1963

The Isolation of Intermediary Metabolites of L-lysine-U-C\textsubscript{p1=s-p4=s} from Tissues of Tumor-Bearing Rats

Robert N. Morris
Loyola University Chicago

Recommended Citation
Morris, Robert N., "The Isolation of Intermediary Metabolites of L-lysine-U-C\textsubscript{p1=s-p4=s} from Tissues of Tumor-Bearing Rats" (1963). Master's Theses. Paper 1807.
http://ecommons.luc.edu/luc_theses/1807

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1963 Robert N. Morris
THE ISOLATION OF INTERMEDIATE METABOLITES
OF L-LYSINE-U-C14 FROM TISSUES
OF TUMOR-BEARING RATS

by

Robert N. Morris

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

January
1963
LIFE

Robert N. Morris was born in Chicago, Illinois, on January 11, 1937. He attended St. George High School, Evanston, Illinois. In June, 1959, he received the degree of Bachelor of Arts from St. Mary's College, Winona, Minnesota.

The period from November, 1959, to May, 1960, was spent with the United States Army. While on active duty the author underwent training as a combat medic at the USAMTC training school, Fort Sam Houston, Texas.

Upon his release from active duty in 1960 the author entered the employ of Baxter Laboratories, Morton Grove, Illinois, as a research pharmacologist. In September, 1960, he entered upon graduate studies at Stritch School of Medicine as a part-time student.

In December, 1960, he was married to the former Miss Martha E. Cox of River Forest, Illinois. They have one child, Mary Kathleen.
ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. Joseph R. Davis for his assistance, inspiration, and patience throughout the course of the work presented in this thesis; to Dr. Alexander G. Karczmar in whose department it has been his privilege to study; to Drs. Fred T. Galysh and Robert M. Gesler of Baxter Laboratories whose constant encouragement and indulgence have contributed greatly to the successful culmination of this work; and to his wife, Martha, for her assistance in the preparation of this manuscript and for her forbearance during the course of the thesis work.
Interest in the catabolism of lysine in neoplastic tissues evolved from studies of the uptake of labeled amino acids by nuclear proteins of transplantable rat tumors (16,17).

In the tumors, the specific activity of the acid-soluble nuclear histone fraction was found to be greater than that of any other cytoplasmic or nuclear protein isolated following the in vivo injection of twelve different C\textsuperscript{14} labeled amino acids. Chromatographic separation of the labeled histones following the injection of L-lysine-U-C\textsuperscript{14} resulted in the isolation of a radioactive protein peak peculiar only to tumor cells (24,25). These findings suggested that part of the increased protein biosynthesis in the nuclei of neoplastic cells may be attributed to the production of proteins not found in the nuclei of other tissues.

The present series of investigations was undertaken in an effort to explore the possibility of a malfunction in the degradation of lysine in tumors. Such a lesion could conceivably result in an accumulation of lysine which in turn would be shunted into protein synthesis to an abnormally high degree.
TABLE OF CONTENTS

Chapter I ••••••••••••••••.•••••••••.••••••••••.••••••••••••••••• 1

INTRODUCTION ................................................................. 1

A. The Catabolism of Lysine in Non-Tumor Tissues .................... 1
   1. Early Studies of Lysine Degradation .......................... 1
   2. $\alpha$-amino adipic Acid ........................................ 5
   3. Pipecolic Acid .................................................. 5
   4. $\alpha$-keto-$\epsilon$-aminocaproic Acid .......................... 8
   5. The Conversion of Pipecolic Acid to $\alpha$-amino adipic Acid.. 11
   6. The Conversion of $\alpha$-amino adipate to Glutarate ........... 11
   7. The Further Catabolism of Glutaric Acid ........................ 12
   8. Glutamic Acid and $\Delta$-aminovaleric Acid .................... 14

B. Amino Acid Catabolism in Pre-Cancerous Tissues .................. 16

C. The Morris Hepatoma 5123 ............................................. 18
   1. The Theory of Enzyme Deletion .................................. 18
   2. Induction of the Primary Tumor .................................. 20
   3. Deoxycytidylate Deaminase and Thymine Reductase ............. 21
   4. Amino Acid Catabolism in Hepatoma 5123 ....................... 23
   5. Metabolic Adaptations in Hepatoma 5123 ....................... 25
   6. Carbohydrate Metabolism in Hepatoma 5123 .................... 28
   7. Catalase Activity of Hepatoma 5123 .............................. 29
   8. Purine Metabolism in Hepatoma 5123 ............................. 30
Chapter II ................................................................. 32

MATERIALS AND METHODS .................................................. 32

A. Animals and Tissues ......................................................... 32

B. Administration of the Tracer .............................................. 33

C. Preparation of the Tissues .................................................. 33

D. Separation of the Radioactive Catabolites of L-lysine-\textsuperscript{14}C

1. Preparation of Dowex-1 Anionic Exchange Resin ................. 33

2. Preparation of Dowex-50 Cationic Exchange Resin ............... 34

3. Addition of Protein-Free Supernate to Ion Exchange
   Columns ........................................................................... 35

4. Elution of Radioactive Peaks from the Ion Exchange
   Columns ........................................................................... 35

5. Desiccation ........................................................................ 36

6. Assay of Radioactivity ....................................................... 36

E. Identification of Radioactive Peaks ................................. 37

1. Dowex-1 ................................................................. 37

2. Dowex-50 ................................................................. 37

Chapter III ............................................................................ 39

RESULTS .............................................................................. 39

Chapter IV ............................................................................ 43

DISCUSSION .......................................................................... 43

Chapter V ............................................................................. 47

SUMMARY ............................................................................ 47

Chapter VI ............................................................................ 49

CHARTS ................................................................................. 49

vi
Chapter VI continued

Chart I  Proposed Catabolic Pathway for Lysine....................... 50

Chart II  Chromatographic Profile of PCA Soluble Radioactivity of Liver of Rat Bearing the Walker 256 Carcinosarcoma. 51

Chart III  Percentage Metabolized Radioactivity of L-Lysine-U-C\textsuperscript{14} Three Hours after Intravenous Injection of Tracer..... 52

Chart IV  Percentage Total Radioactivity in Tissue from L-Lysine-U-C\textsuperscript{14} Three Hours after Intravenous Injection of Tracer.......................... 53

Chart V  Catabolic Pattern: L-Lysine-U-C\textsuperscript{14} Liver................... 54

Chart VI  Catabolic Pattern: L-Lysine-U-C\textsuperscript{14} Walker 256 Carcinosarcoma................................. 55

Chart VII  Percentage Metabolized Radioactivity of L-Lysine-U-C\textsuperscript{14} Three Hours after Intravenous Injection of Tracer..... 56

Chart III  Specific Activity of Chromatographic Peaks Three Hours after Intravenous Injection of L-Lysine-U-C\textsuperscript{14}........ 57

Chapter VII.......................................................... 58

TABLES................................................................. 58

Table I  Distribution of Radioactivity in Tissues Three Hours after the Injection of L-Lysine-U-C\textsuperscript{14}........ 59

Table II  Distribution of Radioactivity in Tissues Three Hours after the Intravenous Injection of L-Lysine-U-C\textsuperscript{14}........................................ 60

Table III  Distribution of Radioactivity in Tissues Three Hours after the Intravenous Injection of L-Lysine-U-C\textsuperscript{14}................................. 61

Chapter VIII.......................................................... 62

BIBLIOGRAPHY....................................................... 62
CHAPTER I

INTRODUCTION

A) THE CATABOLISM OF LYSINE IN NON-TUMOR TISSUES

Osborne and Mendel defined the principle of indispensable amino acids early in the present century (48). Lysine was found by these workers to be a dietary requirement essential for normal growth. Gliadin, a protein prepared from wheat and known to be deficient in lysine was unable to maintain normal growth rates in rats. Addition of crystalline lysine to the gliadin rendered the diet capable of supporting normal development. Utilizing a similar method of study, Berg demonstrated that it was the 1 (+) stereoisomer that was required for growth (2). Supplementation of a zein diet with the d (-) form elicited no growth response.

1. Early Studies of Lysine Degradation:

Early investigations of lysine catabolism established the fact that its degradative sequence involved mechanisms not common to most of the other amino acids. Foster and his coworkers studied the fate of deuterium in tissues of rats maintained on an excess of the isotope (27). All except one of the amino acids of the proteins isolated by hydrolysis of the total body carcasses were found to contain deuterium firmly bound to the carbon chain. The one exception was lysine.

Addition of N^{15} ammonium citrate to the normal rat diet caused the isotope to appear in every amino acid studied except lysine (28).
Further studies by Schoenheimer and his group confirmed the apparent stability of the nitrogen of lysine (69). Tyrosine, containing N\textsuperscript{15} in the amino group, when fed to rats, led to the incorporation of the label by histidine, arginine and several other amino acids, but not by lysine. Similarly, the deuterium and N\textsuperscript{15} labeled atoms of leucine supplied in the diet of rats were found to be taken up by every amino acid isolated from the body tissue proteins with the exception of lysine (70).

Although the amino groups of lysine have been proven to be incapable of incorporating N\textsuperscript{15} from other amino acids, these atoms are not chemically inert. Lysine containing deuterium bound to the carbon chain, and N\textsuperscript{15} in the \(\alpha\)-amino group, was fed to growing rats (78). Most of the labeled lysine was found to be incorporated into tissue protein. However, some of the N\textsuperscript{15} appeared in amino acids other than lysine. Significantly, the deuterium to N\textsuperscript{15} ratio of the lysine of the body tissues was the same as the lysine in the diet. The appearance of N\textsuperscript{15} in other amino acids confirmed the existence of a mechanism for the deamination of lysine. The fact that the deuterium to N\textsuperscript{15} ratio was unaltered in the lysine of the tissue proteins indicated that following the removal of the \(\alpha\)-amino nitrogen, lysine is not capable of accepting nitrogen from other sources. Thus, following its deamination, lysine is apparently not reaminated (78).

The exact mechanism of this deamination is obscure. A purified L-amino acid oxidase isolated from rat liver and kidney was found to catalyze the oxidative deamination of thirteen naturally occurring L-amino acids, but had no effect on dibasic amino acids including lysine (3,4,29). Bender and Krebs (1)
extracted D-amino acid oxidase from sheep kidney and from the mold Neurospora Crassa; and L-amino acid oxidase from cobra venom and from N. Crassa. Upon incubation of these crude preparations with DL-lysine under oxygen, a negligible amount of oxygen uptake was noted, thus confirming the findings of previous investigators.

Transamination, a reaction common to many amino acids, has been investigated by several workers with regard to lysine. Cohen demonstrated that in vitro preparations of pigeon breast muscle were incapable of catalyzing the conversion of \( \alpha \)-ketoglutaric acid to glutamic acid in the presence of lysine (22). Later work by these investigators involving aqueous extracts of livers, kidneys, and hearts of slaughterhouse animals proved the existence of enzymes specific for the interaction of \( \alpha \)-ketoglutaric acid and twenty-five amino acids (19). Lysine, however, was found to be inert in this system. Each transamination reaction appeared to be due to a different transaminase, and all were found to require pyridoxal phosphate for optimal activity. That the reaction in each case was actually due to transamination and not to reductive amination was evidenced by the fact that all of the \( \alpha \)-ketoglutaric acid added to the original reaction mixture could be accounted for in the form of residual \( \alpha \)-ketoglutarate or glutamic acid after the reaction was concluded. Glutamic acid was measured by the evolution of \( \text{CO}_2 \) upon incubation of the reaction mixture with a strain of Clostridium welchii.

Acetone powder preparations of brain, liver, Escherichia coli and A. fumigatus were used by Roberts (58) to study transamination. Similarly, lysine was found not to react with \( \alpha \)-ketoglutaric acid in this system. Citing the
fact that \( \omega \)-amino monocarboxylic acids containing three to five carbon atoms transaminated readily with \( \alpha \)-ketoglutaric acid while an abrupt decrease in activity occurred when the carbon chain was lengthened to six, this author postulated that the loss of ability to transaminate the six carbon acid may be associated with a decreased probability of the molecule existing in a form in which the amino and carboxyl groups lie in close proximity.

Clark and Rittenberg investigated the \( \alpha \)-hydrogen atom of lysine as a possible site for the initiation of lysine catabolism (20). DL-lysine, radioactively labeled with \( N^{15} \) and deuterium in the alpha position, was fed to rats. The \( N^{15} \) to deuterium ratio of the tissue proteins isolated after sacrifice of the rats was found to be unaltered. This evidence indicated that dehydrogenation, if it does occur, proceeds at a very slow rate, and is rapidly followed by hydrolysis of the lysine to the corresponding keto acid.

In the course of a series of studies on the availability of acetyl derivatives of lysine for growth, Neuberger et al. found that \( \varepsilon \)-acetyl lysine was able to entirely supplant the dietary requirement for lysine in rats (46). However, \( \alpha \)-acetyl lysine was unable to support normal growth. These findings were somewhat substantiated by other workers (21). Rats fed \( \alpha \)-\( N^{15} \) acetyl-lysine mixed with their diet incorporated approximately one percent of the isotope into excreted urea and a negligible amount into tissue proteins. The balance of the acetylated lysine was excreted unchanged.

In vitro studies by these workers proved the ability of a crude amino acid oxidase extract of rat kidney and liver to attack \( \varepsilon \)-acetyl-1-lysine (47). On the basis of this finding, a catabolic scheme involving acetylation in the
\( \varepsilon \)-amino position prior to \( \alpha \)-deamination was proposed. It would thus appear that the free terminal basic group of lysine inhibits amino acid oxidase.

2. \( \alpha \)-Amino Adipic Acid:

The first catabolite of L-lysine to actually be isolated and identified was \( \alpha \)-amino adipic acid \((5,6,7)\). Borsook and his coworkers incubated L-lysine-\( \varepsilon ^{14} \) with guinea pig liver homogenates. The non-protein filtrate obtained after boiling the reaction mixture produced two radioactive, ninhydrin positive spots on a paper chromatogram. Co-chromatography with authentic samples indicated that these spots were lysine and \( \alpha \)-amino adipic acid. Subsequent isolation of \( \alpha \)-amino adipate from the reaction mixture as the barium salt, and co-chromatography with authentic sample provided further evidence of its identity. These authors interpreted these data as proof that the initial step in lysine degradation is deamination in the \( \varepsilon \)-position.

3. Pипecolic Acid:

Attention was directed to pипecolic acid by the discovery of its occurrence in extracts of green clover, Trifolium Repens, in amounts comparable to other amino acids. Morrison \((45)\) separated this compound from the alcoholic extract by partition chromatography from powdered cellulose columns with n-butanol plus acetic acid as solvents. Recrystallization from 95% ethanol was effected by cooling and the addition of ether. Mixtures of the crystalline compound and authentic pипecolic acid were inseparable on paper chromatograms.

Other workers extracted pипecolic acid from the aqueous extract of homogenized green beans, Phaseolus vulgaris \((33,80)\). The extract was passed over a Zeo Rex cationic exchange resin, and pипecolic acid was eluted with hydrochloric acid. Recrystallization was effected with pyridine addition in
the cold. The extraction of 175 lbs. of beans resulted in the recovery of 13.4 grams of crystalline pipecolic acid.

The questions of origin and metabolic relationships of pipecolic acid were studied by Grobbelaar and Steward (32). Lysine-\(\varepsilon\)-C\(^{14}\) was injected into the cavities surrounding the fruit ovules of green beans. Radioactive pipecolic acid was found in the alcohol soluble fractions of the fruit ovules and stem tissue.

The occurrence of pipecolic acid as an intermediate in the breakdown of lysine in mammalian species was established by Rothstein and Miller (60). L-lysine-6-C\(^{14}\) and non-radioactive pipecolic acid were injected intraperitoneally into rats. The accumulated 24 hour urine excreted by the rats was passed over IR-4 and IRC-50 ion exchange resins. Following evaporation of the effluent to dryness, the residue was converted to the copper salt by treatment with copper carbonate. Treatment with hydrogen sulfide and hydrochloric acid converted the pipecolic acid to the hydrochloride salt. Two recrystallizations yielded a material which showed only one spot on a ninhydrin treated paper chromatogram (collidine-lutidine-water). The spot corresponded with authentic L-pipecolic acid. The high specific activity of this compound prompted these investigators to propose that pipecolic acid is involved as an intermediate in the conversion of L-lysine to \(\alpha\)-amino adipate.

In a similar manner these workers injected D-lysine-\(\varepsilon\)-C\(^{14}\) into rats (61). The 24 hour urines were found to contain no D-pipecolic acid. Furthermore, insignificant labeling appeared in the plasma and tissue protein of these animals. It thus appeared that unlike most other amino acids, the D-isomer of lysine could not be utilized by the organism. This finding confirmed the
earlier report of Ratner et al. (56) who fed D-lysine-α-N¹⁵ to rats. The label appeared in the urine in the form of lysine, ammonia, and urea. Some of the isotope was present in the non-protein fraction of the body tissues, but no labeling was found in the protein fraction. Although the D-isomer is apparently not utilized in the mammalian organism, the isolation of a lysine racemase from a strain of Pseudomononas has been reported which is capable of converting D-lysine to the L-form (36).

Rothstein and Miller (62,64) conducted further studies on the conversion of L-lysine to pipelicolic acid in order to determine which amino group is lost. In these experiments L-lysine-α-N¹⁵ and L-lysine-ε-N¹⁵ were administered to different rats along with "metabolite overloading" doses of unlabeled piperolic acid. This technique takes advantage of the assumption that the injected non-radioactive compound will equilibrate to a certain extent with similar biologically formed material resulting in a greater yield of radioactive pipelicolic acid in the urine. Labeled piperolic acid was isolated from the urine of these animals only following treatment with L-lysine-ε-N¹⁵. This indicated that deamination occurs in the α-position, contrary to the conclusion of Borsook et al (6).

There is ample evidence to support the belief that piperolic acid represents a point in the primary and major pathway of lysine metabolism. First, the structural relationship is such that the molecule retains the six carbon lysine skeleton. Secondly, the piperolic acid isolated after the administration of L-lysine-ε-C¹⁴ had a very high specific activity. By their method, Rothstein and Miller have reported that a minimum of twenty percent of the N¹⁵ of the ε-C¹⁴ labeled lysine was obtained in the isolated
urinary piperolic acid.

In similar experiments these workers injected DL-lysine-\(\text{N}^{15}\) plus a "metabolite overloading dose" of L-\(\alpha\)-amino adipic acid into rats (64). The urine was collected for 16 hours, diluted, passed through the cation exchange resin, IRC-50, acidified, and evaporated. The residue was dissolved in water, treated with norit, filtered, and evaporated. The residue was dissolved in ethanol, treated with a slight excess of pyridine, and seeded with authentic \(\alpha\)-amino adipic acid in the cold. The precipitate showed only one spot corresponding with the authentic sample on paper chromatograms.

The isolation of labeled \(\alpha\)-amino adipic acid following treatment with labeled lysine confirms the findings of Borsook and his collaborators (6). However, the low specific activity of this intermediate as compared with piperolic acid affords presumptive evidence that \(\alpha\)-amino adipic acid follows piperolic acid in the degradation of lysine.

4. \(\alpha\)-keto-\(\varepsilon\)-aminocaproic acid;

The \(\alpha\)-deamination of L-lysine implies the formation of the \(\alpha\)-keto analogue of lysine, \(\alpha\)-keto-\(\varepsilon\)-aminocaproic acid, in the course of its degradation to piperolic acid. Indirect evidence for such a transformation was obtained by Meister (38, 39). An enzyme obtained by the dialysis of venom from Crotalus adamanteus (rattlesnake) was found to be capable of catalyzing the breakdown of \(\varepsilon\)-N-carbobenzoxylysine to the \(\alpha\)-keto analogue. Removal of the carbobenzoxy group with glacial acetic acid and HBr resulted in the formation of \(\alpha\)-keto-\(\varepsilon\)-aminocaproic acid hydrobromide and the monohydrate of \(\Delta^1\)-piperidine-2-carboxylic acid. Catalytic hydrogenation of these products with palladium black led to the appearance of piperolic acid, the identity of
which was proven by paper chromatography and mixed melting point determinations.

Although such a reaction has not been demonstrated in vivo, two French investigators, Boulanger and Osteaux (8) have isolated an L-amino acid dehydrogenase from turkey liver. This enzyme was found to be active toward the basic amino acids including lysine, and catalyzed a deamination of the classical oxidative type. One mole of oxygen was consumed per one mole of liberated ammonia. Lysine selectively labeled with \( ^{15}N \) in the \( \alpha \)-or \( \omega \)-positions when used in this system established conclusively that deamination occurs in the \( \alpha \)-position (9).

Schweet and his co-workers have incubated lysine requiring strains of Neurospora with \( L \)-lysine-\( 1-C^{14} \) (71,72). The non-protein fraction of the mycelial pads was extracted with water. Column chromatography on the cationic exchange resin Dowex-50 revealed a radioactive peak identified as pipecolic acid by paper chromatography and recrystallization to constant specific activity with authentic carrier pipecolic acid. The anionic constituents were absorbed on the exchange resin Dowex-1. The major peak of radioactivity following elution with acetic acid proved to have an \( R_f \) value similar to glutamic acid, but was non-ninhydrin reactive. Hydrolysis of this unknown compound resulted in the formation of \( \alpha \)-hydroxy-\( \varepsilon \)-aminocaproic acid. Paper chromatography and electrophoresis with authentic sample confirmed its identity. This finding offers more evidence to support the possibility of the \( \alpha \)-keto analogue of lysine occupying a position in its degradation sequence.

Preparation of \( \alpha \)-keto-\( \varepsilon \)-aminocaproic acid by the method of Meister resulted in a compound exhibiting the properties of a cyclized form (38).
Further studies which provide additional evidence that this compound exists mainly in the form of \( \Delta^1 \)-piperidine-2-carboxylic acid are summarized below:

(73)

a) The compound gives only a faint yellow color with ninhydrin. The open chain keto acid would be expected to react more readily due to the free \( \alpha \)-amino group.

b) The compound had an \( R_f \) value on paper chromatograms which was close to the cyclized amino acids proline and pipecolic acid.

c) Catalytic hydrogenation produced pipecolic acid.

All these observations are compatible with the existence of a cyclized structure in equilibrium with a small amount of the open chain keto acid. Therefore, \( \Delta^1 \)-piperidine-2-carboxylic acid was considered to be an intermediate between \( \alpha \)-keto-\( \epsilon \)-amino caproic acid and pipecolic acid (Chart I). Although the catalytic hydrogenation of this intermediate had been accomplished, the ability of living systems to convert the cyclized \( \alpha \)-keto analogue to pipecolic acid was not demonstrated until Meister et al. (39,40) reported the isolation of an enzyme from rat and rabbit liver capable of accelerating this reaction. This enzyme was purified 25 times by differential centrifugation, ammonium sulfate fractionation, and treatment with calcium phosphate gel. Upon incubation of the enzyme with \( \Delta^1 \)-piperidine-2-carboxylic acid in the presence of the reduced pyridine nucleotides DPNH or TPNH, pipecolic acid was formed. The reaction has been followed in this manner and also by the decrease of the characteristic absorption band of the reduced coenzyme at 340 m\( \mu \).

The catabolism of lysine as reviewed in Chart I, is, therefore, thought to involve \( \alpha \)-deamination followed by cyclization of the \( \alpha \)-keto derivative to
piperidine-2-carboxylic acid. Subsequently, this product is reduced to pipercolic acid. Although \( \alpha \)-aminoadipate was the first intermediate of the breakdown of lysine to be reported, it is now generally considered to occupy a position following pipercolic acid.

5. The Conversion of Pipercolic Acid to \( \alpha \)-Aminoadipic Acid:

DL-pipercolic acid-2-\(^{14}\)C incubated with rat liver mitochondria in the presence of ATP, Mg\(^{++}\), and unlabeled L-\( \alpha \)-aminoadipic acid resulted in the appearance of labeled \( \alpha \)-aminoadipic acid in the deproteinized reaction mixture (67,68). Following its elution from the cationic exchange resin, Dowex-50, the isolation of \( \alpha \)-aminoadipate was complicated by trace amounts of contamination by glutamic acid. Conversion of the \( \alpha \)-aminoadipate to ornithine by the Schmidt reaction allowed its clear separation from glutamate by descending chromatography (butanol-pyridine-water). A sufficient amount of radioactivity in the final product was found to ascertain its position as a metabolic product of pipercolic acid. The mechanism of the conversion of pipercolic acid to \( \alpha \)-aminoadipic acid has not been determined. Presumably, pipercolic acid is oxidized to \( \Delta^1 \)-piperidine-6-carboxylic acid, which is in equilibrium with \( \alpha \)-aminoadipic-\( \gamma \)-semialdehyde, and which is further oxidized to \( \alpha \)-aminoadipic acid (Chart I) (30).

6. The Conversion of \( \alpha \)-Aminoadipate to Glutarate:

The further catabolism of \( \alpha \)-aminoadipic acid was studied by Borsook and his collaborators (7). Incubation of the \( \varepsilon \)-\(^{14}\)C labeled amino acid with guinea pig liver homogenates led to the appearance of \( \alpha \)-keto adipic acid and glutaric acid in the non-protein fraction. The \( \alpha \)-keto derivative was isolated as the phenylhydrazone which was recrystallized to constant specific activity.
Mixed melting point determinations contributed to the positive identification of the compound. The presence of labeled glutaric acid in the reaction mixture led these workers to propose the deamination of $\alpha$-aminoadipic acid followed by the decarboxylation of its $\alpha$-keto analogue. These last two steps in the direct catabolic sequence of lysine are indicated in Chart I.

Radioactive glutaric acid has also been isolated from the ether extract of the urine of rats injected with L-lysine-6-\textsuperscript{14}C plus a "metabolite overloading dose" of glutaric acid (68). Recrystallization to constant specific activity with carrier glutarate and mixed melting point determinations confirmed its purity and identity. Similarly, the injection of rats with DL-pipelic acid-2-\textsuperscript{14}C plus an overloading dose of glutaric acid resulted in the production of labeled glutaric acid (63).

7. The Further Catabolism of Glutaric Acid;

Glutaric acid is readily metabolized in the animal body. Phlorhizinized rats were fed meat containing glutaric acid-1, 5-\textsuperscript{14}C and the urine collected for 25 hours.(59). Radioactivity was found in the excreted glucose, acetate, and acetoacetate. Chemical degradation of the labeled glucose was carried out with sodium periodate, and bacterial degradation with a strain of Lactobacillus vulgaricus. Radioactivity assays of the evolved carbon dioxide were carried out in an ionization chamber, and established that the glucose was labeled mainly in the three and four carbon atoms. Acetate and acetoacetate was labeled in the carboxyl position. The position and concentration of the \textsuperscript{14}C in these compounds led to the conclusion that glutarate is probably metabolized for the most part by decarboxylation to butyric acid followed by
conversion to acetate. Conversion of acetate via the tricarboxylic acid cycle would account for the glucose labeling (59,63). However, the quantitative similarity in the radioactivity of the acetate and glucose would seem to indicate a more direct pathway for glucose formation.

The occurrence of $\alpha$-ketoglutarate as a catabolic product of both lysine and glutarate has been reported. Rothstein and Miller (63,65) found that rats treated with a metabolite overloading dose of $\alpha$-ketoglutarate plus a tracer dose of L-lysine-6-C$^{14}$ excreted labeled acetate and $\alpha$-ketoglutarate. The greater amount of radioactivity in the $\alpha$-ketoglutarate was considered to be presumptive evidence of a separate pathway for $\alpha$-ketoglutarate formation not involving acetate.

Data have been presented by Rothstein and Miller (65) in support of the view that the carbon chain of glutaric acid-1, 5-C$^{14}$ remains intact in its conversion to $\alpha$-ketoglutarate. Using the metabolite overloading technique, radioactive glutaconate, $\alpha$-hydroxyglutarate and $\alpha$-ketoglutarate were isolated from rat urine. On this basis the following scheme was proposed:

\[
glu\text{tarate} \rightarrow \text{glutaconate} \rightarrow \alpha\text{-hydroxyglutarate} \rightarrow \alpha\text{-ketoglutarate}.
\]

Contrary to this direct conversion of glutarate to $\alpha$-ketoglutarate, Hobbs and Koepp (35) have hypothesized the degradation of glutarate exclusively via acetate. Glutaric acid-3-C$^{14}$ was injected intraperitoneally into rats. Three hours post-injection liver and carcass protein powders were prepared. Following hydrolysis the amino acids were separated by ion exchange chromatography. Glutamic acid, aspartic acid, and alanine were degraded to carbon dioxide, radioactivity being monitored in a vibrating reed electrometer. These amino acids were labeled almost entirely in the carboxyl carbon position.
The fact that virtually no C$^{14}$ was found in the non-carboxyl portion of these compounds strongly suggested that carbon 3 of glutaric acid is metabolized by way of the carboxyl position of an intermediate of the tricarboxylic acid cycle or the Embden-Meyerhof scheme of glycolysis. The absence of significant amounts of radioactivity in carbon 3 of glutamate apparently eliminates the possibility of a direct conversion of glutarate to $\alpha$-ketoglutarate. The degradation scheme of glutaric acid as proposed by these investigators is as follows: glutarate $\rightarrow$ glutaconate $\rightarrow$ $\alpha$-hydroxyglutarate $\rightarrow$ acetonedicarboxylate. In this manner glutaric acid-3-C$^{14}$ would yield acetonedicarboxylic acid labeled in the carboxyl position. Subsequent decarboxylation to acetoacetate followed by cleavage to acetate would account for the formation of carboxyl labeled acetate. Another source of carboxyl labeled acetate would be a cleavage of acetonedicarboxylic acid to malonic and acetic acids. The resulting carboxyl labeled malonate would in turn yield carboxyl labeled acetate (35).

Menon and Stern (41) demonstrated that animals can synthesize glutaryl CoA from glutarate. Tustanoff and Stern (75) subsequently have demonstrated the reversible carboxylation of crotonyl CoA to form glutaconyl CoA and $\beta$-hydroxyglutaric acid. These workers incubated crotonyl CoA in an in vitro system containing ATP, glutathione, potassium bicarbonate and 9.9 mg. of an enzyme precipitated from rat liver extracts with ammonium sulfate.

8. Glutamic Acid and $\Delta$-aminovaleric Acid;

The appearance of glutamic acid as a catabolite of lysine has been reported by several authors. Grobbelaar and Stewart (32) have isolated the radioactive amino acid from the alcohol soluble fractions of the fruit ovules
and stem tissue of green beans injected with lysine-$\varepsilon$-$\text{C}^{14}$. Miller and Bale (42, 43) found $\text{C}^{14}$ labeled glutamic acid in the protein hydrolysates of the carcasses of dogs fed DL-lysine-$\varepsilon$-$\text{C}^{14}$ in their diet. The catabolite was isolated as the hydrochloride salt by column chromatography with the resin Amberlite IR-4. Degradation by the Schmidt reaction and by ninhydrin proved that the radioactivity resided principally in the $\gamma$-carboxyl position. This is consistent with the findings of Hobbs and Koepppe (35), and suggests the formation of $\gamma$-carboxyl labeled glutamate from carboxyl labeled two carbon fragments via the tricarboxylic acid cycle.

Another possible source of glutamic acid as a breakdown product of lysine has been proposed by several Japanese investigators (34, 37, 74). An enzyme was isolated from a strain of Pseudomonas which was found to be capable of converting L-lysine to $\Delta$-aminovaleric acid in vitro. This catabolite was identified by paper chromatography with authentic sample (34). The addition of $\alpha$-ketoglutarate to the reaction mixture containing $\Delta$-aminovaleric acid resulted in the formation of glutamate (74). Other workers have isolated an enzyme from Pseudomonas acetone powder preparations capable of specifically catalyzing this transamination (37).

The discovery of $\Delta$-aminovaleric acid as a possible intermediate in the breakdown of lysine in Pseudomonas was originally reported by Suda et al., (74). The occurrence of this amino acid in the degradation of lysine in animal tissues was originally suggested by Neuberger and Sanger who proposed the decarboxylation of the $\alpha$-keto derivative of lysine (47). It has been shown that $\alpha$-keto-$\varepsilon$-aminocaproic acid can be decarboxylated with hydrogen peroxide to form $\Delta$-aminovaleric acid (38).
Rothstein and Miller (63) have reported on the isolation of labeled \( \Delta \)-aminovaleric acid from the urine of rats treated with L-lysine-6-C\(^{14} \) plus a metabolite-overloading dose of the proposed catabolite. Its identity and purity were ascertained by paper cochromatography and recrystallization to constant specific activity. Since C\(^{14} \) labeling occurred in \( \Delta \)-aminovaleric acid only following large doses of radioactive lysine, these authors concluded that it represents a product of a lysine degradation scheme of secondary importance. The further degradation of \( \Delta \)-aminovaleric acid was studied by these workers (66). The principle product of its further catabolism appears to be glutaric acid. The high specific activity of the glutarate would suggest a relatively direct pathway.

**3) AMINO ACID CATABOLISM IN PRE-CANCEROUS TISSUES**

Burke and Miller (10-14) have described a series of experiments involving amino acid metabolism in rat liver tissues undergoing experimental carcinogenesis induced by the agents 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) and 2-acetylaminofluorene (2-AAF). Livers from rats maintained on 3'-Me-DAB for a period of 2-3 months, and from rats maintained on 2-AAF for 1-2 months were perfused in situ with rat donor blood containing various amino acids or mixtures of amino acids. There was no histological evidence of true hepatoma formation at the stage at which the livers were used, although such pre-cancerous changes as parenchymal hyperplasia, cytoplasmic vacuolization, fatty degeneration, and biliary duct hyperplasia were observed.

Perfusion of the normal and pre-cancerous livers with a mixture of carbohydrates and amino acids over a period of six hours indicated that the production of urea nitrogen was approximately halved in the latter. Similarly,
the production of CO₂ as measured by the evolution of C¹⁴O₂ during perfusion with L-lysine-6-C¹⁴ was found to be depressed to a similar degree.

Coincident with the marked depression of amino acid catabolism in the 3'-Me-DAB rat livers, the synthesis, or at least the turnover of liver and plasma proteins was increased to almost twice that of normal livers (10).

Perfusion of the preparations with glutamine caused an increase in urea nitrogen production in both normal and pre-cancerous livers. In fact, the urea nitrogen production of the pre-cancerous livers increased to nearly normal levels in the presence of added glutamine (11).

The addition of ammonia to the perfusate in the form of ammonium carbonate induced a normal urea nitrogen production by the 3'-Me-DAB treated liver. However, neither arginine, citrulline, or aspartic acid were capable of restoring normal urea formation (12,14).

The ability of glutamine to increase the production of urea nitrogen has been explained in two ways (11). Glutamine may contribute to the production of urea in the liver by merely providing a means of blood transport and liver cell penetration for amino acid nitrogen of extra hepatic origin. A decrease in the formation of urea in the pre-cancerous liver thus would be accounted for by postulating an impairment of transamination reactions involving the transfer of nitrogen from other amino acids to glutamine.

The second possible explanation for the normal urea nitrogen production in the pre-cancerous liver perfused with glutamine is that the reactions involved in the synthesis of urea from glutamine occur independently of the reactions concerned with the catabolism of most of the commonly occurring amino acids. A pathway for the conversion of glutamine amide nitrogen to
urea by a series of reactions independent of the Krebs-Henseleit cycle has, in fact, been proposed (11). This would explain how the synthesis of urea from glutamine could proceed normally in the presence of a biochemical impairment of the usual pathway of urea synthesis from amino acids.

The ability of ammonia to support normal urea production in livers of 3'-Me-DAB treated rats may indicate a direct conversion of ammonia to urea. However, quite possibly the ammonia is converted to glutamine amide nitrogen prior to its utilization in urea synthesis (12).

The considerable decrease of urea synthesis from arginine and from its precursors citrulline and aspartic acid may indicate a defect involving a marked decrease in arginase activity (12,14). This may be due to some form of enzyme inhibition or to an enzyme loss in accord with the enzyme deletion theory of tumor formation (12).

A further possibility is that those amino acids which show a decreased ability to act as urea precursors during the process of hepatoma formation may be preferentially utilized for other metabolic activities such as protein synthesis (12). The increased uptake of L-lysine-6-C\textsuperscript{14} by pre-cancerous liver proteins indicates that such synthesis is occurring at an accelerated rate (10). Therefore, an accelerated demand for amino acids for anabolic purposes rather than catabolic seems feasible.

C) THE MORRIS HEPATOMA 5123

1. The Theory of Enzyme Deletion:

The concept of enzyme deletion and its role in carcinogenesis has been proposed by Potter (52,54,55). Briefly stated, the deletion hypothesis is intended to mean that a cancer cell lacks some enzyme that is present in the
normal cell from which it was derived. Since alternative pathways of metabolism exist for many individual metabolites, it is possible that the loss of one enzyme could stimulate an alternative metabolic pathway for the corresponding substrate. If a given compound could be used as a "building block" by one enzyme, and be degraded by another, it seems reasonable that a loss of the catabolic enzyme could increase the flow over the other pathway merely by decreased competition. This idea is illustrated in the following diagram (55).

![Diagram showing metabolite flow](image)

One problem that has plagued oncologists studying transplantable animal tumors has been the identity of the cell of origin, that is the normal cell which was the immediate precursor of the first biologically malignant cell. In order to validly compare a neoplasm with normal tissue, the individual cell type which became cancerous must be known. The old approach to the problem was to study a so-called "homologous" tissue, or to pick a tissue that contained dividing cells, or simply to analyze tissue from several different organs and "hope something would come of it" (55). Such an approach is complicated by the following factors: a) organs are composed of different tissues, each tissue having its own cell type, b) each type of normal cell in a multicellular organism has a different and characteristic enzyme pattern, c) the enzyme pattern of normal cells is not constant, but varies widely as age, physiological condition, and environment alter the rate of enzyme synthesis, activation, and destruction, d) the enzyme pattern of a normal cell
type varies even with its position in a tissue or in an organ, and e) cancer
cells do not necessarily have uniform enzyme patterns even when they are mor-
phologically similar (54,55).

There are so many enzyme patterns in different types of cancer cells that
in order to identify the strategic alteration that is carcinogenic in any
given variety of tumor, a type of cancer cell is necessary that: a) is readily
and reproducibly available, b) possesses a stable enzyme pattern, c) possesses
an enzyme pattern which deviates as little as possible from that of the normal
cell of origin, d) is derived from an identifiable cell of origin, and e)
possesses a cell of origin available in adequate amounts for comparison (54,
55).

Potter (52,53) has reported on a biochemical survey of several rat
hepatomas in an attempt to discover "a cancer cell that differs from a normal
prototype as little as possible." It was postulated that many enzymatic
changes seen in transplanted tumors are irrelevant to the problem of carcino-
genesis, and that in these tumors it is impossible to separate the relevant
from the irrelevant. If a sufficient number of such "minimal deviation"
hepatomas could be identified and characterized enzymatically, biochemists
may find the least common denominators of enzymatic change that are associated
with the carcinogenic process (54).

In the course of this survey, the Morris Hepatoma 5123 has been exten-
sively studied by Potter and others.

2. Induction of the Primary Tumor:

The Hepatoma 5123 was originally described by Dr. Harold P. Morris of the
National Cancer Institute (44). Maintenance of female rats of the Buffalo
strain for a period of 9.9 months on a diet containing N-(2-fluorenyl) phthalamic acid (2-FPA), resulted in the formation of liver tumors. The average daily intake of 2-FPA was 4.0 mg/rat/day, the average total intake during the period of treatment being 1.2 gm/rat. Eight months were allowed to elapse between the cessation of 2-FPA treatment and the sacrificing of the animals. The tumor chosen for propagation by transplantation was induced in rat 5123.

The primary tumor was grossly characterized by multiple dark red nodules throughout the liver. Microscopic findings indicated that it was a carcinoma of hepatocellular origin with double cords of cells separated by prominent vascular spaces. Glandular acinar-like formations were observed in some areas. Individual cells were noted to be polygonal, eosinophilic, variable in size, and similar to hepatic parenchymal cells. Prominent nucleoli, sparsity of mitotic figures, and multiple lung metastases further characterize this tumor. These distinguishing features have been retained throughout fourteen generations, transplantation being carried out by means of intraperitoneal, intramuscular, and subcutaneous implantation by trocar (44).

3. Deoxycytidylate Deaminase and Thymine Reductase:

Potter and his co-workers (53) compared the activity of deoxycytidylate deaminase in a number of rat hepatomas in vitro. This enzyme catalyzes the conversion of deoxycytidylic acid to deoxyuridylic acid. The activity of the enzyme in Hepatoma 5123 was found to be of a very low order similar to normal liver, and comparable to another slow growing liver tumor, the Dunning Hepatoma. On the contrary, several rapid growing hepatomas, (the Novikoff, Morris 3683, an ethionine induced hepatoma, and the McCoy Hepatoma) had high d-CMP deaminase activities.
Furthermore, Hepatoma 5123 was found to be capable of degrading thymine-2-\(^{14}\)C to \(^{14}\)CO\(_2\) in a manner quantitatively similar to normal liver, indicating the presence of thymine reductase. In this respect the Hepatoma 5123 was unique in comparison with the other hepatomas studied, none of which were capable of carrying out this reaction (53).

Some ability of the hepatoma to reduce thymidine and uracil to CO\(_2\) was noted, but was too inconsistent to definitely establish the presence of thymidine phosphorylase.

On the basis of thymine catabolism and deoxycytidylic deaminase activity, liver can be differentiated from most other tissues and hepatomas, but not from Hepatoma 5123. The following deletion graph as proposed by Potter et al. (53) summarizes the possible origins of hepatoma strains in terms of the deletion of d-CMP deaminase (D) and thymine reductase (T).

\[
\begin{array}{c}
\text{(embryonic liver)} \\
D^+ T^+ \\
\text{(bile duct epithelium)} \\
D^+ T^+ \downarrow -T \\
D^- T^- (Novikoff Hepatoma) \\
\text{D- T+ (adult liver parenchyma)} \\
\text{-X} \\
\text{D- T+ (Morris Hepatoma)} \\
\text{D- T- (Dunning Hepatoma)}
\end{array}
\]

From this deletion graph it is apparent that the loss of thymine reductase is not a required deletion for the conversion of a normal liver cell to a hepatoma cell since the Morris Hepatoma contains the enzyme. Such a change might be "cancer promoting" in the sense of increased rate of cell division since this hepatoma grows extremely slowly (53).
The Novikoff Hepatoma is thought to be derived from bile duct epithelium (53). The deletion graph presents a possible biochemical correlation with this observation. The Morris tumor, which morphologically resembles liver parenchymal cells is identical to adult liver cells with respect to the two enzymes (D) and (T), and the deletion, X, represents the unknown biochemical deviation.

4. Amino Acid Catabolism in Hepatoma 5123:

To gain further biochemical evidence in support of the cellular origins postulated in the deletion graph, several amino acid catabolizing enzymes, and glucose-6-phosphatase were studied in a series of hepatomas in vitro (49).

Glucose-6-phosphatase (G-6-Pase) activity was found to be present to an extent comparable with normal liver in the hepatoma 5123, and in both primary and transplanted ethionine induced liver tumors. The morphological common denominator of these tumors is that they are all of a large cell variety. In contrast, the Novikoff and Dunning Hepatomas liberated very little phosphate from glucose-6-phosphate in the presence of this enzyme (49).

Similarly, glutamate dehydrogenase (GDH) activity was comparable to liver in the Morris 5123 and ethionine induced tumors, but present at very low levels in the Dunning and Novikoff Hepatomas (49).

With regard to choline oxidase (CO) activity, only the Hepatoma 5123 was found to be capable of converting choline to betaine in vitro (49).

A consideration of these observations further supports the concept of there being at least three distinct classes of hepatic carcinomas in the rat as outlined in the deletion graph.

The first type, exemplified by the Novikoff Hepatoma and designated as D+ T- in the graph is further characterized by the absence of G-6-Pase, GDH,
and CO. These latter three enzymes are markers of renal or hepatic origin. In contrast, CMP deaminase does not occur in liver, but probably does occur in biliary epithelium.

Type two hepatomas, represented in the graph by the Dunning Hepatoma and designated D- T-, also were found to lack G-6-Pase. However, GDH and CO activities were present. This type of tumor resembles normal liver in that it lacks d-CMP deaminase, but differs insofar as thymine reductase and G-6-Pase are deleted.

The Morris Hepatoma 5123 represents a third type of hepatoma, D- T+, which not only resembles liver with regard to d-CMP deaminase and thymine reductase, but also retains demonstrable G-6-Pase, GDH, and CO activity.

Morphologically, the Dunning and 5123 hepatomas are of a large cell, highly differentiated, slow growing type very similar to normal liver. On the contrary, the Novikoff tumor is of a small cell, fast growing type.

Pitot (49) has pointed out that the Morris and Dunning hepatomas can be transplanted successfully only in inbred strains while the Novikoff tumor is less exacting in this respect. He has suggested that perhaps slow growing, highly differentiated neoplasms are able to maintain their growth only when host resistance is low. Anaplastic, rapidly growing neoplasms such as the Novikoff hepatoma will tend to overcome host resistance by their rapid rate of growth.

The activity of glutamic-oxalacetic transaminase (GOT) has been assayed in a series of rat tumors (26). Hepatoma 5123, the most slowly growing of the tumors, was found to have a level of activity from two to five times that of normal and host livers, while all of the other tumors studied possessed
approximately one half of the activity of the control tissues. Among the
tumors analyzed for GOT was the Novikoff Hepatoma, which had the lowest activi-
ty. Since transaminases are concerned with the transfer of the amino group
from certain amino acids to keto acids to produce other amino acids, it might
be predicted that fast growing tissues would contain high levels of these
enzymes in order to facilitate amino acid production for protein synthesis.
However, Cohen et al. have reported that fetal cat tissues, regenerating rat
liver and certain mouse tumors showed a decreased transaminase activity when
compared with control tissues (2).

Thus, the increase in Hepatoma transaminase activity above that of con-
trol and host livers, presumably slow growing tissues by comparison, is not
understood.

Tryptophan pyrrolase activity of the Hepatoma 5123 in vitro was found to
be decreased under control levels by about two thirds (49). Similarly, all
other rat tumors studied by these investigators exhibited a depression of this
enzyme system.

The level of threonine dehydrase studied in the Morris Hepatoma in vitro
was about 40 times that of host liver (49).

5. Metabolic Adaptations in Hepatoma 5123:

The fact that tryptophan pyrrolase and threonine dehydrase levels were
found to be low and high respectively in the Hepatoma 5123, plus the fact
that these enzymes can be induced in normal liver by dietary protein and sub-
strate administration prompted Pitot et al. (50) to study the effects of these
agents on these and other enzyme levels in this tumor.
A high protein diet (91%) caused no significant increase in the already high levels of threonine dehydrase in the hepatoma. However, significant increases were noted in the control and host livers after seven days.

Serine dehydrase which also is present in the tumor in relatively high quantities, likewise was unaffected by the high protein diet while normal liver and host liver did exhibit a demonstrable increase in activity.

Likewise, tryptophan pyrrolase, the activity of which is low in the Morris Hepatoma, was unaffected by dietary protein.

In adrenalectomized tumor-bearing animals, threonine dehydrase and serine dehydrase activities declined to immeasurable levels. Subsequent administration of cortisone caused an increase in the activities of these dehydrases. However, a high protein diet alone had no effect on tumor dehydrase levels in the adrenalectomized animals.

Further studies of tryptophan pyrrolase activity in the Morris Hepatoma have shown that its activity is unaltered by substrate administration (tryptophan injection i.p.) and by cortisone injection, procedures which induced activity increases in normal and host rat livers (51). The availability of the inducer to the tumor was ascertained by dl-tryptophan-3-c14 tracer studies. Chromatograms of tumor and liver confirmed the presence of tryptophan.

Tyrosine alpha ketoglutarate transaminase levels were found to be of a high order of activity in the Hepatoma. Administration of substrate (tyrosine) caused no significant change in tumor levels of the enzyme, but did induce a five to six fold increase in host liver activity. Adrenalectomized tumor-bearing rats demonstrated a decrease in transaminase levels, the host liver remaining stable. Injection of cortisone acetate caused an increase in tumor
and host liver transaminase levels.

All of these enzymes, threonine dehydrase, serine dehydrase, tryptophan pyrrolase, and tyrosine alpha ketoglutarate transaminase are known to be normally induced by dietary, substrate, or hormonal mechanisms.

The dehydrases respond to substrate administration or dietary protein, but not to cortisone administration. However, in the Hepatoma 5123, these normal controlling influences are inoperable. Adrenalectomy caused a decrease in the activities of these enzymes in the neoplasm which was reversible with cortisone, while substrate control was entirely lacking. This represents a reversal of the normal situation and, operationally speaking, suggests a derepression of these enzymes in the tumor (51).

Tryptophan pyrrolase, in contrast, was fully repressed in the tumor, and was subject to none of the controls, hormonal or substrate, that are known to normally influence this enzyme's synthesis. Thus the repression of tryptophan pyrrolase in Hepatoma 5123 appears to lie within the molecular constitution of the tumor itself.

Tyrosine alpha ketoglutarate transaminase can be induced by cortisone or substrate in the normal liver. In the Hepatoma 5123, the activity of this enzyme is high (derepressed) and is unresponsive to the normal controlling influences. However, adrenalectomy of the host results in a repression of the enzyme in the tumor. This decrease is partially reversible by cortisone, but not by substrate administration. It is theorized that possibly, a) the mechanism controlling this enzyme in the tumor is overly sensitive to cortisone, b) the neoplasm is sequestering adrenal hormones, or c) the tumor is not destroying circulating cortisone as rapidly as liver.
All of these enzymes (tryptophan pyrrolase, threonine dehydrase, serine dehydrase, and tyrosine alpha ketoglutarate transaminase) exhibit a lack of substrate control in the Hepatoma 5123. However, with the exception of tryptophan pyrrolase, cortisone sensitivity of the enzymes is definite, though altered.

Therefore, the Hepatoma 5123 has some aspects of a hormonally dependent tumor, although hormones may not be essential to its growth as evidenced by lack of tumor regression following adrenalectomy.

6. Carbohydrate Metabolism in Hepatoma 5123:

Weber et al. have compared the anaerobic glycolytic pathway in normal liver, the Morris Hepatoma 5123, and the Novikoff Hepatoma, and have proposed correlations between growth rate and certain observed alterations (76,77).

The Novikoff tumor was found to lack glucose-6-phosphatase (G-6-Pase) and fructose-1,6-diphosphatase activities, while the activity of glucose-6-phosphate dehydrogenase was elevated by 300 percent. In contrast, the Hepatoma 5123 exhibited decrease levels of these enzymes, but in no case was there a 100 percent deletion.

The absences of G-6-Pase and fructose-1,6-diphosphatase in the Novikoff tumor indicate a lack of ability to mobilize glucose from glycogen and a depression of gluconeogenesis. The concomitant increase in glucose-6-phosphate dehydrogenase suggests a highly active pentose shunt and nucleic acid synthesis. The ability of these systems to function even at their attenuated levels in the Hepatoma 5123 allows the glucose-6-phosphate draining pathway to function. Thus, pentose and nucleic acid formation are more nearly normal in the slow growing Hepatoma 5123 and exaggerated in the faster growing
Novikoff tumor (77).

A deficiency in the incorporation of glucose into glycogen in both tumors due to a decrease in phosphoglucomutase activity was observed. While gluconeogenesis is entirely lacking in the Novikoff tumor, Hepatoma 5123 was found to be capable of converting pyruvate to glucose, although the activity of this system was of a low order. A greater rate of lactate production in the Novikoff tumor furnishes further evidence of a greatly increased glycolysis and, therefore, more energy for rapid cellular metabolism and division (76).

7. Catalase Activity of Hepatoma 5123:

The activity of the enzyme, catalase, has been assayed in the tissues of rats bearing several transplantable hepatomas (57). Catalase activity in the Novikoff and Morris Hepatoma 3683 was comparatively negligible. However, both the Hepatoma 5123 and the ethionine-induced rat hepatomas were found to possess a high level of activity.

The catalase levels of the liver and kidney of the rats bearing these tumors were reduced. There was a progressive decrease in total host liver catalase activity with increasing tumor size, and gradual increase in the total tumor catalase activity.

A protein free diet was found to decrease the liver catalase activity of tumor-bearing animals. However, lack of dietary protein had no significant effect on the catalase levels of the Hepatoma 5123.

These findings are inconsistent with the previously held view that tumor tissues in general contain little or no catalase activity (31). Rechciogl et al. (57) have pointed out the possibility that previous investigators may have failed to observe significant catalase levels due to the loss of either
the catalase producing cells or mechanisms within the cells during tumor transplantation.

The presence of significant catalase activity in the Hepatoma 5123 further confirms the classification of this neoplasm as a minimal deviation tumor. However, the fact that certain ethionine-induced tumors also exhibited considerable catalase activity "points to the need for a re-examination of the (toxohormone) concept since it seems unlikely that such a factor released from tumors would depress the enzyme level of the liver and kidney without markedly affecting the catalase level of the tumor itself" (57).

The possibility remains that toxohormone is released in an inactive state within the tumor cell, and that this might account for its failure to completely depress the catalase activity of certain hepatomas.

8. **Purine Metabolism in Hepatoma 5123:**

One possible causative or contributing factor to neoplasia could be a deficiency of purine-catabolizing enzymes. The occurrence of such a deficiency might promote purine anabolism resulting in uncontrolled growth. In this connection, Wheeler and his co-workers (79) have studied the anabolism and catabolism of purines in vitro in several rat hepatomas and control tissue sonicates.

The Hepatoma 5123 and the Reuber H-35 tumor were found to utilize adenine-8-C$^{14}$ to a similar extent for anabolism and catabolism. In fact, the ratio of the percent total anabolized radioactivity to the percent total catabolized radioactivity (A/C ratio) was not significantly different in the host livers and tumors.

Similarly, the Hepatoma 5123 and the H-35 tumor exhibited patterns of
hypoxanthine-C\textsuperscript{14} anabolism and catabolism which closely resembled host liver. However, the Novikoff Hepatoma was relatively inactive catabolically.

Guanine-C\textsuperscript{14} and Xanthine-C\textsuperscript{14} were also catabolized to an extent comparable to host liver in the Hepatoma 5123 and H-35 tumor while the Novikoff Hepatoma was deficient in this respect.

The catabolism of the ribonucleotides adenylic acid-C\textsuperscript{14}, inosinic acid-C\textsuperscript{14}, guanylic acid-C\textsuperscript{14}, and xanthosine (2'\textsuperscript{+}3') monophosphate-8-C\textsuperscript{14} was also studied in total tissue sonicates \textit{in vitro}. The Hepatoma 5123 and host liver were of the same order of activity while the Novikoff tumor proved to be much less active catabolically.

These studies have shown that although a deficiency of purine catabolism may be a contributing factor to the uncontrolled growth of some neoplasms, this deficiency is not a requisite for neoplastic growth.
CHAPTER II

MATERIALS AND METHODS:

A) ANIMALS AND TISSUES:

Male, Sprague-Dawley rats weighing between 175 and 200 gms. were used for the experiments involving the Walker 256 carcinosarcoma. The tumor was allowed to develop for seven days before each experiment was performed. For transplantation, donor animals bearing the seven day old tumor were sacrificed by ether anesthesia. The tumors were rapidly dissected under sterile conditions, minced in a garlic press fitted with a fine mesh screen, and suspended in 10 to 20 ccs. of sterile saline (0.9%). Five ccs. of this suspension were drawn into a syringe, and 1.25 ccs. were injected in four different subcutaneous abdominal sites in each of the animals inoculated.

Female rats of the buffalo strain weighing approximately 180 gm, bearing the Morris Hepatoma 5123 were obtained from Dr. Harold P. Morris of the National Cancer Institute, Bethesda, Md. The tumors received were in the twenty-fifth transplant generation of subline D, and had developed for approximately two months when used in the present experiments. The primary Hepatoma was induced by the carcinogen N-(2-fluorenyl) phthalamic acid (44) and has been propagated by intraperitoneal transplantation.

Regenerating rat liver experiments were performed on non-tumor-bearing female rats of the buffalo strain weighing approximately 220 gm. Partial hepatectomy was performed and approximately two grams of liver tissue were removed. A small longitudinal incision was made in the upper abdominal region. Two lobes of the liver were extruded through the incision. Each lobe was
ligated as close to its origin as possible, and severed distal to the ligation.
The incision was closed by suture, and the liver was allowed to regenerate for
45 hours prior to the administration of the tracer.

B) ADMINISTRATION OF THE TRACER:

In each experiment 10 μc. of L-lysine-U-C\(^{14}\) were injected intravenously
into the tail vein of rats bearing the 7 day old Walker tumor, the 60 day old
Morris Hepatoma 5123, the 45 hour regenerating liver, and normal rats. The
animals were anesthetized with ether and sacrificed by exsanguination at time
intervals of one, three, or six hours following injection of the tracer. In
the case of the Walker 256 carcinosarcoma, rats were also sacrificed at one
and six hours post injection.

C) PREPARATION OF THE TISSUES:

Immediately after sacrificing, the tumor and liver of each tumor-bearing
animal were rapidly dissected. Livers from non-tumor-bearing rats and from
rats bearing the regenerating liver were similarly removed. The tissues were
weighed wet, immersed in cold 1N perchloric acid (PCA), and homogenized for
2 minutes in a Waring blender. The protein was removed by centrifugation at
600X gravity in an International, Model V centrifuge. The protein-free
supernate was adjusted to pH 8.5 with KOH and allowed to stand overnight in
the cold. The insoluble potassium perchlorate was then removed by centrifuga-
tion.

D) SEPARATION OF THE RADIOACTIVE CATABOLITES OF L-LYSINE-U-C\(^{14}\)

1. Preparation of Dowex-1 Anionic Exchange Resin:

This resin was obtained from the J. T. Baker Chemical Company as Dowex-1
X8 200-400 mesh in the chloride form. In order to convert the resin to the
acetate form, a slurry of 100 gm. was prepared in 500 ccs. of distilled water. After settling for 90 minutes the water was decanted, and 500 ccs. more were added. This operation was repeated at intervals of 75, 60, 45, and 30 minutes. The resin was then placed in a 50 x 5 cm. glass column, and 8 liters of 1M Na acetate were passed through the resin. Four liters of H₂O were then passed through the resin to remove the excess acetate, and the resin was subsequently stored as a water slurry. During this final step, the effluent was tested for the presence of the chloride ion with AgNO₃.

For the separation of the anionic lysine catabolites, the resin in the acetate form was added to glass columns with an inside diameter of 0.9 cm. and was allowed to pack by gravity to a height of 12.5 cm. A 10 cc. water wash was then passed through the column.

2. Preparation of Dowex-50 Cationic Exchange Resin:

This resin was obtained from the J.T. Baker Chemical Company as Dowex-50W-X4, 200-400 mesh H⁺ form. In order to prepare this resin in the hydrogen form, a slurry of 150 gm. of the dry powder was prepared in 500 ccs. of H₂O, and placed on a 5 x 50 cm. glass column. The following solutions were then passed through the column: a) 8 liters H₂O, b) 5 liters 2N HCl, c) 5 liters 1N HCl, d) 1 liter H₂O, e) 5 liters 2N NH₄OH, f) 1 liter H₂O, g) 5 liters 1N HCl, and h) 5 liters H₂O. The resin was stored as a water slurry.

In the case of the Dowex-50W resin, columns 17 x 0.9 cm. were prepared by packing the resin slurry with 5 lbs. of air pressure. Thirty ccs. of water under 5 lbs. of air pressure were forced through the resin as a wash.
3. Addition of Protein-Free Supernate to Ion Exchange Columns:

The PCA soluble supernate at pH 8.5 was allowed to flow through Dowex-1 resin by gravity. Since a large portion of the radioactivity was not initially absorbed to the anionic column the effluent was adjusted to pH 2.5 with HCl, and then placed on the Dowex-50W-X2 cationic exchange column with 5 lbs. of pressure. A 25 cc. water wash was then passed through the column under 5 lbs. of pressure.

4. Elution of Radioactive Peaks from the Ion Exchange Columns:

A uniform, gradual increase in acidity in the resin beds of both exchange resins was established by the use of an apparatus which consisted of a reservoir of acid, a mixing flask, and a glass column containing the resin. Drops of eluate from the resin column were collected in 10 cc. test tubes with a fraction collector.

A controlled air inlet equipped with a manostat and an air filter admitted air to the system at a pressure sufficient to force fluid from the acid reservoir, through the mixing flask, and finally through the resin column. The volume of eluate collected in the tubes was controlled by manipulation of the air pressure in the system. To insure thorough mixing of the acid entering the mixing flask, and the aqueous solution within, a teflon coated magnetic stirring bar was placed within the mixing flask and spun by a rotating magnetic field. A glass manifold interposed between the mixing chamber and the resin columns allowed for two columns to be eluted simultaneously. Therefore, the elution of amino acids from a given resin was carried out under identical conditions for the tumor and host liver in each experiment.

Elution from the Dowex-1 acetate resin was carried out by an acetate
gradient, a modification of the method described by Busch et al. (15). Four hundred ccs. of triple distilled water were placed in the mixing chamber. The reservoir contained 180 ccs. of 4 N acetic acid during the collection of 40 2-ml. fractions; 250 ccs. of 8 N acetic acid for the collection of 20 6-ml. fractions, and 300 ccs. of a mixture of 8 N acetic acid and 4 N ammonium acetate for the collection of 30 6-ml. fractions (18).

The Dowex-50 H+ columns were eluted with an HCl gradient. The acid reservoir contained 400 ccs. of 4 N HCl and the mixing chamber held 400 ccs. of H2O. The timer was set for 2 minutes and the air pressure was regulated to deliver 2 ccs. in this interval. Ninety fractions were collected.

5. Desiccation:

The fractions were evaporated in heated vacuum desicicators containing a mixture of calcium chloride and sodium hydroxide in a ratio of 2:1. Heat was supplied by infra-red lamps suspended above the desicicators.

6. Assay of Radioactivity:

Each dried fraction was dissolved in 1 cc. of 0.5 N HAc. Aliquots (0.5 cc.) of each effluent fraction were directly pipetted onto stainless steel planchets (1 inch diameter) and dried on a rotating turntable by a stream of warm air produced by hair dryers and an infra-red lamp. Plates made with these solutions were generally infinitely thin. However, plates of peak 10 were consistently coated with a considerable residue. Therefore, corrections for self absorption were made for the radioactivity of these plates.

Radioactivity was determined in a Nuclear Chicago system consisting of a Model C-110B Automatic Fraction Changer, a Model 183 Scaling Unit, and a Model C-111B Printing timer.
E) IDENTIFICATION OF RADIOACTIVE PEAKS

1. Dowex-1:

Authentic samples of α-amino adipic acid, glutamic acid, and aspartic acid were chromatographed on columns of Dowex-1. Ninhydrin positive peaks corresponding to the radioactive peaks 2, 3, and 4 respectively, confirmed the identity of the radioactive peaks (See Chart II). Authentic samples of glutaric acid and succinic acid were found to correspond with radioactive peaks 6 and 7 respectively. These unlabeled, authentic samples were located by titration with 0.1 N NaOH.

Authentic samples of α-ketoglutaric acid and α-ketoaspartic acid were chromatographed and found to correspond with peak 9 (Chart II). The compounds were identified by their color reaction with 2,4-dinitrophenylhydrazine.

2. Dowex-50:

Authentic, unlabeled samples of pipecolic acid and lysine were chromatographed on Dowex-50 resin, and were found to correspond to radioactive peaks 11 and 13 respectively. The locations of these peaks were ascertained by their color reactions with ninhydrin.

In addition, the identities of these two compounds were further established by carrier recrystallization to constant specific activity.

Authentic, unlabeled pipecolic acid was added to dried pooled samples of radioactive peak 11. The mixture was dissolved in absolute methanol, and recrystallized with acetone. An aliquot was plated, counted, and corrected for self absorption. This recrystallization procedure was performed a total of three times, and the following specific activities were determined: 263, 268, and 283 counts/minute/mg.
An authentic sample of L-lysine was added to pooled samples of peak 13, dissolved in hot water, and recrystallized three times with absolute ethanol. The following specific activities were found: 69, 64, and 64 counts/minute/mg.
CHAPTER III

RESULTS

Chart II shows the pattern of radioactivity eluted from the Dowex-1 and Dowex-50 ion exchange columns. The values expressed in this chart represent the average of two experiments utilizing the liver of Walker tumor bearing rats. This host liver was removed three hours following the injection of the precursor, L-lysine-U-C\textsuperscript{14}. A total of thirteen peaks of radioactivity were found to occur in the PCA-soluble fractions of these tissues. Nine peaks were eluted from the Dowex-1 columns, and four from the Dowex-50 ion exchange resin columns.

Seven of these peaks have been identified by cochromatography and recrystallization to constant specific activity, and represent known catabolites of lysine. These are, peak 2-\(\alpha\)-aminoadipic acid, peak 3-glutamic acid, peak 4-aspartic acid, peak 6-glutaric acid, peak 7-succinic acid, peak 11-pipeolic acid, and peak 13-lysine. Peak 9 has not been identified, but appears to be a mixture of \(\alpha\)-ketoacidate and \(\alpha\)-ketoglutarate. These two compounds cannot be resolved due to the very low amount of radioactivity in these fractions. \(\Delta\)-aminovaleric acid was found not to correspond with any of the peaks of radioactivity isolated. Peaks 1, 5, 8, 10, and 12 do not correspond chromatographically to any of the known catabolic products of lysine which were chromatographed. These peaks might possibly represent newly isolated intermediates of lysine catabolism.

Chromatograms of the PCA soluble fractions of the Walker tumor removed from these same animals three hours following the injection of L-lysine-U-C\textsuperscript{14}
revealed radioactive peaks which coincided with the tube numbers as shown for the liver in Chart II with one exception. Peak 12 was found not to occur in the tumor.

Chart III represents a quantitative comparison of the amount of radioactivity appearing in each peak in both the Walker tumor and the host liver at three hours following injection of the isotope. The most outstanding quantitative difference observed is in the percentage of metabolized radioactivity which appears in the pipecolic acid fraction. In the host liver 31% of the metabolized L-lysine-U-C\textsuperscript{14} was converted to pipecolic acid while 67% of the isotope metabolized by the tumor was in the form of pipecolic acid.

Peak 12, which appeared only in the liver, accounted for almost 10% of the metabolized radioactive lysine in this organ. The sections of the diagram labeled organic acids include peaks 1, 5, 8, and 9 which are unknown compounds. In the liver, these acids account for 18% of the metabolized isotope while in the Walker tumor 13% of the utilized radioactivity appeared in these peaks.

A comparison of the percentage of the total radioactivity occurring in each peak 3 hours after the injection of L-lysine-U-C\textsuperscript{14} appears in Chart IV. This chart includes not only the labeled catabolic intermediates of lysine, but also the percentage of radioactivity attributable to the precursor. In both the host liver and the Walker tumor, 61% of the total radioactivity of the PCA soluble fraction was due to L-lysine-U-C\textsuperscript{14}.

The kinetics of lysine catabolism in the Walker tumor and host liver in vivo are presented in Charts V and VI. Experiments were performed in which the tumor and liver were removed at one, three, and six hours following injection of the isotope. The total catabolized radioactivity of lysine,
expressed as counts per minute per gram of tissue (CPM/g.) attained a maximum at three hours post injection in both tissues. A greater percentage of labeled picheolic acid was found in the tumor as compared with the host liver at each of the time points studied. Also, peak 12 was found to be absent from the tumor and present in the liver at all of the time points.

The 3 month old Morris Hepatoma 5123 was studied in a manner identical to the Walker tumor. Chart VII summarizes the percentages of the labeled catabolites of L-lysine-U-\textsuperscript{14} occurring in the tumor and in the host liver. The percentage of the metabolized radioactivity occurring as picheolic acid was found to be almost identical to the Walker tumor bearing rats with regard to both the tumor and the liver. Furthermore, peak 12 was present in the host liver, but absent in the tumor.

In order to extend the comparison of the Morris Hepatoma, 3 hour experiments were also performed on control livers (livers from non-tumor bearing buffalo rats) and on the rapidly growing, non-malignant 48 hour regenerating liver. Chart VIII presents a comparison of the specific activities of each radioactive peak found in the three tissues three hours after injection of the isotope. In this chart, specific activity is expressed in counts per minute per gram (CPM/g.) With the exception of peaks 1 and 2 which represent an unknown intermediate and \( \alpha \)-aminoacidic acid respectively, the normal and regenerating livers exhibited essentially the same specific activity in each peak. However, the Hepatoma 5123 was found to be quantitatively dissimilar to the normal and regenerating livers in the majority of the chromatographic peaks. This is especially apparent with regard to picheolic acid, the specific activity of which was nearly 10 times as great as that found in either
of the other two tissues.

The hepatoma is further differentiated from normal and regenerating liver by the absence of peak 12 from the tumor.
CHAPTER IV

DISCUSSION

The occurrence of pipecolic acid as a catabolite of lysine has been well documented (60,61,62,64). The increased amount of radioactivity which is incorporated into pipecolic acid of the tumor in the course of lysine degradation can be explained in several ways.

First, an acceleration of the metabolic events leading from lysine to pipecolic acid (Chart I) could account for the excessive labeling of the latter in tumor tissues. However, little is known of the enzymes involved in this sequence, and in fact, neither the alpha-keto acid of lysine nor its cyclized form have ever been proven to occur in vivo as catabolites of lysine. Furthermore, such an acceleration of lysine catabolism in the tumor would tend to drain lysine away from protein synthesis. Several investigators have demonstrated the opposite to be true. Busch et al. have reported an increased incorporation of $^{14}C$ labeled amino acids into tumor histones (16,17). Likewise, Burke and Miller have shown that the uptake of L-lysine-6-{$^{14}C$} by liver and plasma proteins was enhanced in precancerous livers perfused with the isotope (10). An accompanying depression in the evolution of $^{14}CO_2$ during the perfusion is further evidence against an acceleration of lysine catabolism.

Although there was 1.5 times more total PCA soluble radioactivity and 1.5 times more precursor lysine found in the tumor as compared with the liver, the greater amount of pipecolic acid found in the tumor does not appear to be readily explained on the basis of merely more isotope reaching the tumor, inasmuch as the corresponding ratio for pipecolic acid was considerably higher.
A third explanation for the excessive accumulation of pipecolic acid in the tumors would be the existence of a depression of one of the metabolic steps following pipecolic acid. The excessive accumulation of pipecolic acid might tend to shunt lysine into protein synthesis, thus causing a suppression of the further catabolism of lysine. Such a depression would account for both the increased incorporation of lysine into protein, and the decreased evolution of CO₂ by precancerous livers. The occurrence of less labeled α-aminoadipic acid in the Walker tumor as compared with the host liver provides further evidence for a depression occurring in the metabolic sequence between these two intermediates. The depression of an enzyme normally involved in the breakdown of potential "building blocks" agrees well with the deletion theory (52,54,55).

Potter has pointed out that altered enzyme functions in hepatomas appear to be of two types; deleted enzymes and derepressed enzymes. Enzymes that have been deleted cannot be induced by administration of the corresponding substrate. Derepressed enzymes are those enzymes which operate at accelerated levels in tumor tissues. Since both types of altered function have been found to occur in the same tumors, it has been postulated that the deleted enzymes normally are involved in the production of "repressor" substances which normally exert a constant check on enzymes involved in the synthesis of DNA. Therefore, the loss of repressor synthesizing enzyme systems would allow DNA production to proceed at an uncontrolled rate resulting in increased protein synthesis and accelerated growth (52,54,55).

The possibility exists that lysine catabolites are in some way involved in the synthesis of substances which exert a depressor effect on anabolic
enzymes in normal tissues. The deletion of an enzyme in the degradation sequence of lysine could, therefore, contribute to the derepression of DNA synthesis in neoplastic tissues. Uncontrolled growth would be the result of this derepression.

Depression of the degradation of amino acids in the precancerous rat liver undergoing carcinogenesis has been reported (10-14). A decrease of urea formation in this tissue has been attributed to a deletion or inhibition of the enzyme arginase. Other investigators (49) have demonstrated the depression of several amino acid catabolizing enzymes in various rat hepatomas. Among these enzymes are glutamate dehydrogenase, glutamic oxalacetic transaminase (26), and tryptophan pyrrolase (49).

The isolation and identification of labeled glutamic acid following the injection of L-lysine-U-C\(^{14}\) further substantiates the findings of other investigators (42,43). The fate of glutaric acid-3-C\(^{14}\) strongly suggests that it is metabolized via acetate to form glutamic acid rather than by way of a direct conversion of the five carbon chain of glutaric acid (35). Conversion to acetate would account for the recovery of the labeled Krebs Cycle intermediates, succinate and \(\alpha\)-ketoglutarate. It is possible that several of the minor, unidentified peaks of radioactivity are intermediates of the TCA cycle or the Embden-Meyerhof scheme of glycolysis. In fact, labeled glucose has been isolated in trace amounts following the injection of L-lysine-6-C\(^{14}\) and glutaric acid-1, 5-C\(^{14}\) (59,63).

The Morris Hepatoma 5123 was included for comparison in these studies on the basis of its classification as a slow growing "minimal deviation" tumor as opposed to the rapidly growing Walker tumor. The percentage of metabolized
radioactivity occurring in pipecolic acid in the Hepatoma 5123 bears a very close resemblance to the Walker tumor (Chart VII). This so called minimal deviation tumor is compared with normal liver and the rapidly growing, non-malignant regenerating rat liver in Chart VIII. Pipecolic acid was found to accumulate to the extent of about ten times that of normal or regenerating liver. This further affirms the closer identity of the Hepatoma 5123 to the Walker tumor rather than to normal liver.

The unidentified radioactive peak 12 was found to be present in all of the host, control, and regenerating livers studied, but absent in both the Hepatoma 5123 and the Walker tumor. This catabolite might possibly represent an intermediate of a pathway for lysine catabolism present in the liver, but deleted from the tumor.

Although eight intermediates have been postulated in the immediate degradation sequence of lysine, only pipecolic acid, $\alpha$-amino adipic acid, $\alpha$-keto adipic acid, and glutaric acid have been isolated and identified (Chart I). In the present studies all of these intermediates plus glutamic acid and two Krebs Cycle intermediates have been isolated. However, five major peaks of radioactivity, peaks 1, 5, 8, 10, and 12 remain unidentified. It is possible that these peaks may represent other TCA or Embden-Meyerhof intermediates; or that some of these peaks may be the postulated intermediates in the immediate degradation of lysine (Chart I). The high specific activity of peaks 10 and 12 suggests that these compounds might occur relatively early in the catabolism of lysine. The possibility also exists that these radioactive peaks may represent newly isolated intermediates in the catabolic fate of lysine.
CHAPTER V

SUMMARY

1) The catabolism of L-lysine-U-C\textsubscript{14} \textit{in vivo} has been studied in five rat tissues. These are a) normal liver, b) the Walker 256 Carcinosarcoma, c) the Morris Hepatoma 5123, d) the regenerating liver, and e) host liver from tumor-bearing animals.

2) Pipelicolic acid, an early intermediate of lysine degradation, was found to accumulate in the two neoplastic tissues to a much greater extent than in the normal, host, and regenerating livers.

3) It is postulated that a lesion exists in the degradative sequence of lysine in the tumors. This lesion might occur in one of the metabolic steps immediately following pipelicolic acid. This catabolic deficiency may also cause an accumulation of lysine which would favor increased protein anabolism, a phenomenon common to all tumors.

4) The catabolism of lysine in tumors is also considered from the viewpoint of Potter's deletion hypothesis. End products of lysine breakdown might function as "repressor" substances with respect to DNA synthesis in normal tissues. A loss of the repressor substance would then result in an accelerated DNA synthesis, and ultimately an increase in protein synthesis.

5) An unidentified chromatographic peak of radioactivity was isolated in the normal, host, and regenerating livers following the injection of L-lysine-U-C\textsubscript{14} \textit{in vivo}. However, this intermediate was not found to
be present in the Walker 256 Carcinosarcoma or in the Hepatoma 5123.

6) The increased accumulation of pipecolic acid and the absence of the unidentified intermediate are common to both tumors studied. With respect to these two parameters it would appear that the minimal deviation Hepatoma 5123 bears a closer resemblance to the multiple deviation Walker Carcinosarcoma than to non-malignant liver tissue.
CHAPTER VI

CHARTS
This pathway for lysine degradation has been proposed by Rothstein and Greenberg (67). Although eight intermediates are shown, only pipecolic acid, \(\alpha\)-aminoadipic acid, \(\alpha\)-ketoacidic acid and glutaric acid have been proven to be catabolites of lysine. The other compounds shown in the scheme are postulated, but unproven intermediates.
Three hours prior to the removal of the liver, 10 μc. of L-lysine-U-C\textsuperscript{14} were injected i.v. The PCA soluble fraction was placed on a column of Dowex-1 at pH 8.5 and eluted with an acetate gradient. The neutral effluent from this column was adjusted to pH 2.5, placed on a column of Dowex-50 and eluted with an HCl gradient. Fractions were collected in test tubes on an automatic collector. The ordinate represents the counts per minute per tube, and the abscissa is the tube number.
Percentage Metabolized Radioactivity of L-Lysine-U-C\textsuperscript{14}  
3 Hours after Intravenous Injection of Tracer

CHART III

Percentage of the PCA soluble radioactivity incorporated into each chromatographic peak of the Walker tumor and host liver. The percentage of radioactivity attributable to the precursor, L-lysine-U-C\textsuperscript{14} is not considered in this diagram. The area labeled "organic acids" includes peaks 1, 5, 7, 8, and 9.
Percentage Total Radioactivity in Tissue from L-Lysine-U-C\textsuperscript{14} 3 Hours after Intravenous Injection of Tracer

In both the Walker tumor and the Host Liver, 61\% of the total PCA soluble radioactivity was due to the precursor, L-lysine-U-C\textsuperscript{14}. Consequently, the percentage of the total radioactivity metabolized was the same in each tissue (39\%).
The top line labeled Total CPM represents the total counts of metabolized, PCA soluble radioactivity. The distinctively shaded areas under the curve represent the following catabolites: I-Pipolic acid, II-α-aminoadipate, III-Glutamate, IV-Unknown peak 10, V-Unknown peak 12, and VI-Organic acids, (peaks 1 and 4-9). The amount of radioactivity appearing in each peak at each time point following the injection of the isotope can be readily observed on this chart. The total catabolized radioactivity of L-lysine-U-C14 attained a maximum at three hours.
The top line labeled Total CPM represents the total counts of metabolized, PCA soluble radioactivity. The distinctively shaded areas under the curve represent the following catabolites: I-Pipeolic acid, II-α-aminoadipate, III-Glutamate, IV-Unknown peak 10, V-Unknown peak 12, and VI-Organic acids, (peaks 1 and 4-9). The amount of radioactivity appearing in each peak at each time point following the injection of the isotope can be readily observed on this chart. The total catabolized radioactivity of L-lysine-U-C\textsuperscript{14} attained a maximum at three hours.
Percentage Metabolized Radioactivity of L-Lysine-U-C\textsuperscript{14} 3 Hours after Intravenous Injection of Tracer

CHART VII

Percentage of the PCA soluble radioactivity incorporated into each chromatographic peak of the Hepatoma 5123 and host liver. The percentage of radioactivity attributable to the precursor, L-lysine-U-C\textsuperscript{14}, is not considered in this diagram. The area labeled "organic acids" includes peaks, 1, 5, 7, 8, and 9.
Specific activity of chromatographic peaks found in the PCA soluble fraction of the Morris Hepatoma 5123, normal liver, and regenerating liver three hours after the intravenous injection of L-lysine-U-C\textsuperscript{14}.
CHAPTER VII

TABLES
Table I
DISTRIBUTION OF RADIOACTIVITY IN TISSUES THREE HOURS AFTER
THE INTRAVENOUS INJECTION OF L-LYSINE-U-\text{C}^{14}

<table>
<thead>
<tr>
<th>Chromatographic Peak</th>
<th>WALKER 256 CARCINOSARCOMA</th>
<th>LIVER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/gm. Tissue</td>
<td>% Metabolized cpm</td>
</tr>
<tr>
<td>1. (Unknown)</td>
<td>166</td>
<td>7.5</td>
</tr>
<tr>
<td>2. (\text{\alpha}-amino Adipate)</td>
<td>77</td>
<td>3.4</td>
</tr>
<tr>
<td>3. (Glutamate)</td>
<td>161</td>
<td>7.0</td>
</tr>
<tr>
<td>4. (Aspartate)</td>
<td>32</td>
<td>1.4</td>
</tr>
<tr>
<td>5. (Unknown)</td>
<td>49</td>
<td>2.1</td>
</tr>
<tr>
<td>6. (Glutarate)</td>
<td>36</td>
<td>1.6</td>
</tr>
<tr>
<td>7. (Succinate)</td>
<td>16</td>
<td>0.8</td>
</tr>
<tr>
<td>8. (Unknown)</td>
<td>63</td>
<td>2.8</td>
</tr>
<tr>
<td>9. (Unknown)</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>10. (Unknown)</td>
<td>132</td>
<td>5.8</td>
</tr>
<tr>
<td>11. (Pipolic Acid)</td>
<td>1486</td>
<td>66.6</td>
</tr>
<tr>
<td>12. (Unknown)</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>13. (Lysine)</td>
<td>3455</td>
<td>60.5</td>
</tr>
<tr>
<td>Chromatographic Peak</td>
<td>Morris Hepatoma 5123</td>
<td>Liver</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>cpm/gm. Tissue</td>
<td>% Metabolized</td>
</tr>
<tr>
<td>1. (Unknown)</td>
<td>112</td>
<td>2.7</td>
</tr>
<tr>
<td>2. (α-amino Adipate)</td>
<td>131</td>
<td>3.1</td>
</tr>
<tr>
<td>3. (Glutamate)</td>
<td>222</td>
<td>5.4</td>
</tr>
<tr>
<td>4. (Aspartate)</td>
<td>124</td>
<td>3.0</td>
</tr>
<tr>
<td>5. (Unknown)</td>
<td>108</td>
<td>2.9</td>
</tr>
<tr>
<td>6. (Glutarate)</td>
<td>111</td>
<td>2.5</td>
</tr>
<tr>
<td>7. (Succinate)</td>
<td>43</td>
<td>1.1</td>
</tr>
<tr>
<td>8. (Unknown)</td>
<td>250</td>
<td>5.3</td>
</tr>
<tr>
<td>9. (Unknown)</td>
<td>36</td>
<td>0.8</td>
</tr>
<tr>
<td>10. (Unknown)</td>
<td>192</td>
<td>4.7</td>
</tr>
<tr>
<td>11. (Piperolic Acid)</td>
<td>2935</td>
<td>67.9</td>
</tr>
<tr>
<td>12. (Unknown)</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>13. (Lysine)</td>
<td>3920</td>
<td>48.8</td>
</tr>
<tr>
<td>Chromatographic Peak</td>
<td>NORMAL LIVER</td>
<td>REGENERATING RAT LIVER</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td></td>
<td>(BUFFALO RAT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cpm/gm. Tissue</td>
<td>% Metabolized cpm</td>
</tr>
<tr>
<td>1. (Unknown)</td>
<td>94</td>
<td>5.0</td>
</tr>
<tr>
<td>2. (O(-amino Adipate)</td>
<td>117</td>
<td>5.7</td>
</tr>
<tr>
<td>3. (Glutamate)</td>
<td>95</td>
<td>5.0</td>
</tr>
<tr>
<td>4. (Aspartate)</td>
<td>58</td>
<td>2.9</td>
</tr>
<tr>
<td>5. (Unknown)</td>
<td>230</td>
<td>13.8</td>
</tr>
<tr>
<td>6. (Glutarate)</td>
<td>55</td>
<td>2.9</td>
</tr>
<tr>
<td>7. (Succinate)</td>
<td>50</td>
<td>2.3</td>
</tr>
<tr>
<td>8. (Unknown)</td>
<td>177</td>
<td>8.5</td>
</tr>
<tr>
<td>9. (Unknown)</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>10. (Unknown)</td>
<td>564</td>
<td>27.7</td>
</tr>
<tr>
<td>11. (Pipecolic Acid)</td>
<td>317</td>
<td>15.9</td>
</tr>
<tr>
<td>12. (Unknown)</td>
<td>185</td>
<td>9.4</td>
</tr>
<tr>
<td>13. (Lysine)</td>
<td>2780</td>
<td>55.7</td>
</tr>
</tbody>
</table>
CHAPTER VII

BIBLIOGRAPHY


APPROVAL SHEET

The thesis submitted by Robert N. Morris has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Jan. 18, 1963

Date

Joseph R. Davis, M.D., Ph.D.

Signature of Adviser