-3 hours after intravenous administration. It does not pass readily from vascular to tissue space. Clearance of the enzyme appears to depend upon its source. For example, guinea pig asparaginase requires 3 or more days to be cleared, whereas yeast asparaginase is cleared in less than 1 hour. Escherichia coli L-asparaginase has a half-life of 8-24 hours, depending on the preparation. The enzyme seems not to be excreted in appreciable quantities in urine or bile nor eliminated by the reticuloendothelial system.

Nonessential amino acids can be removed from an animal (including humans) only by in vivo administration of the appropriate enzymes. However, essential amino acids can be depleted simply by dietary restriction. This has been shown to effectively suppress a variety of solid tumors.

Dietary cystine deficiency (47) and lysine deficiency (48) have been shown to inhibit the development of spontaneous mammary tumors in C3H mice. More recently, diets low in phenylalanine have been reported to markedly inhibit the development of spontaneous mammary hyperplastic alveolar nodules and tumors in female C3H female virgin mice (49). In another study on the effects of amino acid restriction on the growth of female C57BL mice and their implanted BW10232 adenocarcinomas (50), low dietary levels of tryptophane, threonine, leucine, or methionine significantly inhibited tumor growth but also depressed host weight. In contrast, low dietary levels of phenylalanine, valine, or isoleucine significantly inhibited tumor weight without affecting host weight.

Moreover, low phenylalanine-tyrosine diets suppressed S91 mouse melanomas in DBA/2 mice (51,52); the same diets used in treating patients with advanced malignant melanoma caused a cessation of tumor growth and an absence of new clinically evident metastases. In some patients, definite tumor regression occurred. Low phenylalanine diets were also shown to cause significant tumor inhibition against a variety of animal tumors, including mouse hepatoma (BW7756), mouse mammary adenocarcinomas (H2712 and $CaD_2$) and pleomorphic sarcoma S180 (53). In humans, the same diet inhibited a variety of solid tumors (54). A minimal level of phenylalanine was found which had no apparent adverse effects upon the host while inhibiting tumor growth. These results were obtained with metastatic vulvar malignant melanoma, severe chronic Hodgkins disease, and advanced pelvic squamous cell carcinoma. A period of 4 to 6 weeks of the deficient diet was required to significantly lower the serum amino acid levels. Since normal cells as well as tumor cells require essential amino acids, the inhibition of tumor growth might be dose-dependent; in one study (55), the amino acid concentration of tumors was found to be 1.2-11 times that of surrounding normal tissue. However, a minimal level of essential amino acids is required by normal cells. In animal experiments (56) forced feeding of diets deficient in certain essential amino acids caused varying amounts of tumor inhibition but also resulted in a negative nitrogen balance.

## Human Normal and Leukemic Lymphocytes in Culture

Lymphocytes can be isolated from other blood leukocytes by separation on glass-wool columns. Separation by this method is excellent because lymphocytes do not readily adhere to glass, while other leukocytes such as polymorphonuclear cells are easily adsorbed. Light microscopy studies (57) have indicated that the population of leukocytes in blood separated on glass-wool columns comprises 95-97% small lymphocytes (diameter 7-10 μ) and less than 1% large lymphocytes (12-15 μ). Later studies using the electron microscope (58) have shown that control cultures incubated up to 8 days contain less than 1% large cells. The leukocyte population remains unchanged during this period, with no significant alteration in lymphocyte fine structure; cell diameter ranges from 5 to 8 μ. The small lymphocytes themselves seem to be heterogeneous, consisting of both short-lived and long-lived cells (59, 60). Long-lived blood lymphocytes constitute approximately 65% of the small peripheral blood lymphocytes; the percentage of long-lived cells ranges from 90% in the thoracic duct to 0-5% in the thymus and bone marrow.

Peripheral blood lymphocytes can be maintained in tissue culture in a viable state for several days. Small but measurable rates of RNA and protein synthesis are observed, but essentially no nuclear DNA synthesis occurs in these cultures (61,62).

Studies by Hungerford *et al.* (63) and later by Nowell (64) showed that the addition of phytohemagglutinin (PHA), an extract from the red kidney bean (*Phaseolus vulgaris*) stimulates lymphocytes to undergo DNA synthesis and cell division. Normal lymphocytes reach

maximum DNA synthesis at 3 to 4 days after PHA stimulation (65). Lymphocytes treated in this fashion will undergo 3-4 cell divisions, with a lifespan of approximately 2 weeks (66).

In contrast to lymphocytes from normal donors, which do not synthesize DNA, lymphocytes from patients with acute lymphocytic leukemia do incorporate $^{3}$H-thymidine into DNA, due to immature lymphocytes (blast cells) in the peripheral blood. The generation time of the dividing leukemic cells appears to vary considerably, the shortest total generation time usually being 60-70 hours (67). Studies (68,69) have shown that the labeling of blast cells with $^{3}$H-thymidine in acute lymphoblastic leukemia is low; the labeling index is uniformly and distinctly lower in blood than in marrow. These results indicate that not all leukemic blast cells are actively synthesizing DNA and undergoing mitosis. However, recent studies (70,71) indicate that the leukemic blast cells not in the active growth cycle are not all end-stage cells; at least some of these may potentially enter the active generative cycle. Prolonged cultivation of lymphoblasts in culture results in their maturation to mature lymphocytes which are not labeled with $^{3}$H-thymidine (72). Prolonged cultivation of the leukemic cells in culture is possible using a supporting layer of cells that have adapted to growth in tissue culture (73,74). Cell lines of lymphoblasts have been derived from normal persons as well as from patients with malignancies (75,76). Biochemical properties of normal and leukemic leukocytes have been reviewed elsewhere (77,78).

## L5178Y Murine Leukemic Lymphoblasts

Murine leukemic lymphoblasts require a supporting layer of mouse fibroblasts for successful propagation (79). However, in 1957, a murine lymphoblastic leukemia cell line was isolated that would grow in suspension culture in the complete absence of nonleukemic cells (80). The origin of the L5178Y line was reported to have been produced by methylcholanthrene in an adult DBA/2 mouse (81). The L5178Y will grow as an ascites tumor in DBA/2 mice. However, if injected intraperitoneally into mice of other strains, the cells initially grow but are rejected by a homograft reaction after approximately 10 days. A more detailed description of the growth of L5178Y cells in vivo is given elsewhere (82). The growth of the L5178Y cells in culture requires a high level of folic acid, as well as the presence of a peptone. The peptone requirement has since been shown (85) to be partially due to a requirement of these cells for L-asparagine. Fischer (86) cloned the L5178Y cells on a feeder layer of chick fibroblasts, yielding seven pure lines by two consecutive cloning procedures. Time-lapse photography has demonstrated that, although cells from one pure line (L5178Y) settle to the bottom of the culture flask, they remain spherical and do not attach to glass throughout the life cycle (87).

The generation time of the cells in media supplemented with horse serum (8%) has been reported to be 7.6-14 hours, depending on the particular lot of horse serum (88). The generation time of the cells has also been shown to be temperature-dependent with the $G_1$ and S phase of the cell cycle most markedly affected (89). At the optimum growth

temperature of 37°C, the S and $G_1$ phases of cell cycle in cells with a 10.8 hour generation time occupy 66.3% and 22% of the generation time, respectively. The $G_2$ and M phases occupy only 8% and 3.6%, respectively (90). Other requirements for optimum growth of the cells include a pH maintained in the region of 6.6-7.7 and a volume ratio of air to medium of 6:1 (86).

The L5178Y cells have a stable diploid model chromosome number of 43, which remains the same in vivo or in vitro (91). The karyotype of the L5178Y contains no metacentric chromosomes and in this respect resembles the normal mouse cell (92). These findings in L5178Y murine lymphoblastic leukemia contrast with the findings in many human acute leukemias (93) where abnormalities range from minor rearrangements in a diploid chromosome arrangement to extensive alterations in both chromosome number and morphology; the most marked changes have been observed in some of the acute lymphoblastic leukemias of childhood.

Electron micrographs of the L5178Y cells in culture have demonstrated the existence of Type-A and Type-C virus-like particles (94,95). The cells have also been shown to have an RNA-dependent DNA polymerase (96). Other biochemical studies have been performed on the L5178Y murine leukemic lymphoblasts, including a) protein, glycoprotein, and lipid synthesis in intact cells (97-99), b) protein, glycoprotein, RNA, and DNA synthesis in isolated mitochondria (100), and c) RNA (101,102) and DNA (103,104) synthesis in intact cells. Apparently the cells can also rejoin single-strand breaks induced in DNA without necessitating DNA, RNA or protein synthesis (105-107).

The growth of L5178Y leukemic lymphoblasts in culture is inhibited by a variety of agents, including radiation (108-114), alkylating agents (115,116), pyrimidine analogs (117-121), hydroxyurea or formamidoxime (122), hydroxyguanidine (123), 6-mercaptopurine, 6-(methylmercapto)purine ribonucleoside (124), methotrexate (83), amethopterin (86), antibodies (125), treatment with enzymes to remove folic acid (126), and asparagine (127) from the medium. The respiration of these cells is inhibited by the common respiration inhibitors (128).

## Phenylalanine Ammonia-Lyase

Phenylalanine ammonia-lyase (E.C. 4.3.1.5) is an enzyme that catalyzes the deamination of phenylalanine to trans-cinnamic acid and ammonia. The enzyme has been found in a wide variety of plants (129-134) and fungi (135,136). In plants, the product of phenylalanine deamination, cinnamic acid, is used to make cell wall structures in a process generally referred to as lignin formation (137). Activity of the enzyme has been shown to be inducible in pea pods by the addition of poly-L-lysine, spermidine, or histone fractions (138). Potato tuber phenylalanine ammonia-lyase has been purified more than 300-fold (133). It has an estimated molecular weight of 330,000; and its activity is inhibited by carbonyl reagents (e.g. $NaBH_4$) but unaffected by sulfhydryl reagents.

Phenylalanine ammonia-lyase has also been found in bacteria (139) and in yeast-- primarily Sporobolomyces pararoseus (140,141) and Rhodotorula glutinis (142-144). The enzyme from Sporobolomyces pararoseus has been purified more than 450-fold (145). It has a molecular weight between 275,000 and 300,000, with four subunits of equal molecular weight between 70,000 and 74,000. The enzyme deaminates both

phenylalanine and tyrosine and is inactivated by carbonyl, amino, and sulfhydryl reagents. Dehydroalanine has been shown to be present at the active site and is essential for catalytic activity of the enzyme.

Phenylalanine ammonia-lyase has been purified approximately 48.5 fold from Rhodotorula glutinis by Hodgins (143,144) by salt fractionations and Sephadex chromatography. The enzyme has an approximate molecular weight of 275,000. It is inactivated by borohydride or cyanide and by reagents attacking either amino or sulfhydryl groups. Evidence suggests that dehydroalanine is at the active site of the enzyme. The enzyme has been shown to have a primary substrate affinity for L-phenylalanine and L-tyrosine. However, it will deaminate a variety of substrate analogs, including DL-3-hydroxy-phenylalanine and D-phenylalanine. The ability of the Rhodotorula glutinis enzyme to deaminate both phenylalanine and tyrosine has also been demonstrated by Uchiyama et al. (146).

## Specific Aims

The aims of this research were to: a) isolate and purify phenylalanine ammonia-lyase from Rhodotorula glutinis for use in tissue culture with normal and leukemic lymphocytes, b) ascertain the effects of the enzyme on the growth and viability of both normal human lymphocytes and lymphocytes from patients with acute lymphoblastic leukemia, c) test the effects of this enzyme on murine leukemic lymphoblastic (L5178Y) growth and on macromolecular synthesis in these cells, and d) compare phenylalanine ammonia-lyase treatment with L-asparaginase treatment; and dietary deprivation cf phenylalanine with phenylalanine ammonia-lyase treatment in the ability of each to inhibit cell division of the murine leukemic lymphoblasts.

# CHAPTER II

# MATERIALS AND METHODS

## Materials

### Human Lymphocytes

For the studies on normal lymphocytes, 500-ml samples of peripheral blood were collected from normal female donors who had fasted overnight. Each sample was collected in bottles containing heparin to prevent coagulation.

For the studies on acute lymphocytic leukemia (ALL) lymphocytes, 50-ml blood samples were collected from patients with ALL. These, too, were treated with heparin. All patients had elevated white blood cell counts with differing amounts of immature lymphocytes (lymphoblasts) in the peripheral blood. The patients either had received no prior treatment for their disease, or had been treated at least 3 months prior to the time of blood collection.

### Murine Leukemic Lymphoblasts (L5178Y)

For the studies on murine leukemia, the L5178Y murine leukemic lymphoblastic cell line was obtained from Dr. Judson Spalding, National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina.

## Cell Culture

Heparin was purchased from the Upjohn Co. (Kalamazoo, Michigan). A column packed with glass wool (Owens Corning, Corning, New York) was used to separate human lymphocytes from other white cells. During the lymphocyte isolation step, cells were suspended in Eagle's minimal essential medium (MEM). McCoy's modified 5A medium without antibiotics, glutamine, or serum was used to culture the human lymphocytes. Fischer's medium without antibiotics but with glutamine was used to culture the murine leukemic lymphoblasts. For incubation of the human lymphocytes, the McCoy's 5A medium was supplemented with glutamine and 10% fetal calf serum. Fischer's medium supplemented with 10% horse serum and 1% penicillin/streptomycin was used to culture the murine L5178Y lymphoblasts. Eagle's minimal essential medium, McCoy's modified 5A medium, Fischer's medium, media supplements, and Earle's balanced salt solution were purchased from Grand Island Biological Co. (Grand Island, New York). Cell culture manipulations were performed in a laminar flow hood (Pure Aire Corp., Van Nuys, California) to reduce contamination by air borne organisms. The cell cultures were incubated in a water-jacketed $CO_2$ incubator (National Appliances Co., Cherry Hill, New Jersey). For the human lymphocytes, bone-dry grade $CO_2$ (Matheson Co., East Rutherford, New Jersey) was used to regulate the incubator at 5% $CO_2$ in balanced air. Five percent $CO_2$ (Matheson Co., East Rutherford, New Jersey) was added to the Fischer's medium, antibiotics, and supplements to adjust the pH to approximately 7.0 before the L5178Y lymphoblasts were incubated. Phytohemagglutinin-P was purchased from Difco Laboratories (Detroit, Michigan). Rhodotorula glutinis was

purchased from P-L Biochemicals, Inc. (Milwaukee, Wisconsin). L-asparaginase was purchased from Merck, Sharp, and Dohme (West Point, Pennsylvania).

L-amino acids and nucleosides (L-tyrosine-3,5-$^{3}$H, L-leucine-4,5-$^{3}$H, thymidine-methyl-$^{3}$H and uridine-6-$^{3}$H) were obtained from New England Nuclear Corp. (Boston, Massachusetts). L-phenylalanine-$^{14}$C(UL) was obtained from Amersham-Searle (Arlington Heights, Illinois). Cinnamic acid and coumaric acid were obtained from Sigma Chemical Co. (St. Louis, Missouri).

## Other Experimental Materials

All common chemical reagents were purchased from Baker Chemical Co. (Phillipsburg, New Jersey). Quantitative studies on DNA, RNA, and protein were performed with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Paper chromatography was performed on Whatman paper No. 1 (Curtin Scientific Co., Houston, Texas). Ultraviolet-absorbing material on the paper chromatographs was located with a Mineralight UV lamp (Ultra-violet Products, Inc., San Gabriel, California). Radioactivities were determined either on a Vanguard strip counter (Vanguard Instruments, North Haven, Connecticut) or with a liquid scintillation counter (Nuclear Chicago, Chicago, Illinois). Cell counts were determined with a Coulter Counter Model B (Coulter Electronics, Hialeah, Florida) or a hemacytometer (Curtin Scientific Co., Tulsa, Oklahoma) and an Olympus light microscope (Actino Rex, Springfield, Missouri). The pH was measured on a Radiometer Copenhagen pH meter (London Co., Westlake, Ohio). Absorption spectra of cinnamic and coumaric acids were determined on a Cary recording spectrophotometer

(Applied Physics Corp., Monrovia, California). Small amounts of known standards were weighed on a Cahn microbalance (Ventron Instruments Corp., Paramount, California).

## Methods

### Preparation of Phenylalanine Ammonia-Lyase

Phenylalanine ammonia-lyase (PAL) was prepared by the method of Hodgins (143) from the yeast Rhodotorula glutinis. One unit of phenylalanine ammonia-lyase is defined as the amount of enzyme necessary to produce 1 μmole of cinnamic acid per minute at 30°C. The reaction mixture (1 ml) consisted of 0.833 mM L-phenylalanine, 0.1 M Tris-HCl (pH 8.5) and 10 μl of enzyme. Five separate preparations gave enzymes ranging in specific activity from 0.48 to 1.25 units per milligram of protein. The activity of highly purified phenylalanine ammonia-lyase varied in specific activity from 0.5-1.2 units/mg protein (143). The enzyme preparation was dialyzed against 0.05 M Tris-HCl (pH 8.5) for 24 hours and sterilized with a Millipore filter (0.2 μ). The enzyme was stored at 4°C before use in tissue culture at 37°C.

### Stability of PAL

The stability of PAL at 4°C in different concentrations was determined at various intervals until the enzyme was depleted. Aliquots (10 μl) of the enzyme preparation were assayed for enzyme activity by the method of Hodgins (143).

The stability of PAL at 37°C in Fischer's medium plus 10% horse serum (FHS) with and without cells was also determined at various intervals during incubation. Cells were removed from the incubation medium by centrifugation at 1000 x g for 10 minutes and the supernatant was decanted for use in the enzyme assay.

Preparation of Cinnamic Acid-$^{14}$C and Coumaric Acid-$^{3}$H

Cinnamic acid-$^{14}$C(UL) and coumaric acid-3,5-$^{3}$H were prepared enzymatically. A 1-ml reaction mixture containing L-phenylalanine-$^{14}$C (50 μCi) in 0.05 M Tris-Cl, pH 8.5, was incubated with 1.2 units of phenylalanine ammonia-lyase for 64 hours. Another mixture containing L-tyrosine-3,5-$^{3}$H (1 mCi) was incubated with 0.6 units of phenylalanine ammonia-lyase for 52 hours. In the latter reaction, unlabeled L-tyrosine (1 mg) was added to provide additional substrate. The reactions were stopped by the addition of concentrated HCl (0.1 ml). The material was then cooled to 4° for 30 minutes and centrifuged at 23,500 x g for 5 minutes. Each acidified supernatant (0.5 ml) was diluted to 1.5 ml with distilled water and extracted with diethyl ether (3 ml). The aqueous layer was reextracted once more with ether. The two ether extracts were combined and reextracted with 0.1 N sodium hydroxide (2 ml). This procedure was a modification of the method of Uchiyama et al. (146).

Cinnamic acid-$^{14}$C(UL) and coumaric acid-3,5-$^{3}$H were separated by descending paper chromatography in 1-butanol:acetic acid:water (450:50:125) (147). A Vanguard paper chromatogram scanner was used to find the radioactivity on the paper, and UV light was used to locate the cinnamic and coumaric acids. Each preparation contained only one radioactive peak; these peaks corresponded to cinnamic acid and coumaric acid standards, respectively. The absorbance spectra of the cinnamic acid and coumaric acid used as standards were determined by dissolving known amounts of both acids in NaOH (0.1 N) and using a Cary recording spectrophotometer. The absorption spectra thus determined were compared to those shown by Uchiyama et al. (146) for cinnamic acid and coumaric acid in NaOH (0.1 N).

## Determination of Rate of Conversion of L-Phenylalanine and L-Tyrosine to Cinnamic Acid and Coumaric Acid by Phenylalanine Ammonia-Lyase

FHS (15 ml), either with or without added leukemic cells, was treated with phenylalanine ammonia-lyase at various concentrations. The rates of deamination of phenylalanine and tyrosine were determined spectrophotometrically; the formation of cinnamic acid and coumaric acid was measured after their extraction from the media by a modification of the method of Uchiyama et al. (146). The efficiency of extraction was determined by adding the prepared radioactive products, cinnamic acid-$^{14}C$ (UL)(6,600 dpm) and coumaric acid-3,5-$^{3}H$ (20,700 dpm) to the media (0.5 ml) before extraction. These radioactive standards did not contribute significantly to the final absorbances of the unlabeled cinnamic and coumaric acids (<0.05 A). The concentrations of cinnamic and coumaric acid produced by treatment with phenylalanine ammonia-lyase were measured spectrophotometrically at 268 and 333 mμ, respectively, against an absorbancy curve with known amounts of cinnamic and coumaric acids. An additional correction was used to compensate for the coumaric acid absorbance at 268 mμ (24). The concentrations of cinnamic and coumaric acids were then corrected for the efficiency of extraction to determine the actual amounts of each produced. The efficiency of extraction was determined by adding 0.5-ml aliquots of the medium to 15 ml Bray's solution after the final extraction with 0.1 N NaOH (150). Radioactivity was counted on a Nuclear Chicago liquid scintillation counter using a barium external standard quench correction curve for counting doubly labeled $^{3}H$ and $^{14}C$ samples (151). The standards used in determining the quench correction curve were obtained by adding different

amounts of 0.1 N NaOH to Bray's solution containing known amounts of $^{14}C$ and $^{3}H$ labeled standards.

The total phenylalanine and tyrosine concentrations in FHS were determined independently by the enzymatic procedure described below and by fluorometry (148,149). In the enzymatic determination, L-phenylalanine-$^{14}C$ (135,000 dpm) and L-tyrosine-3,5-$^{3}H$ (131,000 dpm) were added to 1 ml of FHS. The pH of the medium was adjusted to 8.5 with 1 N NaOH for optimal enzyme activity. The medium was then incubated with 5.0 units of phenylalanine ammonia-lyase (1 ml) for approximately 65 hours. The reaction was terminated, and cinnamic and coumaric acids were determined as described above. Both methods gave essentially the same values for phenylalanine (55 µg/ml) and tyrosine (115 µg/ml) in FHS.

## Preparation of Dialyzed Horse Serum

Twenty milliliters of horse serum (HS) were dialyzed against Earle's balanced salt solution (EBSS) for approximately 65 hours with a volume ratio of EBSS to HS of 20:1. The EBSS was changed at 24-hour intervals. To determine the phenylalanine remaining after dialysis, L-phenylalanine-$^{14}C$(UL) (3.5 x $10^6$ dpm) was added to the horse serum before dialysis. Aliquots of the horse serum were then assayed for radioactivity using Bray's (150) or Gordon's (152) solution and liquid scintillation spectroscopy at the times indicated.

## Cell Culture

Human normal lymphocytes. Blood lymphocytes from normal female donors were prepared for culture by the procedure of Abell et al. (65), which is a modification of the method of Cooper and Rubin (153). One

unit (500 ml) of heparinized blood (10,000 units heparin/unit blood) was drawn from a fasting normal female donor and transferred to sterile plastic 50-ml centrifuge tubes with twist caps. The blood was allowed to settle for 1 hour in an incubator at 37°C. The leukocyte-rich plasma layer was separated and mixed with an equal volume of Eagle's minimal essential medium (MEM) prewarmed to 37°C. A purified lymphocyte population was obtained by passing the cell suspension through a column containing glass wool. Wright stain smears showed that the effluent contained 97-99% lymphocytes (165).

Lymphocyte pellets were obtained by centrifugation at 280 x g for 30 minutes at room temperature. The supernatant was removed and the lymphocytes were resuspended in a small volume of modified McCoy's 5A medium. The cells were counted on a hemacytometer and resuspended at a concentration of $10^7$ cells per 5 ml of modified McCoy's 5A medium supplemented with 10% fetal calf serum and 1% L-glutamine in 2-ounce prescription bottles. The lymphocyte cultures in bottles with loose lids were incubated at 37°C in an atmosphere of 5% $CO_2$ in balanced air.

Human leukemic lymphocytes. For ALL cells, the total leukocyte population was used in the tissue culture procedure. The procedure was the same as that used for the preparation of normal lymphocytes except that only 50 ml of heparinized blood (1000 units heparin/50 ml blood) was drawn from patients with ALL, and the separation step using the glass wool column was omitted. The leukocytes were cultured in 2-ounce prescription bottles with loose lids at a concentration of $10^7$ cells per 5 ml of modified McCoy's 5A medium supplemented with 10% fetal calf serum and 1% L-glutamine at 37°C in an atmosphere containing 5% $CO_2$ in balanced air.

Murine leukemic lymphoblasts. Murine leukemic lymphoblasts (L5178Y) were cultured in the medium described by Fischer (88). Immediately before use, 10% horse serum and 1% penicillin/streptomycin (750 units penicillin, 750 µg streptomycin) were added to this medium in 4-ounce prescription bottles, to a total volume of 15 ml. The complete medium was gassed with 5% $CO_2$ until it turned amber (pH 7.0). After rubber stoppers were inserted tightly into the bottles, the media were incubated at 37°C for 30-60 minutes. L5178Y cells (1000/ml) were then added and allowed to reach the logarithmic growth phase before being used for experimental determinations.

Assessment of cell division and cell viability. Cell division of the L5178Y murine leukemic lymphoblasts was determined by use of a hemacytometer or a Coulter Counter Model B. Cell viability was determined by the Erythrosin B dye exclusion test (154) using 0.2 ml of 0.4% Erythrosin B per milliliter of cell suspension. The preparation was examined microscopically within 30 minutes after staining. Dye uptake was associated with a loss of cell viability.

Assessment of macromolecular synthesis. DNA synthesis was estimated by the uptake of tritiated thymidine (Th-Me-$^3$H, 2.0 and 6.7 Ci/mmole, 2 µCi/ml), which was added to the cultures 2 hours before the reaction was stopped. RNA and protein synthesis were estimated by the uptake of tritiated uridine (Ur-6-$^3$H, 10.4 Ci/mmole, 2 µCi/ml) and tritiated leucine (L-leu-4,5-$^3$H, 5.0 Ci/mmole, 2 µCi/ml), respectively, which were added to the cultures 30 minutes before the reaction was stopped. The incorporation rate of the radioactive precursors into nucleic acid and protein was determined by liquid scintillation spectrometry, as described in the next section.

Isolation and measurement of DNA. At the end of the 30-minute and 2-hour incubation periods, the cells and media were poured into chilled centrifuge tubes and centrifuged at 1000 x g for 10 minutes at 0°C. The supernatant was then decanted and 1 ml of a hypotonic phosphate buffer solution (0.4 mM potassium phosphate, pH 6.7, and 2 mM magnesium chloride) was added to the cell pellet. The pellet was resuspended by use of a Vortex mixer and fast-frozen in acetone and dry ice. Subsequent procedures to remove unwanted cellular constituents and unincorporated radioactive material were performed at 0°C.

The wash procedure was briefly described. The cells were broken by freezing and thawing seven times in a solution of acetone and dry ice. The nucleic acids and protein were precipitated with 1.6 N cold perchloric acid. The standard centrifugation after the addition of the various reagents was 1000 x g for 10 minutes. After each centrifugation, the supernatant was decanted, the next solution was added, and the resuspended pellets were dispersed with glass stirring rods. The solutions added sequentially in 3-ml aliquots were 0.5 N PCA (three times), 95% ethanol, chloroform:ethanol:ether 2:2:1 (v/v) and acetone. The pellet obtained after acetone treatment was hydrolyzed in 0.5 ml of 0.5 N PCA at 90°C for 30 minutes and centrifuged at 1000 x g for 10 minutes.

After centrifugation, 0.2 ml of the supernatant was added to 15 ml of Bray's solution (150) for counting the radioactivity incorporated into nucleic acid. Another 0.2 ml of the supernatant was used to assay total DNA content by Burton's diphenylamine method (155). Radioactivity incorporated into protein was determined by adding 0.2 ml of 1 N NaOH to the pellet remaining after removal of the aliquot for the Burton

procedure. The NaOH-treated pellet was heated at 100°C for 30 minutes to dissolve it and 0.2 ml was counted in 15 ml of Gordon's solution (152). Precursor incorporation of each sample was expressed in disintegrations per minute (dpm); the dpm was calculated by dividing the counts per minute (cpm) by the efficiency of the counting system. The addition of tritiated water of known radioactivity to several samples after the initial counting made it possible to determine the counting efficiency. DNA content was based on a standard calf thymus preparation. The results were expressed in specific activities, i.e., labeled precursor incorporated per microgram of DNA.

### Effect of PAL or L-Asparaginase on Human ALL Cells

Leukocytes were obtained from five patients with acute lymphoblastic leukemia and prepared for tissue culture as indicated previously. These cells were treated with different concentrations of PAL or L-asparaginase (one unit of L-asparaginase is defined as that quantity of enzyme which catalyzes the formation of 1 μmole of L-aspartic acid per minute at 37°C) and their effects on DNA synthesis and DNA content were determined.

### Effect of PAL on Normal Human Lymphocytes

The effect of PAL on normal human lymphocytes in the resting state was determined by incubating the cells with different concentrations of enzyme for 1 day. The cells were separated from the medium by centrifugation (300 x g) and washed twice with fresh medium to remove the enzyme. The cells were then suspended in fresh medium and stimulated to divide with PHA (40 μg). DNA synthesis and DNA content were determined.

The effect of PAL on normal human lymphocytes in the dividing state was determined by stimulating the cells to divide with PHA (40 μg) and treating them with different concentrations of PAL. The effect of enzyme treatment on DNA synthesis and DNA content was determined.

### Effect of PAL or Asparaginase on Cell Growth and Viability of Murine Leukemic Lymphoblasts

The effect of different concentrations of PAL or L-asparaginase on cell growth of L5178Y murine leukemic lymphoblasts was determined by treating the cells with different concentrations of enzyme and assessing cell numbers with either a hemacytometer or a Coulter Counter Model B. The effect of higher concentrations of PAL on total cell count and viable cell count was also determined.

### Effect of Removal of PAL from L5178Y Cells and Resuspension of Cells in Fresh Medium

L5178Y cells were treated with relatively high concentrations of PAL for 70 hours. At the end of the treatment procedure, cells were centrifuged at 280 x g for 5 minutes and washed twice with fresh medium; they were resuspended in complete medium, and their numbers were determined.

### Effect of PAL on Macromolecular Synthesis in L5178Y Cells

The effect of PAL on DNA, RNA, and protein synthesis was determined. In separate experiments, 5 ml of cells from logarithmically growing cultures in 4-ounce bottles were transferred to 2-ounce bottles and treated with the same concentration of PAL. Incorporation of thymidine-methyl-$^{3}H$, uridine-6-$^{3}H$, or L-leucine-4,5-$^{3}H$ into DNA, RNA, or protein and DNA content were determined.

### Effect of PAL Treatment on Phenylalanine Concentration and Cell Viability of L5178Y Cells in FHS

The effect of phenylalanine ammonia-lyase on phenylalanine concentration in media plus L5178Y cells was determined by the spectrophotometric procedure described previously. Briefly, the procedure involved extracting cinnamic acid from the medium at various times after enzyme treatment to determine the amount of phenylalanine deaminated. The phenylalanine remaining in the medium was then determined by subtracting the amount of phenylalanine converted from the initial concentration in the medium before enzyme treatment. Total cell count and cell viability were determined by direct counting on a hemacytometer and by microscopic examination with the Erythrosin B dye exclusion test.

### Effect of Phenylalanine Deprivation on the Viability of L5178Y Murine Lymphoblasts in Culture

Logarithmically growing L5178Y cells cultured in complete Fischer's medium were centrifuged at 280 x g for 5 minutes and transferred to a special Fischer's medium lacking phenylalanine but supplemented with either 10% dialyzed or undialyzed horse serum. At the times indicated, the cells were counted on a hemacytometer and cell viability was determined using the Erythrosin B dye exclusion test. The results were compared to those of L5178Y lymphoblasts in complete medium treated with different amounts of PAL.

### Effect of 0.05 M Tris-Cl (pH 8.5), Cinnamic Acid, Coumaric Acid and Ammonia, and Inactivated Enzyme on the Growth of Murine Leukemic Lymphoblasts

Since PAL was diluted in 0.05 M Tris-Cl (pH 8.5), the effect of this solution on the cell growth of the L5178Y cells was determined. In

addition, the products of phenylalanine and tyrosine deamination, cinnamic acid, coumaric acid, and ammonia were tested for their effects on cell growth. Finally, PAL was treated with sodium borohydride ($NaBH_4$) and the inactivated enzyme was tested for its effect on cell growth.

### Electron Microscopy of Untreated L5178Y Cells and Cells Treated with PAL in Culture

Electron micrographs of untreated and PAL-treated L5178Y cells in culture were prepared by Dr. Robert Nordquist. The L5178Y lymphoblasts were first concentrated by centrifugation and placed in cold 6.25% glutaraldehyde fixative in 0.1 M phosphate buffer at pH 7.2 with 5% sucrose for 30 minutes. To remove aldehyde from the cells, specimens were transferred to pH 7.2 phosphate for 18 hours. The cells were then transferred to 1% phosphate-buffered osmium tetra-oxide ($OsO_4$) at pH 7.2 for 30 minutes at $4^{\circ}$. They were rinsed in pH 7.2 phosphate buffer for 5 minutes to remove excess $OsO_4$, dehydrated in graded alcohols, embedded in epoxy resin (Araldite), and heated at $60^{\circ}C$ for 48 hours to produce polymerization. Sections were cut on a microtome (Porter-Blum) and placed on naked copper grids. The sections were floated on a uranyl acetate-methanol staining solution and then on lead citrate. Sections were examined with a Hitachi Hu-11B electron microscope.

# CHAPTER III

## RESULTS

### Phenylalanine Ammonia-Lyase: Purification, Stability and Action of the Enzyme from Rhodotorula Glutinis

The studies reported in this section were: (a) the preparation of PAL according to the procedure of Hodgins (143), (b) the stability of the enzyme at 4°C and in tissue culture at 37°C, and (c) the action of the enzyme on phenylalanine and tyrosine in tissue culture at 37°C.

Table 1 shows the results obtained in one preparation of the enzyme purified from approximately 450 g of yeast. This procedure yielded two highly active fractions with 110 and 47 units of enzyme activity, respectively. The fractions had a 62 and 52-fold increase in specific activity, respectively. However, the increase in specific activity was accompanied by about an 80% decrease in total enzyme units. The greatest losses of total units in this preparation occurred after fractionation with sodium citrate and Sephadex chromatography. Subsequent dialysis of these fractions against Tris-Cl and sterilization through a Millipore filter appeared not to alter the specific activity of the enzyme. Approximately 900 units of enzyme for use in this research project were prepared by this procedure.

The sterilized PAL preparations were stored at 4°C in different concentrations. Table 2 indicates their stability. The enzyme stored

TABLE 1

PURIFICATION OF PHENYLALANINE AMMONIA-LYASE
FROM RHODOTORULA GLUTINIS

| | Protein (mg) | Enzyme (Units) | Specific Activity Units/mg |
|---|---|---|---|
| (1) Sonic Extract | 48,000 | 780 | 0.02 |
| (2) Protamine Sulfate | 19,200 | 765 | 0.04 |
| (3) First Ammonium Sulfate Fractionation | 1,748 | 700 | 0.38 |
| (4) Sodium Citrate Fractionation | 1,093 | 491 | 0.45 |
| (5) Sephadex G-200 Eluent | 193 | 202 | 1.04 |
| (6) Second Ammonium Sulfate Fractionation | (a) 88<br>(b) 45 | 110<br>47 | 1.25<br>1.04 |

TABLE 2

STABILITY OF DIFFERENT CONCENTRATIONS
OF PAL AT 4°C

| Time (Days) | Enzyme Activity (Units/ml) | |
|---|---|---|
| 0 | 0.050[a] | 12.5[b] |
| 13 | 0.040 | 12.5 |
| 28 | 0.030 | --- |
| 56 | 0.025 | --- |
| 87 | --- | 12.2 |
| 232 | --- | 12.6 |

[a]Specific Activity of 1.04
[b]Specific Activity of 1.25

at 12.5 U/ml appeared to lose no activity after 8 months of storage. When PAL was stored in dilute solution, however, 45-50% of the enzyme activity was apparently lost after only about 2 months of storage.

The stability of PAL in complete Fischer's medium at 37°C with and without cells was also determined (Table 3). The presence of L5178Y cells seemed not to alter enzyme activity. The enzyme lost no activity during the 2-week culture period.

The action of PAL on L-phenylalanine and L-tyrosine is shown in Figure 1. For use in determining the rate of deamination of these amino acids in culture, labeled cinnamic acid and coumaric acid were prepared and compared to commercial standards. Figure 2 compares the absorbance spectrum of the commercial standards (5 µg/ml) with the absorbance spectrum of cinnamic and coumaric acids, as reported by Uchiyama *et al.* (146). Both preparations yielded essentially the same results indicating comparable purity. The alkaline solution of cinnamic acid had a primary absorption maximum at 265-270 mµ and an alkaline solution of coumaric acid had a primary absorption maximum at approximately 335 mµ.

A concentration curve of known standards of cinnamic acid and coumaric acid was plotted. An alkaline solution of each acid was weighed on a Cahn microbalance and its absorbancy determined at 268 mµ (cinnamic acid) or 333 mµ (coumaric acid). The optical density for alkaline solutions of the organic acids was linear over the concentrations used (Figure 3).

The rate of deamination of L-phenylalanine and L-tyrosine by PAL was determined by extracting cinnamic acid and coumaric acid from the medium. The amount of each acid present was determined

TABLE 3

STABILITY OF PAL AT 37°C IN FHS WITH AND WITHOUT L5178Y LYMPHOBLASTS

| Time (Hours) | Enzyme Activity (Units/ml) | | |
|---|---|---|---|
| | FHS | FHS with Cells | |
| 0 | 0.016[a] | 0.020[a] | 0.088[b] |
| 6 | 0.021 | 0.021 | -- |
| 21 | 0.017 | 0.016 | -- |
| 45 | 0.017 | 0.017 | -- |
| 70 | 0.016 | 0.016 | -- |
| 123 | -- | -- | 0.070 |
| 350 | -- | -- | 0.075 |

[a]Specific Activity of 1.00
[b]Specific Activity of 1.04

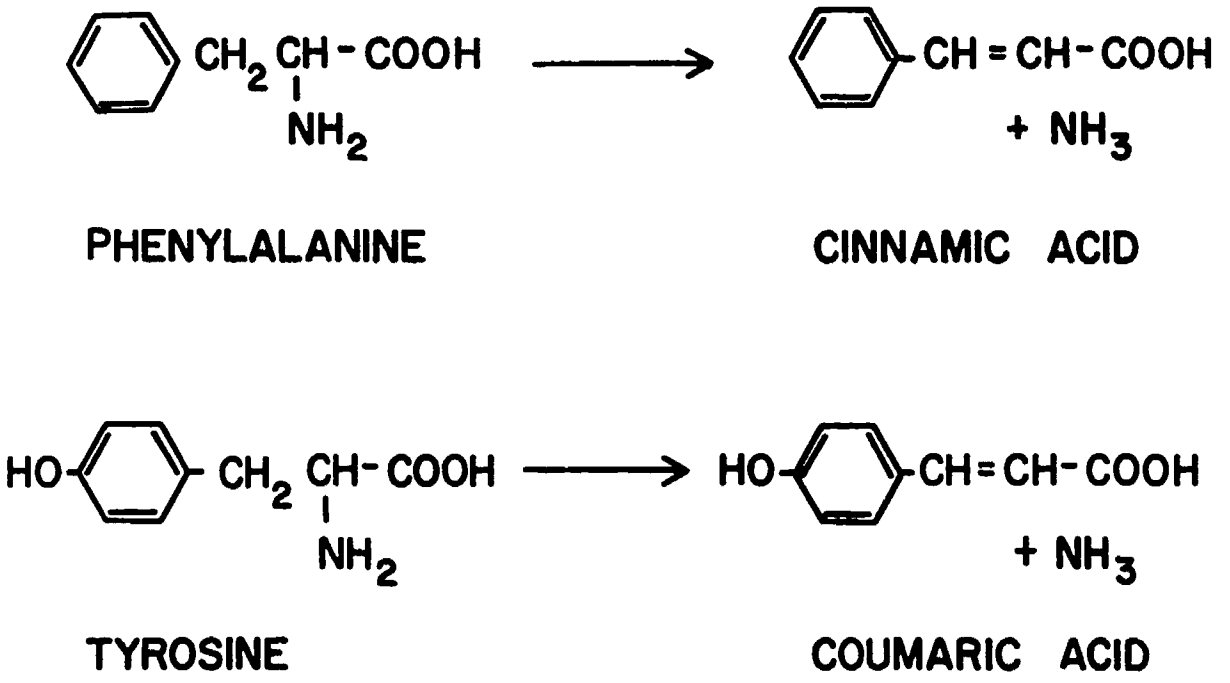


Figure 1 - The action of phenylalanine ammonia-lyase on the amino acids, phenylalanine and tyrosine.

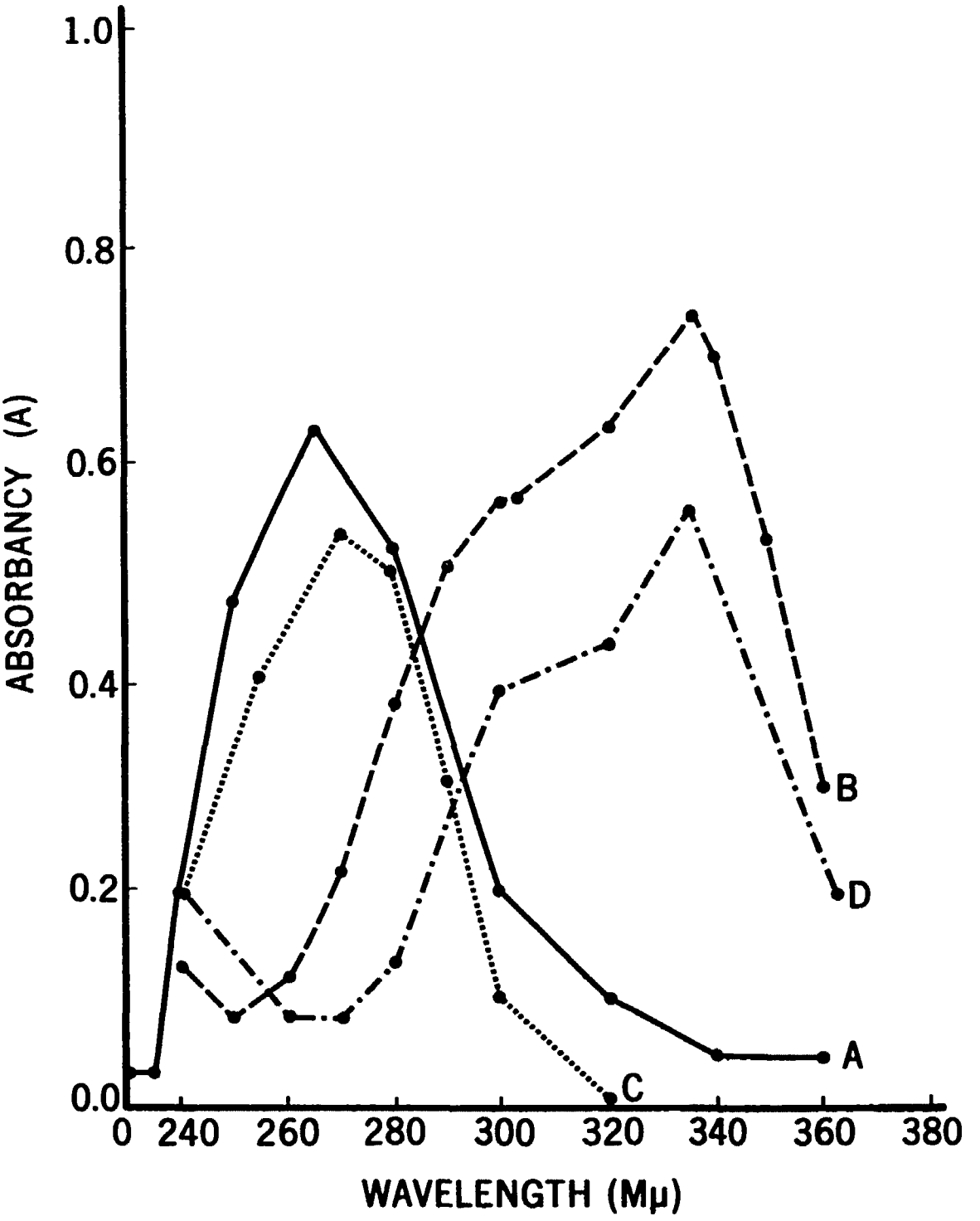


Figure 2 - A comparison of the spectra of commercial preparations of cinnamic acid or coumaric acid in alkaline solution with those reported by Uchiyama, et al.(146). Curves A and B represent spectra of 5 microgram per ml commercial standards of cinnamic acid and coumaric acid in 0.1 normal sodium hydroxide, respectively; curves C and D represent the spectra of cinnamic acid and coumaric acid in 0.1 normal sodium hydroxide respectively, as reported (146).

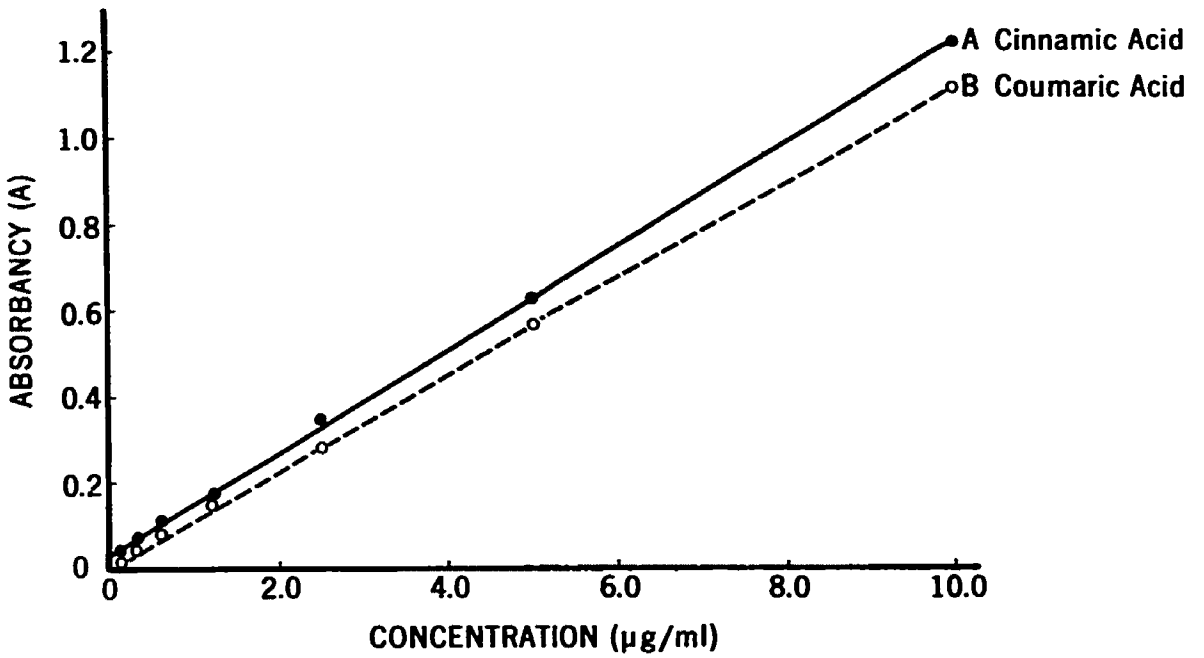


Figure 3 - Absorbancy of standard solutions of cinnamic acid and coumaric acid. Curves A and B represent the absorbancy of standard solutions of cinnamic acid or coumaric acid at 268 mµ and 333 mµ, respectively.

spectrophotometrically by use of Figure 3. To determine how efficiently these acids were extracted from the medium, labeled cinnamic acid and coumaric acid were added to the medium prior to extraction. The concentrations of the extracted cinnamic and coumaric acids were then corrected for the efficiency of extraction to determine the actual amounts of each produced. The efficiency of extraction varied from 81-93% for cinnamic acid and from 83-96% for coumaric acid. These results, shown in Figure 4, indicated a 100% conversion of phenylalanine to cinnamic acid in the medium with or without cells by about 45 hours after PAL addition. Consistent with this observation was the finding that the enzymatic activity of PAL remained constant throughout the experiment. Although phenylalanine was completely converted to cinnamic acid, PAL deaminated tyrosine more slowly, with only 75% of the tyrosine converted to coumaric acid during the 71 hours of incubation with the enzyme.

## Effect of PAL/Asparaginase on Normal Human Lymphocytes and ALL Leukocytes in Culture

The experiments reported in this section were designed to determine the effects of different concentrations of PAL or asparaginase on cell division in ALL leukocytes and the effects of PAL on normal resting and dividing lymphocytes.

The ALL leukocytes were maintained in tissue culture as described in Methods. They were treated with either PAL or L-asparaginase at various concentrations. The effects of these enzymes on cell division were determined by measuring DNA synthesis and DNA content. The effect of PAL on leukocytes obtained from one of the leukemic patients is shown in Figure 5. This patient had a white blood cell count of 237,000/$mm^3$. The differential count revealed the presence of 99% lymphoblasts in the

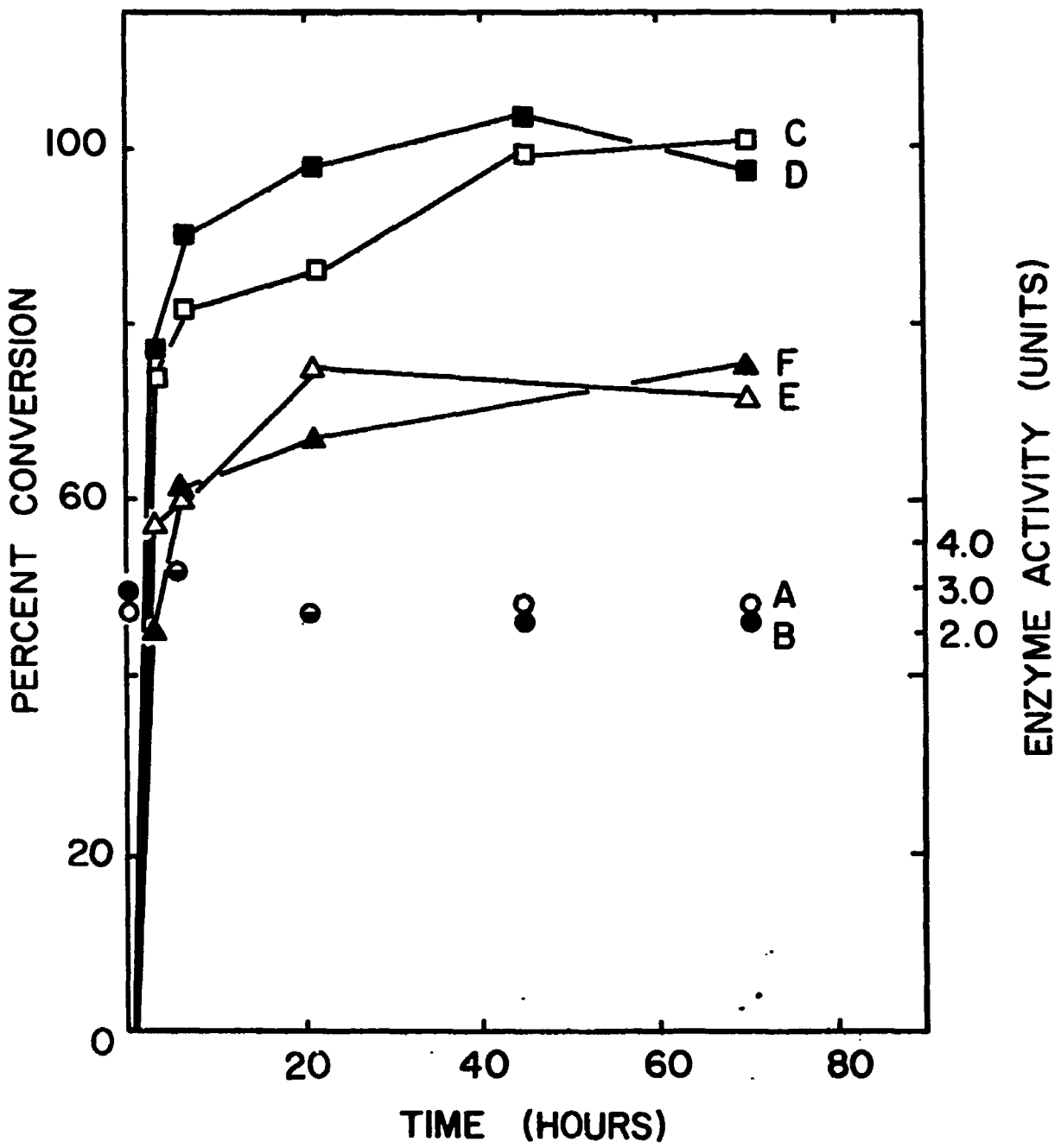


Figure 4 - Rate of formation of cinnamic acid and coumaric acid by phenylalanine ammonia-lyase in complete Fischer's medium in the presence and absence of L5178Y lymphoblasts. Curves A and B represent PAL activity in the presence and absence of cells, respectively. Curves C and D represent formation of cinnamic acid in the medium in the presence and absence of cells, respectively. Curves E and F represent formation of coumaric acid in the medium in the presence and absence of cells, respectively.

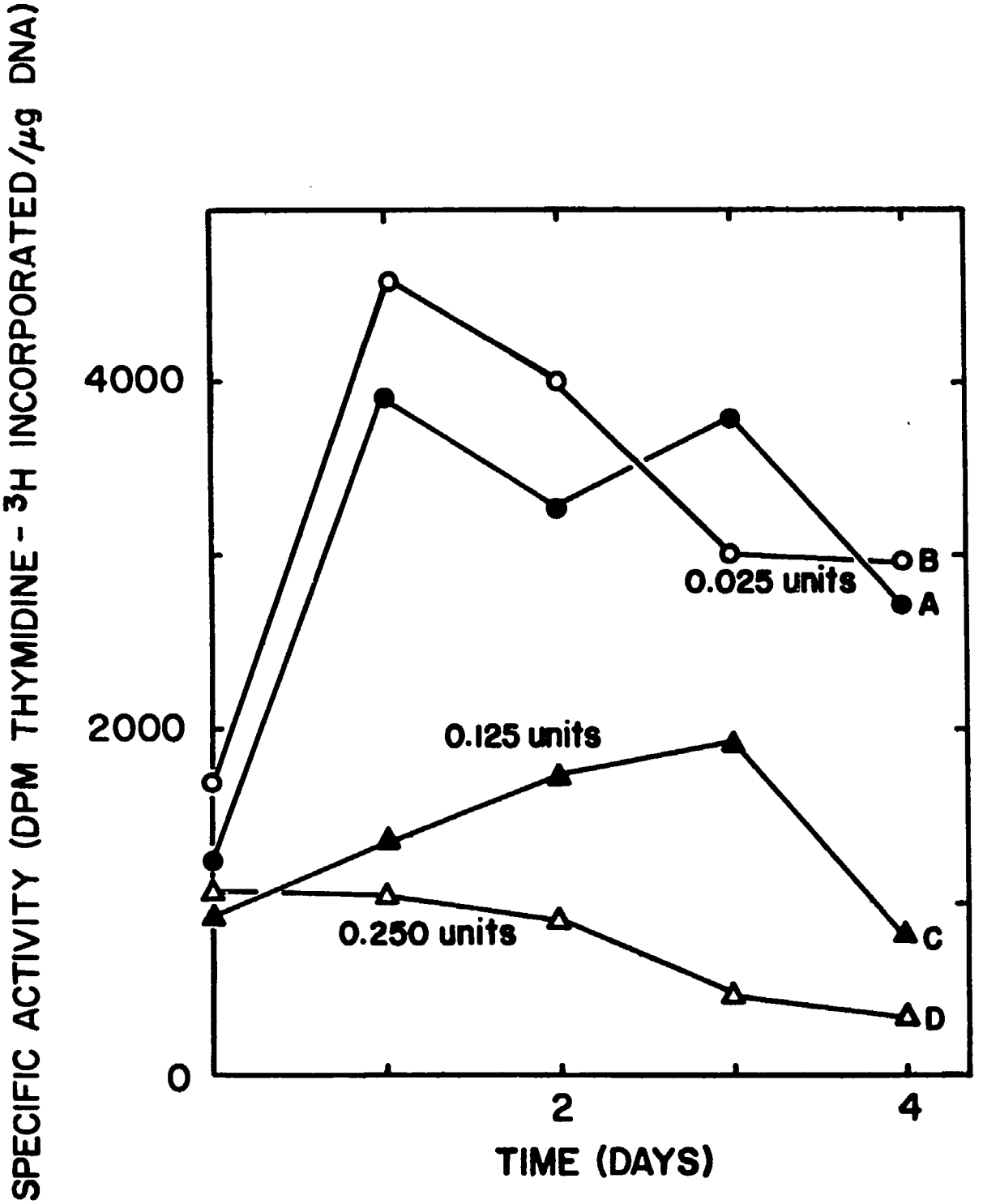


Figure 5 - The effect of phenylalanine ammonia-lyase on DNA synthesis in leukocytes from a patient with acute lymphoblastic leukemia. Curve A, untreated cells; Curves B, C, and D, cells that were treated with 0.025, 0.125, and 0.250 units of PAL per 5 ml media, respectively.

peripheral blood. Untreated acute lymphoblastic leukemia cultures exhibited appreciable DNA synthesis during the first 6 hours in culture (1,200 dpm of thymidine incorporated per microgram of DNA). The rate almost tripled during the next 4 days. Phenylalanine ammonia-lyase in a concentration of 0.025 units had little effect on DNA synthesis, while addition of 0.125 and 0.250 units resulted in 65% and 80% inhibition, respectively, at Day 4. Although the extent of DNA synthesis varied somewhat in lymphocytes from the five leukemic patients studied, the inhibition pattern obtained with phenylalanine ammonia-lyase was similar in all cases.

A similar study was performed to determine the effects of asparaginase on DNA synthesis in acute lymphoblastic leukemia leukocytes (Figure 6). DNA synthesis in ALL leukocytes dropped markedly after treatment with asparaginase.

The effect of PAL treatment on normal lymphocytes in the non-dividing state was determined by pretreating these cells with enzyme for 1 day, changing the media to remove the enzyme, and measuring the proliferative capacity as a function of PHA stimulation (Table 4). Pre-treatment of these normal cells in the nondividing state with relatively high enzyme concentrations (0.500 unit) had no effect on the DNA synthesis resulting from subsequent PHA stimulation.

The effect of PAL treatment on normal lymphocytes in the dividing state was also determined by treating these cells with enzyme after PHA addition (Table 5). Phenylalanine ammonia-lyase both delayed the onset of DNA synthesis and markedly inhibited it once synthesis had started. Additional experiments demonstrated that DNA synthesis remained

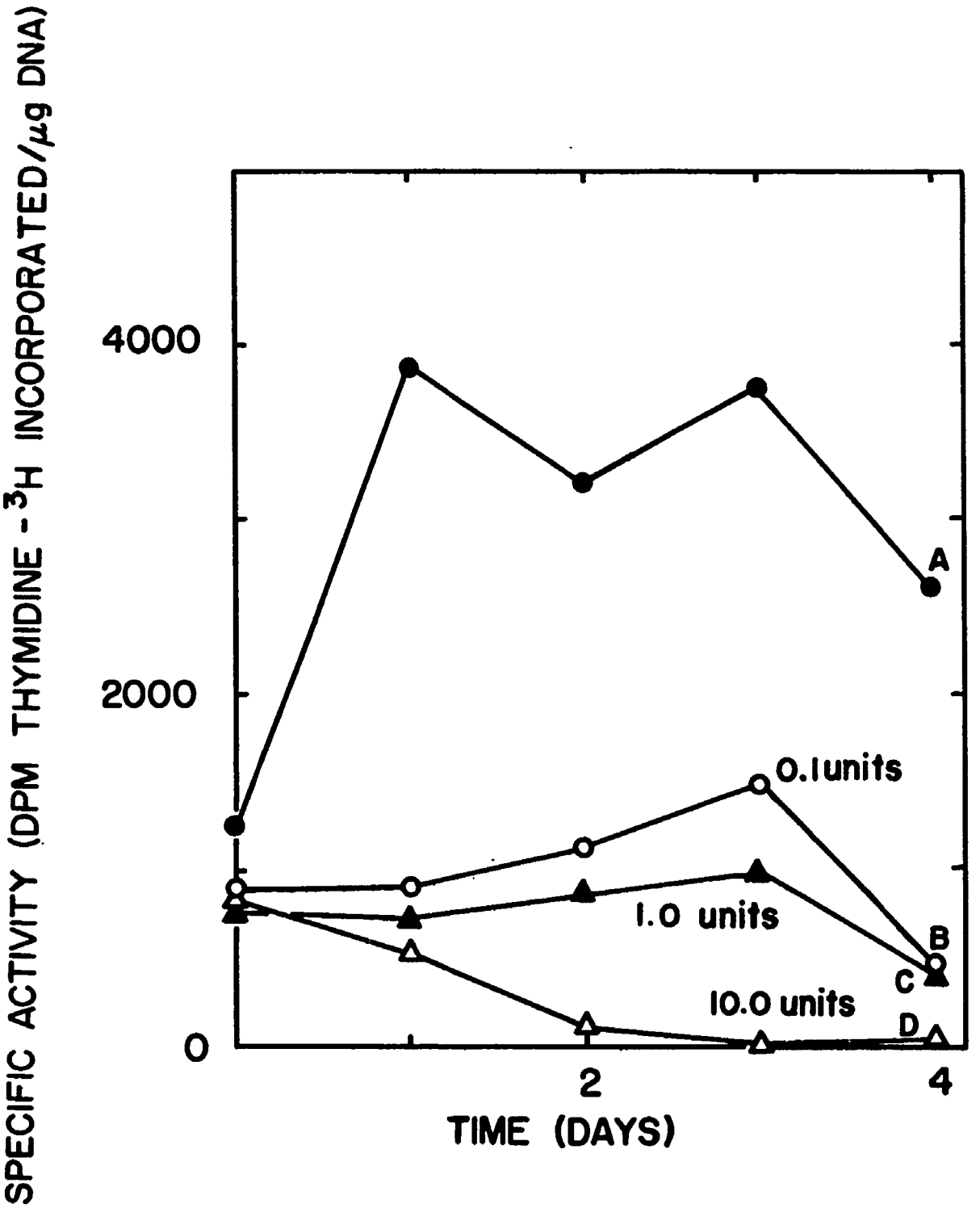


Figure 6 - The effect of asparaginase on DNA synthesis in leukocytes from a patient with acute lymphoblastic leukemia. Curve A, untreated cells; Curves B, C, and D cells that were treated with 0.1, 1.0, and 10.0 units of asparaginase per 5 ml media, respectively.

TABLE 4

EFFECTS OF DIFFERENT CONCENTRATIONS OF PHENYLALANINE AMMONIA-LYASE ON DNA SYNTHESIS IN NORMAL HUMAN LYMPHOCYTES IN THE RESTING STATE

| Enzyme Concentration (Total Units) | Thymidine-$^3$H Incorporated (dpm/μg DNA) | | |
|---|---|---|---|
| | Day 2 | Day 3 | Day 4 |
| None | 26,100 | 40,700 | 26,000 |
| 0.112 | 21,600 | 41,100 | --- |
| 0.225 | 30,000 | 37,500 | 21,000 |
| 0.500 | 29,700 | 42,900 | 15,500 |

TABLE 5

EFFECTS OF DIFFERENT CONCENTRATIONS OF PHENYLALANINE AMMONIA-LYASE ON DNA SYNTHESIS IN NORMAL HUMAN LYMPHOCYTES IN THE DIVIDING STATE

| Enzyme Concentration (Total Units) | Thymidine-$^3$H Incorporated (dpm/μg DNA) | | |
|---|---|---|---|
| | Day 2 | Day 3 | Day 4 |
| None | 19,500 | 28,100 | 19,400 |
| 0.225 | 640 | 2,200 | 6,000 |
| 0.500 | 38 | 250 | 4,800 |

subnormal (i.e., at Days 5, 6, and 8). When the effect of phenylalanine ammonia-lyase on nondividing and dividing lymphocytes was compared under identical conditions (0.500 units of phenylalanine ammonia-lyase), DNA synthesis was four times less by Day 4 in the lymphocyte cultures which were treated with enzyme after PHA stimulation.

## Effect of PAL, Asparaginase, and Phenylalanine Deprivation on Murine Leukemic Lymphoblasts (L5178Y) in Culture

The studies reported in this section were designed to: (a) compare the effects of PAL and asparaginase treatment upon inhibition of cell growth of L5178Y cells in culture, and (b) determine the mechanism of action by which PAL inhibits leukemic cell division. The L5178Y cells were maintained in complete Fischer's medium and were treated with different concentrations of PAL or asparaginase while the cells were in the logarithmic growth phase.

The effect of relatively high concentrations of PAL on the L5178Y cells (100,000/ml initial inoculum) in FHS (15 ml) is shown in Figure 7. Treatment of these lymphoblasts with 3.25 units of PAL rapidly inhibited cell growth (within 24 hours). Treatment with higher concentrations of PAL also caused a slight decrease in total cell number. In contrast to these growth patterns, the control preparation had a doubling time of 10.5 hours.

The effect of treating L5178Y cells in FHS (10 ml) with low concentrations of PAL or asparaginase is shown in Figure 8. Four preparations of cells were treated with 1/8, 1/10, 1/2, and 1 unit of PAL, respectively. Another four preparations of cells were treated with

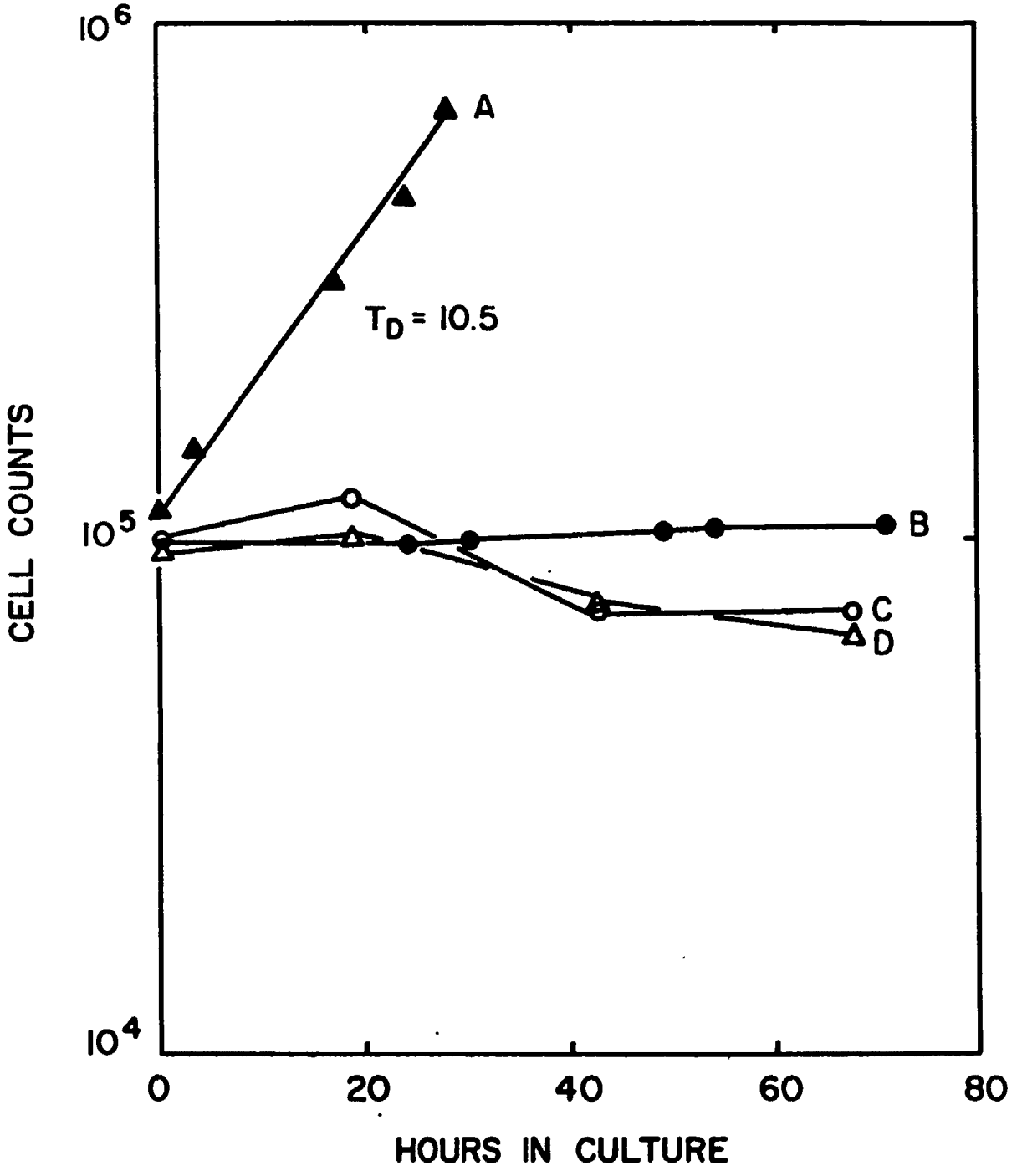


Figure 7 - The effect of phenylalanine ammonia-lyase on cell division of L5178Y lymphoblasts. Curve A, untreated cells; Curves B, C, and D, cells that were treated with 3.25, 5.25 and 7.50 units of PAL per 15 ml media, respectively.

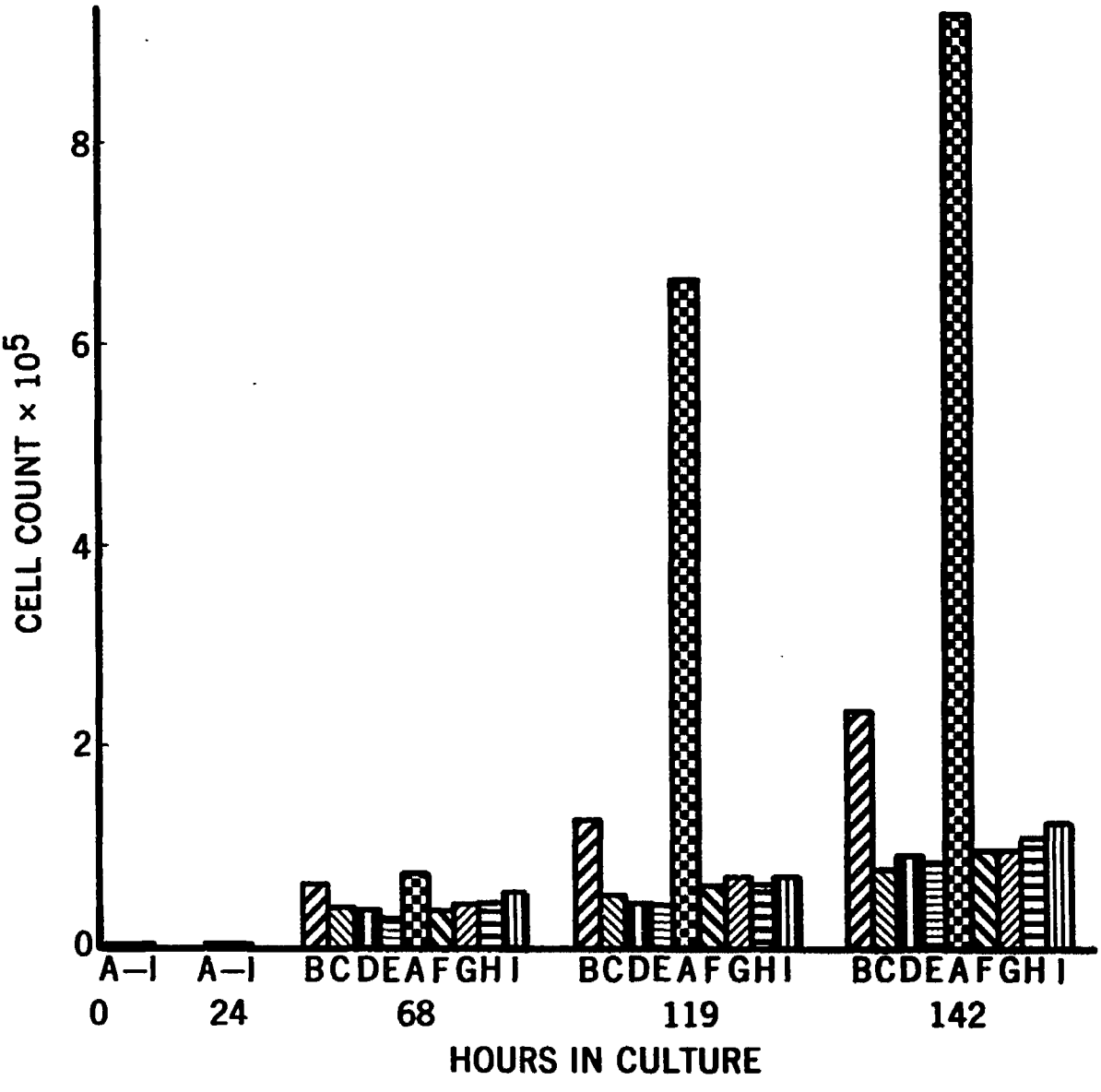


Figure 8 - A comparison of the effect of treatment with low concentrations of PAL or asparaginase on the growth of L5178Y lymphoblasts. Bar A represents untreated cells. Bars B, C, D, and E represent cells treated with 1/8, 1/4, 1/2, and 1 unit of PAL per 10 ml media, respectively. Curves F, G, H, and I represent cells treated with 1/2, 1, 2, and 4 units of L-asparaginase per 10 ml media, respectively.

1/2, 1, 2, and 4 units of L-asparaginase, respectively. Treatment with low concentrations of either PAL or asparaginase markedly inhibited cell growth.

When the L5178Y lymphoblasts were checked for viability after treatment with PAL, a marked difference emerged between total cell count and viable cell count (Figure 9). Untreated cells had a doubling time of approximately 10 hours and maintained a 99±1% viability during logarithmic growth. The results showed that treatment of the cells with a relatively high concentration of enzyme (4.2 U/15 ml FHS) inhibited cell division and stabilized cell number after 4-6 hours of PAL treatment. In contrast to the total cell count, which remained constant, the viable cell count decreased after approximately 10-12 hours of PAL treatment, eventually dropping to less than 2% of the total cell count.

The effect of removal of the enzyme on the subsequent growth of L5178Y cells suspended in fresh medium is shown in Figure 10. These cells had been pretreated with PAL (3.75 U/15 ml FHS) for 70 hours and determinations indicated only 2% viable cells. When these cells were washed and resuspended in fresh medium, regrowth of the cells was observed. After approximately 64 hours in the fresh medium, these cells resumed a normal 10-hour doubling time.

## Effect of PAL on Macromolecular Synthesis of L5178Y Cells in Culture

Further experiments were performed to determine the mechanism of action of PAL by studying the effects of this enzyme on DNA, RNA, and protein synthesis in L5178Y leukemic lymphoblasts. Cells were grown in FHS (initial inoculum $10^7$ cells/5 ml of medium), treated with PAL

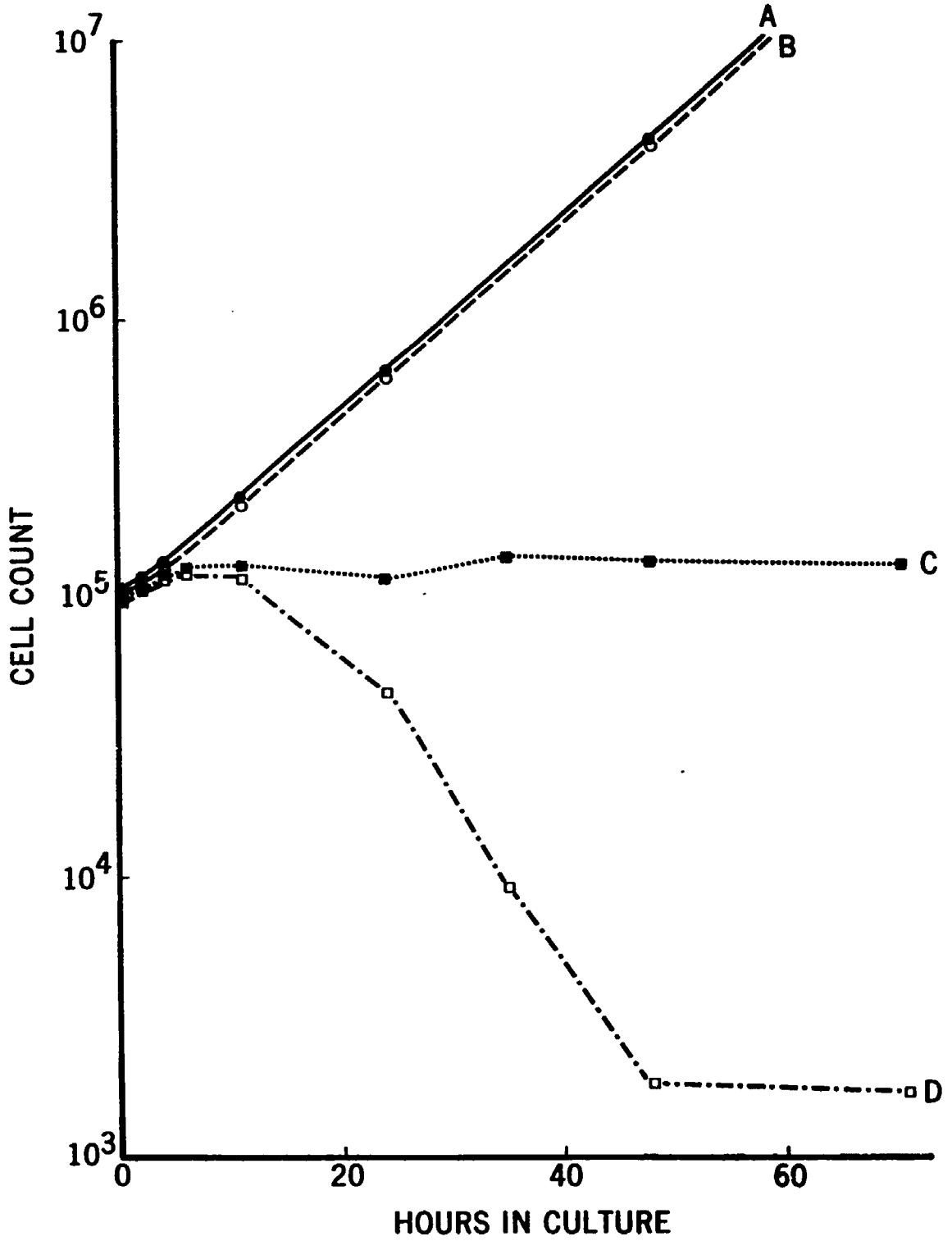


Figure 9 - The effects of phenylalanine ammonia-lyase on the growth and viability of L5178Y lymphoblasts. Curves A and B represent total cell and viable cell count of untreated cells, respectively; curves C and D represent total cell count and viable cell count of cells treated with 4.2 units of PAL per 15 ml media, respectively.

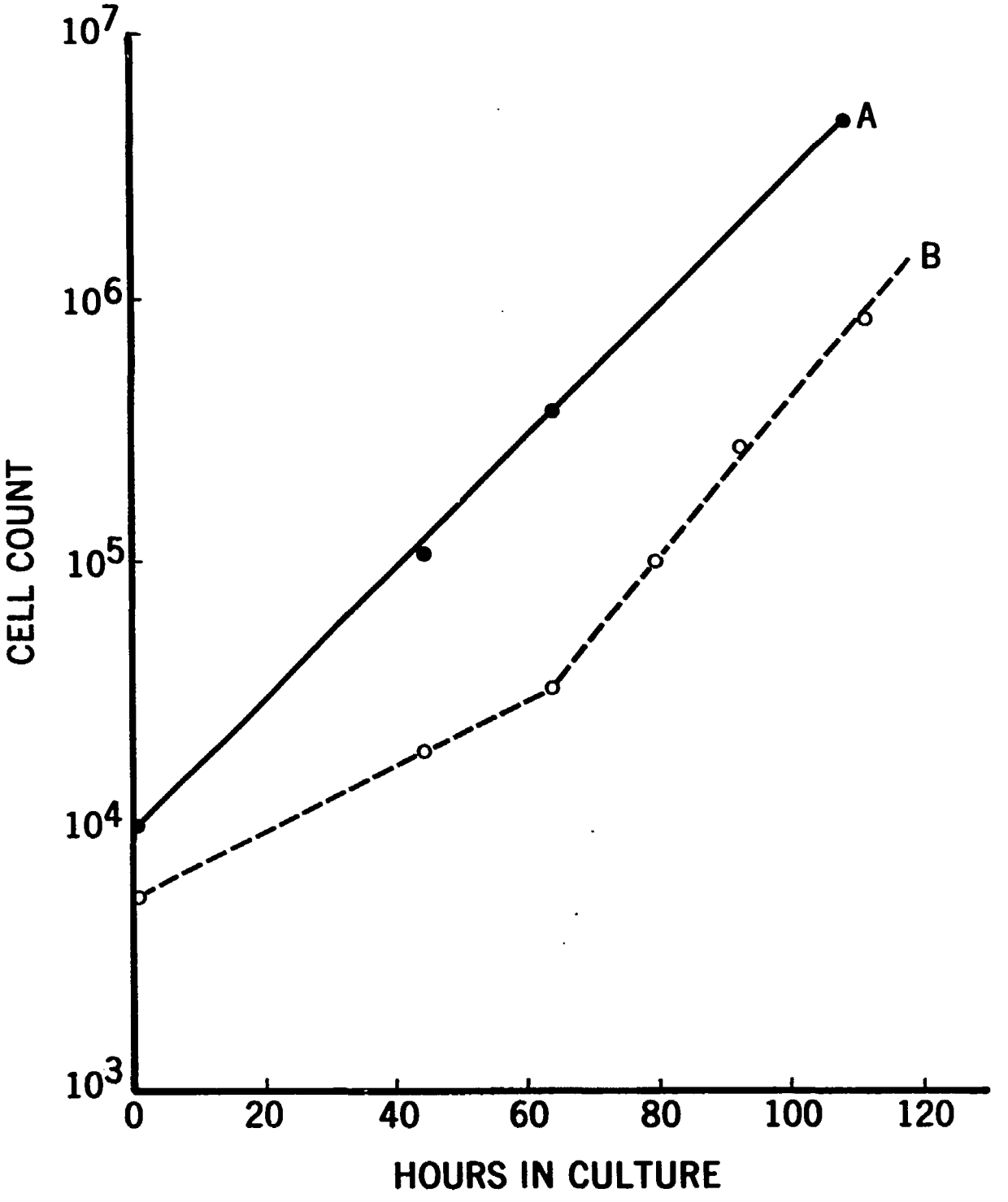


Figure 10 - Effect of treating L5178Y lymphoblasts with phenylalanine ammonia-lyase, removing the enzyme, and resuspending the cells in fresh media. Curve A represents untreated cells; curve B represents cells treated with PAL (3.75 U/15 ml media) for 70 hrs, the cell suspension centrifuged (280 X g/5 min), and the cell pellet resuspended in 15 ml fresh media.

(1.3 U/5 ml FHS), and incubated at 37°C. DNA synthesis was determined by a 2-hour incorporation of $^{3}$H-thymidine into DNA. RNA and protein synthesis were determined by a 30-minute incorporation of $^{3}$H-uridine and $^{3}$H-leucine into RNA and protein, respectively. DNA content was also assayed so that the results of these determinations could be expressed as dpm labeled precursor incorporated per microgram of DNA.

The effect of PAL treatment on cellular DNA synthesis is shown in Figure 11. Treatment with PAL produced relatively little inhibition for approximately 4-6 hours, followed by an appreciable decrease in the rate of DNA synthesis.

The effect of PAL on RNA synthesis is shown in Figure 12. As in the case of DNA synthesis, little inhibition of RNA synthesis occurred during the first 4-6 hours of treatment. However, uridine incorporation into RNA was inhibited 50-70% after treatment with the enzyme for 24 hours.

The effect of PAL on protein synthesis is shown in Figure 13. Both the untreated and PAL-treated cells underwent an increased rate of incorporation of leucine into protein during the first 4 hours in culture. However, treatment with PAL markedly inhibited leucine incorporation into protein after approximately 4 hours of enzyme treatment. Not shown on the graphs is the fact that enzyme treatment for 3 days depressed DNA synthesis to zero. However, some RNA and protein synthesis was observed after similar times of enzyme treatment. This suggests that some RNA and protein synthesis may continue in cells that are severely damaged. A complete understanding of these observations, however, is not possible since uridine or leucine pool sizes and RNA or protein content were not measured in these experiments.

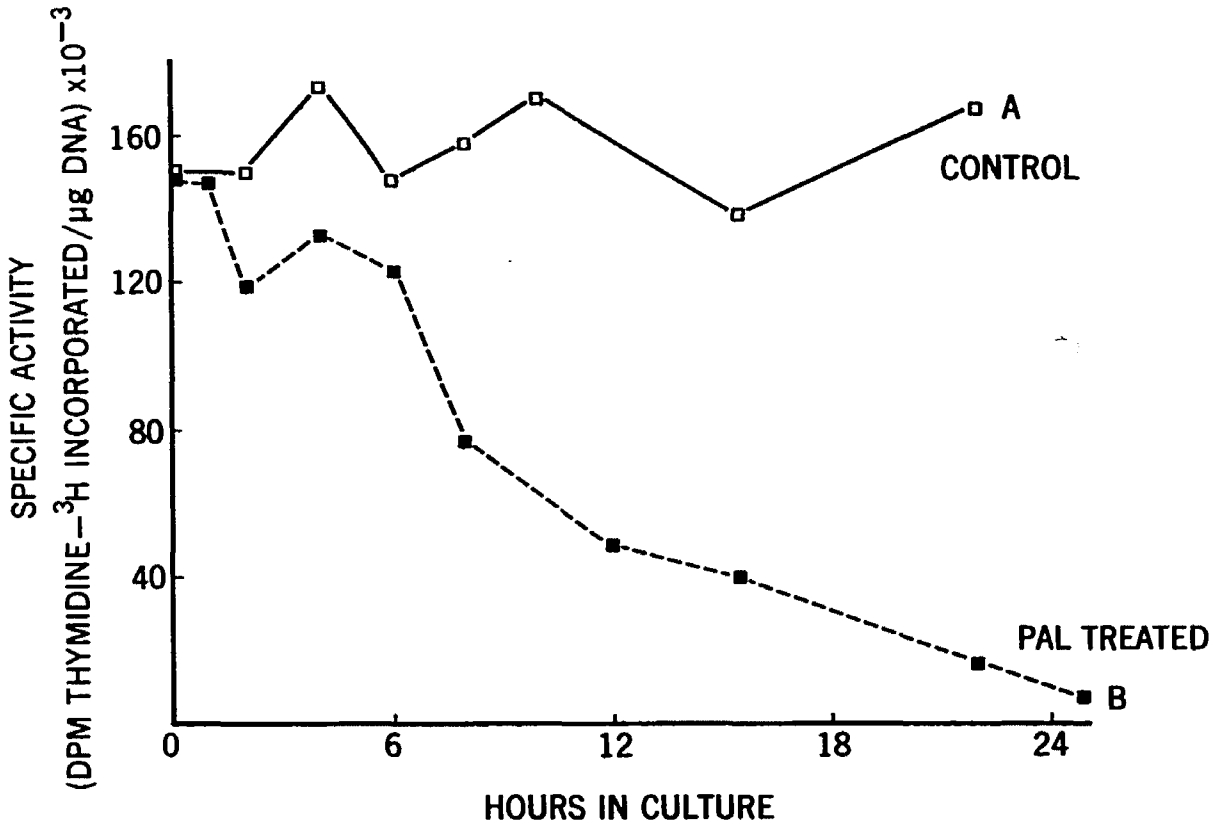


Figure 11 - The effect of phenylalanine ammonia-lyase on DNA synthesis in L5178Y lymphoblasts. Curve A represents untreated cells; and Curve B, cells treated with PAL (1.3 units/5 ml media).

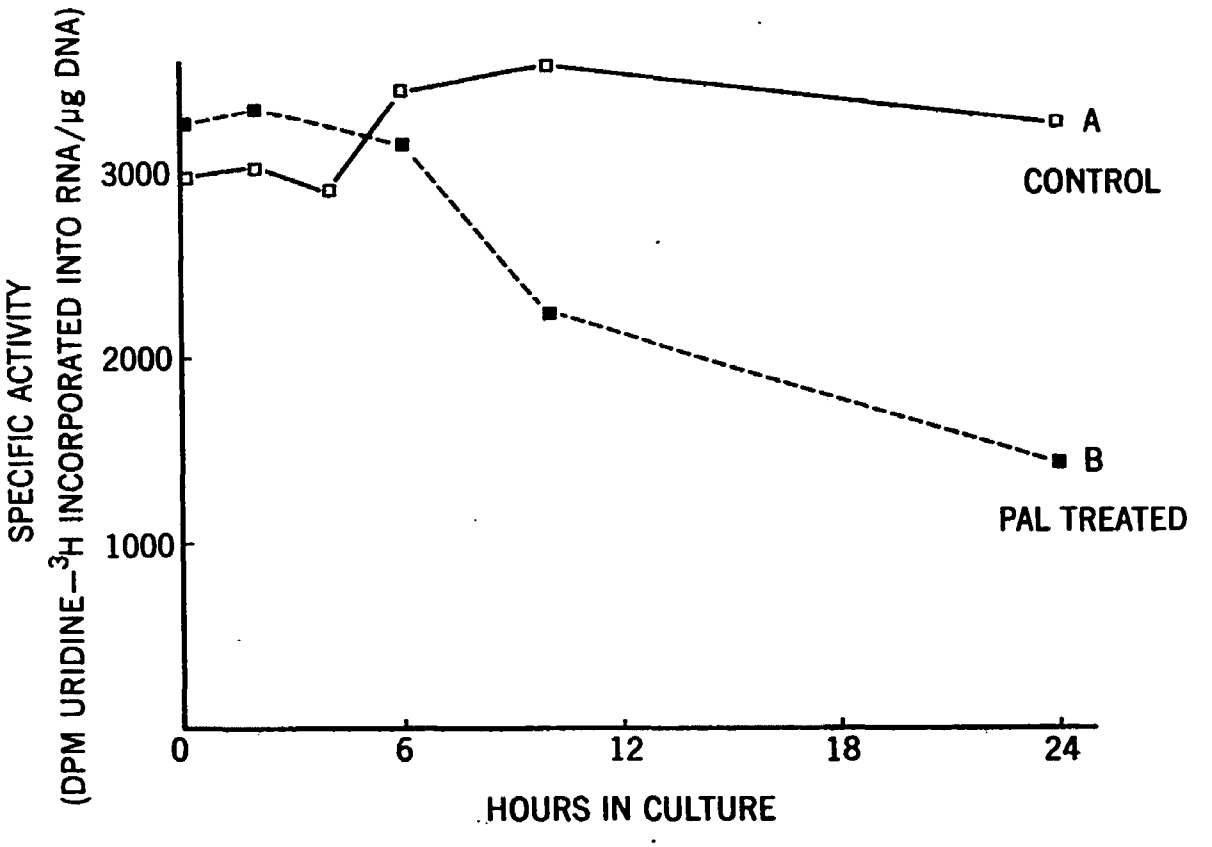


Figure 12 - The effect of phenylalanine ammonia-lyase on RNA synthesis in L5178Y lymphoblasts. Curve A represents untreated cells; and Curve B, cells treated with PAL (1.3 units/5 ml media).

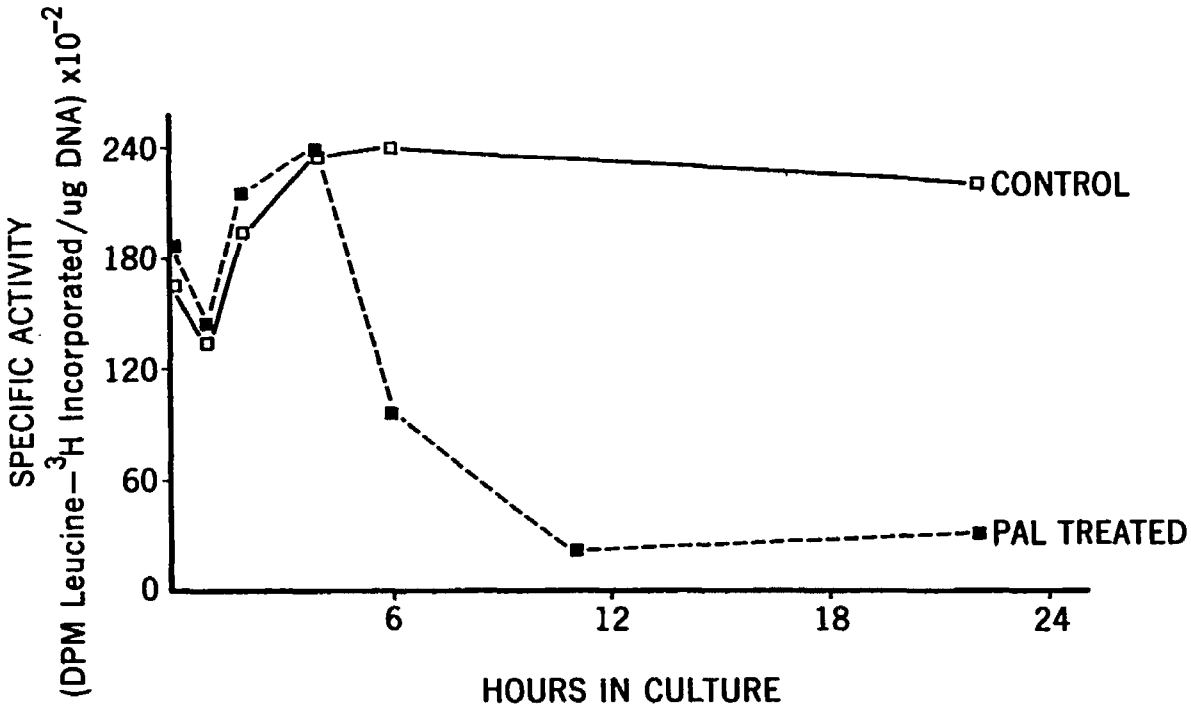


Figure 13 - The effect of phenylalanine ammonia-lyase on protein synthesis in L5178Y lymphoblasts. Curve A represents untreated cells; and Curve B, cells treated with PAL (1.3 units/5 ml media).

## Comparison of the Effects of PAL Treatment with Phenylalanine Deprivation on Viability of the L5178Y Lymphoblasts

The earliest effect of PAL treatment appeared to be decreased protein synthesis, presumably due to the lowering of phenylalanine levels. Therefore, further studies were performed to compare the effects of PAL treatment with phenylalanine deprivation on cell viability.

The effects of PAL treatment (3.35 U/15 ml) on phenylalanine concentration and cell viability of the L5178Y lymphoblasts are shown in Table 6. PAL treatment caused a decrease in phenylalanine concentration in the medium. After 10-12 hours of PAL treatment, approximately 82-84% of the phenylalanine in the culture medium had been deaminated and the viable cell count had dropped. The phenylalanine concentration required for cell viability based on these results is approximately 21.7 pg per cell.

In order to culture L5178Y cells in phenylalanine-deficient medium, dialyzed horse serum had to be prepared. To determine the concentration of phenylalanine remaining after dialysis, $^{14}$C-phenylalanine was added to the horse serum before dialysis. Aliquots of the horse serum were assayed for radioactivity at the times indicated (Figure 14). Dialysis removed more than 99% of the phenylalanine from the horse serum. Spectrofluorometry had shown that this lot of horse serum contained 2.7 mg of phenylalanine per 100 ml of serum. An additional 0.6 µg/ml was contributed by the $^{14}$C-labeled phenylalanine. Thus, removal of 99.32% of the total phenylalanine from the serum left less than 0.28 µg/ml in the horse serum after dialysis.

TABLE 6

EFFECT OF PAL TREATMENT (3.35 U/15 ML MEDIA) ON PHENYLALANINE CONCENTRATION AND VIABLE CELL COUNT OF L5178Y LYMPHOBLASTS

| Hours in Culture | Phenylalanine Concentration (µg) | Viability | Viable Cell Count x $10^6$ | Phenylalanine Conc./Viable Cell (pg) |
|---|---|---|---|---|
| 0 | 825.0 | 97 | 4.4 | --- |
| 2 | --- | 97 | 5.2 | --- |
| 4 | --- | 99 | 6.8 | --- |
| 6 | 148.5 | 99 | 6.2 | 24.0 |
| 8 | 144.4 | 99 | 6.8 | 21.2 |
| 10 | 143.6 | 99 | 7.9 | 18.3 |
| 12 | 132.0 | 99 | 6.5 | 20.4 |
| 25 | 103.1 | 65 | 4.8 | 21.2 |
| 35 | 57.8 | 33 | 2.3 | 25.3 |
| | | | | Average= 21.7 |

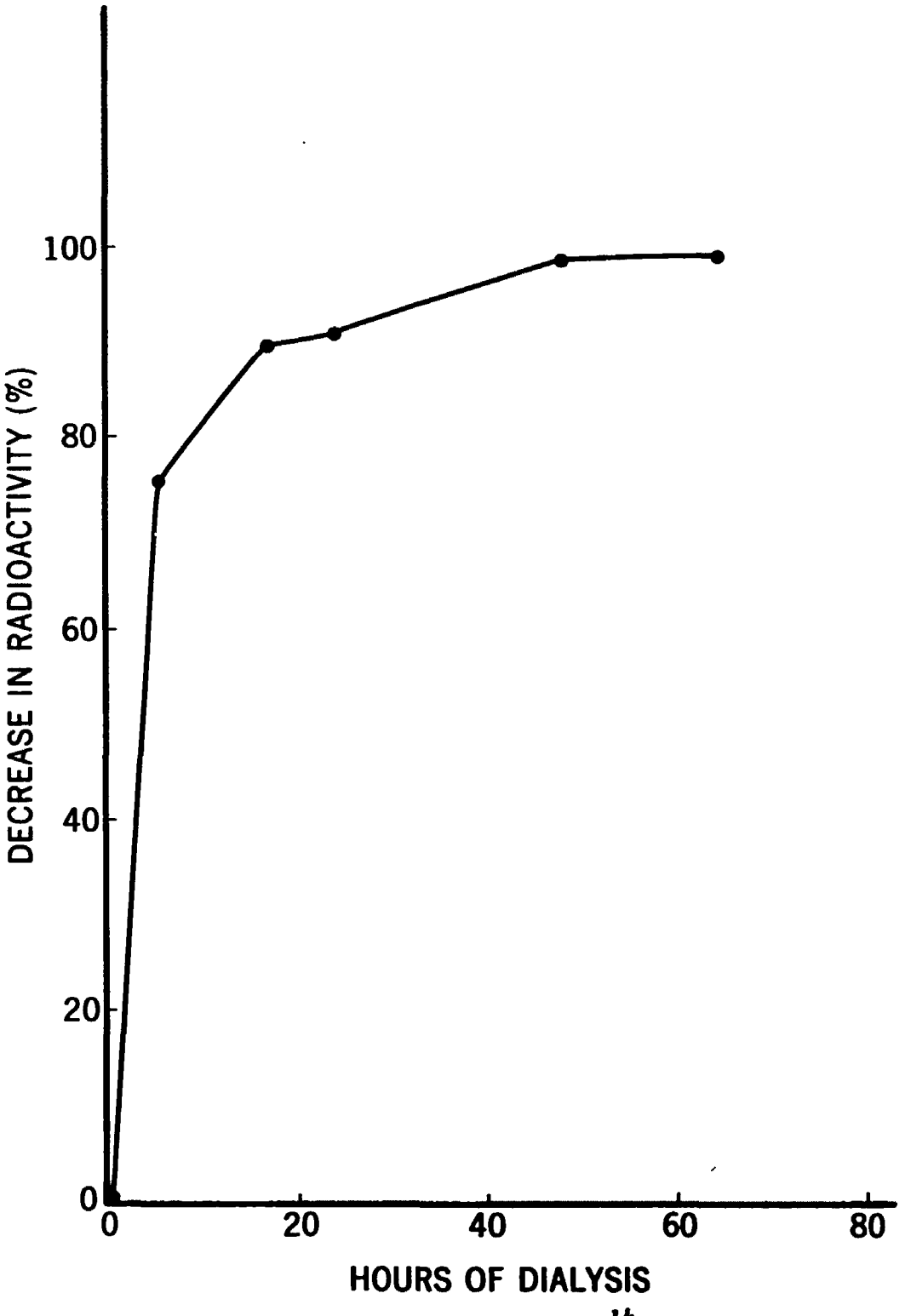


Figure 14 - Rate of removal of L-phenylalanine-$^{14}$C(UL) from horse serum by dialysis against Earle's balanced salt solution. The curve represents the decrease in labeled phenylalanine from 20 ml of horse serum dialyzed against 400 ml of Earle's balanced salt solution, with the balanced salt solution changed at 24-hour intervals.

L5178Y lymphoblasts were cultured in a special Fischer's medium deficient in phenylalanine, with either 10% undialyzed or 10% dialyzed horse serum. As shown in Figure 15, logarithmically growing cells suspended in special Fischer's medium, with phenylalanine added before culture, maintained 98±1% viability and had a doubling time of 10.5 hours. Logarithmically growing cells suspended in special Fischer's medium without phenylalanine, with undialyzed horse serum, maintained the same doubling time and viability as the control for about 32 hours in culture. Subsequently, however, the viability began to decline rapidly. Logarithmically growing cells suspended in special Fischer's medium without phenylalanine, with dialyzed horse serum, decreased in viability after about 18 hours in culture. Logarithmically growing cells treated with 1.1 units and 3.38 units of phenylalanine ammonia-lyase decreased in viability after about 20 hours and 12 hours, respectively. Both phenylalanine deprivation and phenylalanine ammonia-lyase treatment produced similar decreases in cell viability. The ultimate effect of phenylalanine ammonia-lyase treatment was to decrease the viability of the lymphoblasts to less than 2% viable cells. Further experiments (not depicted in the Figures) showed that while treatment of these cells with additional enzyme caused no further decrease in cell viability, no cells grew in the presence of enzyme for at least 1 month.

Further experiments were performed to insure that the effect of PAL on cell growth was due to neither the enzyme buffer (0.05 M Tris-Cl, pH 8.5) nor to the deaminated products of phenylalanine and tyrosine.

The effect of different concentrations of 0.05 M Tris-Cl on cell growth of the L5178Y lymphoblasts is shown in Figure 16. Cells were

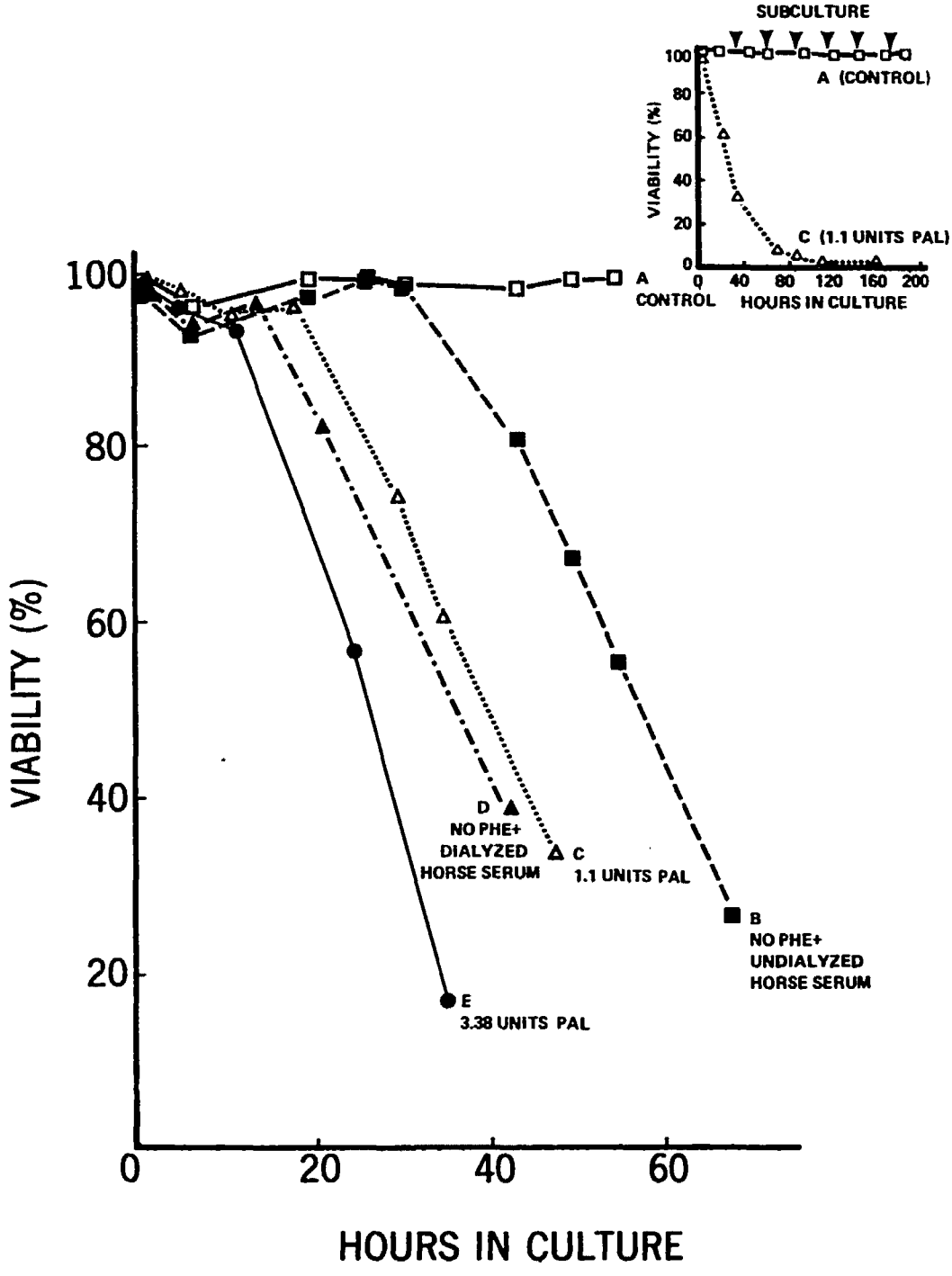


Figure 15 - Comparison of the effects of treatment with phenylalanine ammonia-lyase with phenylalanine deprivation on L5178Y cells. Curve A untreated cells; Curve B, cells cultured in media lacking phenylalanine with 10% undialyzed horse serum; Curve C, cells treated with PAL (1.1 units/15 ml media); Curve D, cells cultured in media lacking phenylalanine with 10% dialyzed horse serum; and Curve E, cells treated with PAL (3.38 units/15 ml media). The inset shows viability following treatment with PAL (1.1 units/15 ml media) for 175 hours.

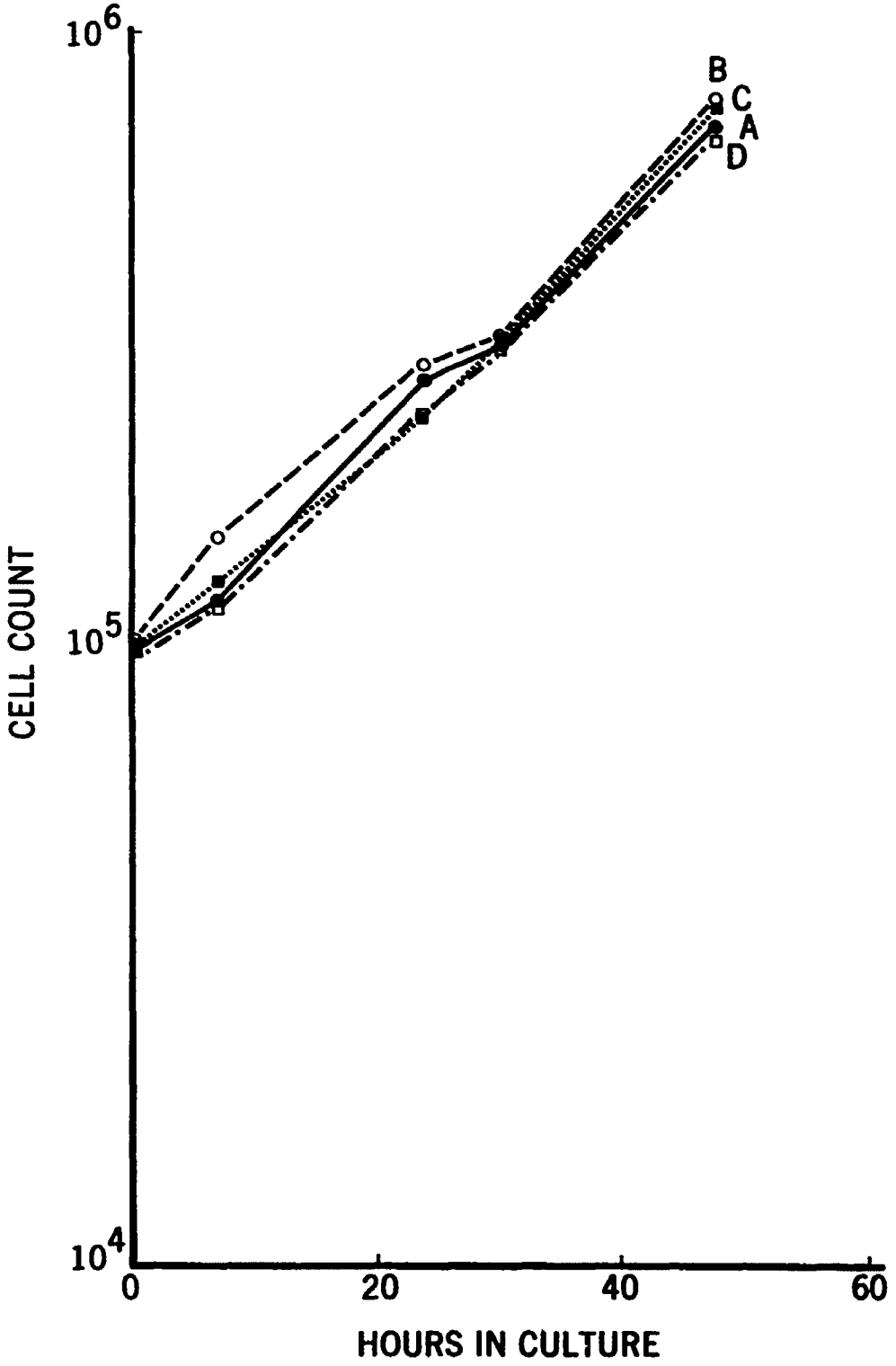


Figure 16 - Effect of different concentrations of Tris-Cl buffer on the growth of L5178Y lymphoblasts. Curve A represents untreated cells; curves B, C, and D represent cells treated with 0.1, 0.5, and 1.0 ml of 0.05M Tris-Cl buffer (pH 8.5) per 15 ml media, respectively.

treated with 0.1, 0.5, and 1.0 ml of Tris-Cl per 15 ml FHS, respectively. A control preparation was untreated. The data indicated that the buffer used to dilute the enzyme had no effect on growth of the lymphoblasts in culture.

The effect of cinnamic acid, coumaric acid, and ammonia (as ammonium chloride) on the cell growth of L5178Y lymphoblasts in culture was determined. The amounts of each compound added corresponded to those obtained by complete deamination of phenylalanine and tyrosine in 15 ml of medium. Cells were treated with 1.61 mg cinnamic acid, 1.63 mg coumaric acid, and 0.557 mg ammonium chloride. A control preparation was untreated. The data indicated that the deaminated products of phenylalanine and tyrosine had no effect on the growth of L5178Y lymphoblasts in culture (Figure 17).

In another experiment (not depicted in the Figures), PAL was inactivated by sodium borohydride and its effect on cell growth of the lymphoblasts was determined. The results showed that the inactivated enzyme had no effect on cell growth of the L5178Y lymphoblasts in culture.

## Electron Microscopy of L5178Y Lymphoblasts After Treatment with PAL

Electron micrographs of both untreated and PAL-treated L5178Y lymphoblasts were prepared by Dr. Robert E. Nordquist. Figure 18 shows a typical L5178Y lymphoblast with Type-C virus particles near the cell surface. After treatment of these cells with PAL, intermediate steps of degradation appeared to involve breakdown of the cellular membrane, formation of lipid bodies, and a generalized lysis of cellular structure. Figure 19 shows a typical cell that had been treated with PAL (1.1 U/15

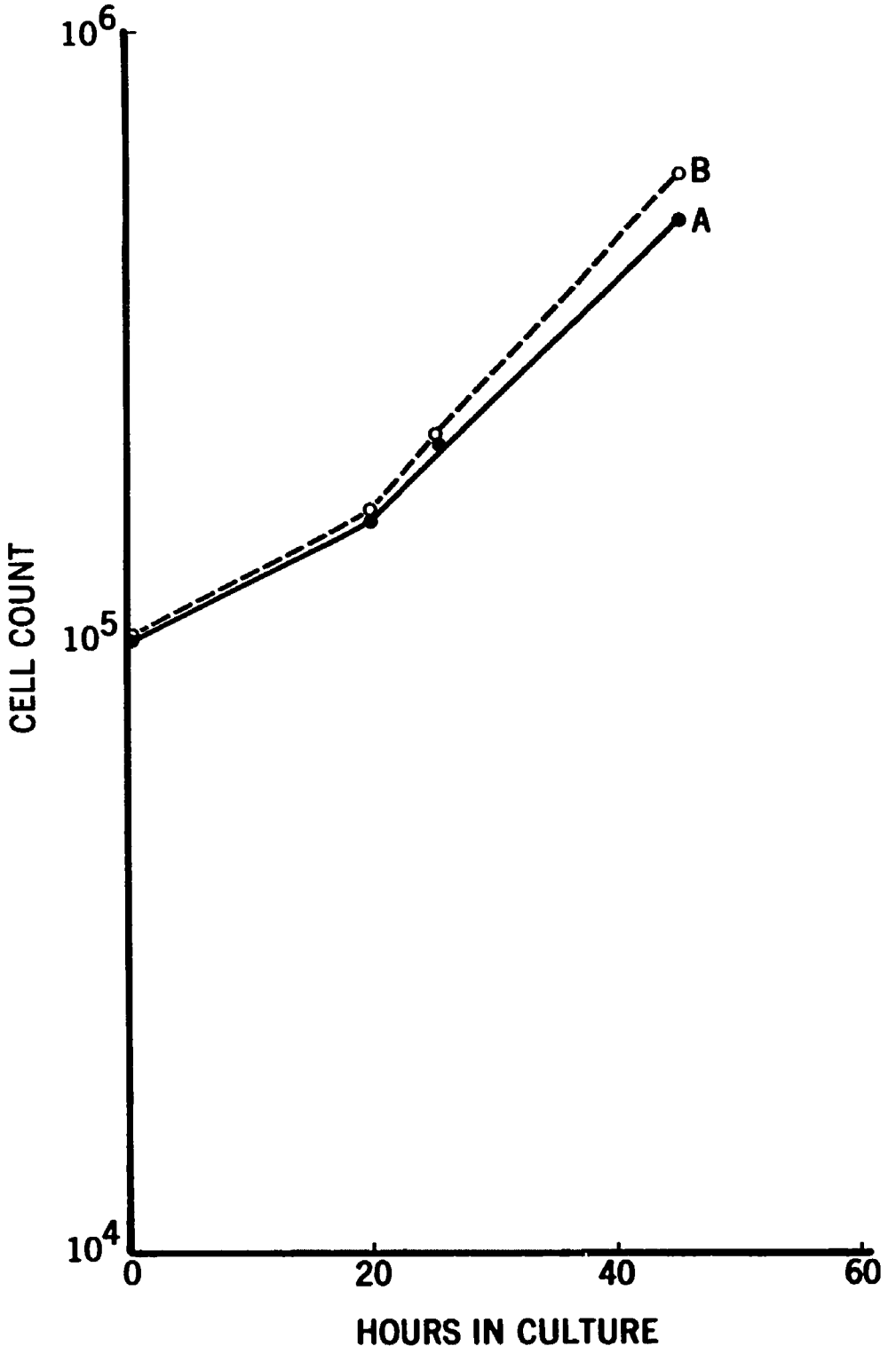


Figure 17 - Effect of deamination products of L-phenylalanine and L-tyrosine on the growth of L5178Y lymphoblasts. Curve A represents untreated cells; curve B represents cells treated with 1.63 mg coumaric acid, 1.61 mg cinnamic acid, and 0.557 mg ammonia (as ammonium chloride) per 15 ml media.



Figure 18 - Electron micrograph of an untreated L5178Y lymphoblast (x 20,000 ).



Figure 19 - Electron micrograph of an L5178Y lymphoblast which had been treated for 350 hours with phenylalanine ammonia-lyase (1.1 U/15 ml media) (x 25,000 ).

ml FHS) for approximately 350 hours. At the time these micrographs were taken, less than 2% of the cells were viable and approximately 80-90% of the cells present exhibited degenerative changes resembling those shown in Figure 19.

# CHAPTER IV

## DISCUSSION

The treatment of leukemic lymphocytes with PAL appears to result in a sequential series of events: first, inhibition of protein synthesis, then inhibition of RNA and DNA synthesis, then inhibition of cell division, and finally decreased cell viability.

In protein synthesis, PAL rapidly inhibits leucine incorporation into acid-insoluble material, with an eventual, gradual tapering off. The enzyme's mechanism of action occurs through depletion of phenylalanine, an essential amino acid for mammalian cells. The deamination rate of phenylalanine can be correlated with decreases in $^{3}H$-leucine incorporation, since the enzyme also deaminates 82% of the exogenous phenylalanine in the medium within the first 6 hours. Deamination of the remaining phenylalanine requires an additional 40 hours.

The amount of phenylalanine (21.7 pg/cell) required to maintain actively metabolizing cells as determined after PAL treatment appears to be lower when phenylalanine is excluded and cell viability is measured. Special Fischer's medium without phenylalanine but with 10% undialyzed or 10% dialyzed horse serum containing 40 µg and 0.28 µg of phenylalanine, respectively, can support population growth to a maximum of 9.9 and 5.1 x $10^{6}$ cells, respectively, before cell viability diminishes. The cell counts are at least five times higher than the calculated values would

be on the basis of 21.7 pg/cell. These results could be explained on the basis of carry-over of phenylalanine from the previous medium, containing 55 μg/ml of phenylalanine, or a slower depletion of phenylalanine stores in the cell since dietary restriction may not deplete phenylalanine as rapidly as PAL treatment.

As mentioned earlier, the L5178Y cell line is known to require exogenous asparagine (85). Treatment of these cells with L-asparaginase inhibits cell growth similar to treatment of the cells with PAL. Bosmann and Kessel (127) have shown that treatment of these cells with L-asparaginase rapidly inhibits glycoprotein and protein synthesis. However, they also reported that high levels of asparaginase do not completely inhibit protein synthesis. We, too, found this after treatment of the cells with PAL for 71 hours. Those proteins whose synthesis was not inhibited by enzyme treatment may represent membrane proteins which are constantly being replenished. Eagle et al. (156,157) have shown that in cultured HeLa cells a specific intra- and extracellular concentration of each amino acid is necessary to sustain protein synthesis and cellular growth. However, labeled precursors can be incorporated into protein even in the absence of net protein synthesis. Winkler (158) has also reported that, in protein synthesis in normal leukocytes, extracellular amino acids are used preferentially over internal pool amino acids. When the extracellular amino acid supply is inadequate, continuous protein synthesis is supported by the internal pool, at least for a short period of time.

In other cell lines, dietary deprivation of required amino acids also inhibits protein synthesis. In the asparaginase sensitive Gardner

lymphoma (6C3HED cells), for example, deprivation of asparagine inhibits protein synthesis (159). Likewise, valine deprivation of HeLa cells causes inhibition of protein synthesis and disaggregation of polysomes (160). Synthesis of the 50S ribosomal subunit, particularly the ribosomal proteins, is also inhibited (161). Polysome breakdown has also been shown to occur in stringent strains of Escherichia coli deprived of phenylalanine (162).

Following inhibition of protein synthesis, PAL treatment appears to result in an inhibition of DNA synthesis. This enzyme appears to act primarily on dividing cells, since normal lymphocytes in the resting state are unaffected by PAL, but DNA synthesis is inhibited in cells stimulated to divide with PHA. L-asparaginase has also been shown to have a similar effect on dividing cells as evidenced by its ability to inhibit mitogen-induced blastogenesis, represented by a decreased incorporation of $^{3}$H-thymidine into DNA (163,164).

Various dividing tissues appear to be affected differently by asparaginase treatment. Becker et al. (165) reported that this enzyme delays DNA synthesis in regenerating liver after partial hepatectomy. On the other hand, it does not affect intestinal and lymphoid cells. Nor does it affect renal tubular cells stimulated to divide by unilateral nephrectomy or folic acid injection, nor salivary acinar cells stimulated by isoproterenol. As mentioned in the Introduction, the enzyme seems to cause little bone marrow impairment (27,28,34,35).

In L5178Y murine leukemic lymphoblasts, PAL appears to act by inhibiting first protein synthesis and then RNA and DNA synthesis. The dependence of nucleic acid synthesis on protein synthesis observed in

these cells has also been shown in sensitive cells treated with asparaginase. Treatment of Gardner lymphosarcoma (6C3HED-Asn$^-$) cells with asparaginase rapidly inhibits protein synthesis, then DNA synthesis, then RNA synthesis (166). Ellem et al. (167) showed that these effects are due to deceleration of DNA synthesis in each cell by prolongation of the S phase. The inhibition of RNA synthesis after inhibition of DNA synthesis also occurs in a definite sequence. Ribosomal RNA is inhibited first, followed by inhibition of DNA-like RNA, and finally inhibition of transfer RNA. Both the transcription and processing of ribosomal RNA are inhibited. Saunders (168) has also shown that asparaginase treatment of patients with acute lymphoblastic leukemia blocks entrance of the cells into the S phase of the cell cycle. The cells in the S phase which are synthesizing DNA go on to complete cell division. The population of cells in mitosis does not decrease until cells are unable to synthesize DNA.

A similar dependence of nucleic acid synthesis on protein synthesis has been shown when Gardner lymphoma (6C3HED) and Jensen sarcoma cells are deprived of asparagine (166,169), or when an amino acid-deficient medium plus dialyzed serum is used (170). In Ehrlich ascites tumor cells, an amino acid deficient diet also inhibits ribosomal RNA synthesis. However, neither the maturation, processing, and transport of nuclear precursors into cytoplasmic ribosomal RNA nor the synthesis of rapidly labeled RNA is affected (171). In cultured human liver cells (Chang's), deprivation of either glutamine or leucine depresses the formation of both ribosomal RNA and transfer RNA; the phenomenon is independent of a decreased uptake of precursors into the acid-soluble nucleotide pool (172,173).

Inhibition of protein synthesis in mammalian cells by puromycin also inhibits subsequent DNA synthesis (174-176). After DNA synthesis begins, the cells are much less sensitive to puromycin, suggesting the requirement for synthesis of a particular protein(s) for initiation of replication.

PAL treatment decreases cell viability, and although we did not determine cell size, the enzyme also appears microscopically to cause the cell to become smaller. Cell viability correlates with phenylalanine concentration in the medium. PAL treatment and phenylalanine deprivation result in similar decreases in cell viability.

Electron micrographs of cells treated with PAL show many degenerative changes involving breakdown of cellular membrane, formation of lipid bodies, and a generalized lysis of cellular structure. When these cells are suspended in fresh medium, however, even after 350 hours of treatment with PAL, the cells regrow. This regrowth could be due to the fact that even though almost all of the phenylalanine in the medium is converted to cinnamic acid, the assay is significant only to within 3-4%. Thus, a small but undetectable amount of phenylalanine (< 0.2 μM) might be present in the medium and be sufficient to maintain a small population of cells. This possibility is further supported by the fact that the equilibrium constant for the deamination reaction predicts that approximately 0.02% of the original phenylalanine (< 0.08 μM) remains in the medium.

Dietary deprivation of amino acids in other cell lines has also been shown to decrease cell viability. Eagle (177) found that mouse L and HeLa cells deprived of single amino acids develop microscopic changes

indicative of cell injury within 2-3 days. The cells eventually die, with the changes depending on the particular amino acid deficiency. Cells exposed to a medium deficient in a single amino acid, which have largely degenerated, can be revived on restoration of the missing component. Cohen et al. (178) found that HeLa cells deprived of valine immediately stop growing, undergo a series of degenerative changes in cell structure, and then decrease in population. Readdition of valine promotes repair of surviving cells, and they resume normal growth.

Littlefield (179) found that prolonged lack of feeding of L cells results in a progressive loss of cell viability, with the remaining cells becoming partially synchronized. Phenylalanine deprivation of L5178Y cells resulted in a progressive loss of cell viability but did not appear to produce a synchronized population. Cells pretreated with PAL and transferred to fresh medium showed regrowth even after 300 hours of enzyme treatment. However cell counts done at hourly intervals, showed that the cells increased progressively in number over a 12 hour period, indicating an asynchronous population.

The results of previous studies showing that amino acid deprivation decreases cell viability contrast with the data of Tobey and Lay (180-182). They used isoleucine deprivation to synchronize Chinese hamster (Line CHO), mouse L, and Syrian hamster BHK21 cells in the $G_1$ phase of the cell cycle. The $G_1$ state is characterized by reduced cell division, stabilization of cell number, and DNA synthesis dropping to zero. These Chinese hamster cells can be maintained in the stationary phase of the growth cycle, exhibiting a 95% viability for at least 80 hours, with essentially all the cells in $G_1$. In the presence of

sufficient glutamine, approximately 2 x $10^{-6}$ M isoleucine is required for all cells to initiate DNA synthesis in a population initially containing 1.5 x $10^5$ cells/ml. Under similar conditions, about 4 x $10^{-6}$ M isoleucine is required for all $G_1$ arrested cells to progress through cell division in synchrony. The results of Tobey and Lay also indicate that a concentration of 4 μm isoleucine is required to maintain cell division of 1.5 x $10^5$ cells; only half this amount (2 μm) is required to initiate DNA synthesis in the same number of cells. Their lower value is approximately 100 times higher than the phenylalanine concentration (0.02 μm) determined by PAL treatment required to maintain viability of a similar volume of L5178Y cells. However, as shown by McCarty (183), a selective quantitive utilization and liberation of amino acids occurs that is characteristic of different established cell lines in culture.

In other studies with Chinese hamster cells (pseudodiploid line C14FAF28), Freed and Schatz (184) found that deprivation of any single required amino acid inhibits the division cycle during the S phase. After restoration of the amino acid, the division cycle is completed. Furthermore, the amino acid deprivation produces chromosomal abnormalities including multiple breaks and exchanges. The number of aberrations increases as a function of the time the cells are held in the inhibited state. Since chromosomal analysis were not performed in cells treated with PAL, it could not be determined if treatment with PAL resulted in a similar effect.

In other cell lines, Ward and Plageman (185) found that the transition of suspension cultures of Novikoff rat hepatoma cells from the exponential growth phase to the stationary phase is accompanied by a 90%

decrease in the rate of synthesis of DNA, RNA, and protein and by a minimum cell size. Cells stainable with trypan blue, indicating decreased cell viability, appear early in the stationary phase and thereafter increase progressively although the total number of cells remains constant.

Comparable results were obtained by Watanabe and Okada (186) working with L5178Y cells in the stationary phase. They found that the transition from the exponential growth phase to the stationary phase consists of an early phase characterized by a decline in the mitotic index followed by stabilization of cell number. Several hours after flattening of the growth curve, the number of nonviable cells begins to increase as measured by the eosin dye exclusion test. Furthermore, the dying or dead eosin-stained cells persist in the medium for several days and, after this, are only slowly eliminated. They have postulated that the stationary phase is probably caused by a depletion of essential nutrients, accumulation of toxic wastes, or both.

The results of the present study indicate that PAL treatment of leukemic cells deprives the rapidly growing cells of phenylalanine. This then causes a transition from the exponential to the stationary phase of growth. The early part of the stationary growth phase could be characterized by decreases in protein, RNA, and DNA synthesis, resulting in an inhibition of cell division and a stabilization of cell number. Inhibition of macromolecular synthesis eventually produces chromosomal abnormalities and cell degradation; the severity of the effects depend on the duration of phenylalanine deprivation. The ultimate effect of phenylalanine restriction is decreased cell viability.

In conclusion, the results of this study indicate that PAL is an effective chemotherapeutic agent <u>in vitro</u> because it inhibits leukemic

cell growth. Further studies investigating the effects of PAL on neoplastic cell growth in tumor bearing animals are required to establish the enzyme as a useful agent for the treatment of cancer in man.

# CHAPTER V

## SUMMARY

Human peripheral blood lymphocytes obtained from both normal donors and patients with acute lymphocytic leukemia (ALL) and L5178Y murine leukemic lymphoblasts were maintained in tissue culture. The cells were treated with either phenylalanine ammonia-lyase (PAL) or L-asparaginase. The effects of each treatment were compared to evaluate the effectiveness of PAL in vitro as a possible cancer chemotherapeutic agent.

The results demonstrated that PAL and asparaginase inhibited cell division in human leukemic cells and L5178Y murine leukemic lymphoblasts. However, PAL was shown to have little or no effect on resting (non-dividing) normal human lymphocytes.

The mechanism by which PAL inhibits leukemic cell growth was further investigated using L5178Y murine leukemic lymphoblasts. PAL treatment initiated a definite sequence of events: The first effect of enzyme treatment appeared to be an inhibition of protein synthesis. This was followed by inhibition of RNA and DNA synthesis, and later an inhibition of cell division and a stabilization of cell number. The ultimate effect of enzyme treatment was a decrease in cell viability.

Further studies showed that cell viability of the L5178Y murine leukemic lymphoblasts in culture could be correlated with phenylalanine

concentration in the medium. Similar decreases in cell viability were observed by culturing the cells in a phenylalanine-deficient medium. The leukemic lymphoblasts were not affected by cinnamic acid, coumaric acid, and ammonia, the products of phenylalanine and tyrosine deamination, nor inactivated phenylalanine ammonia-lyase. These results suggest that PAL inhibits cell division by depriving these rapidly growing cells of the amino acid phenylalanine.

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