HEPATIC ORNITHINE METABOLISM IN HYDRAZINE-TREATED RATS

Van Saxton Hubbard
ABSTRACT

Ornithine plays an important role in mammalian intermediary metabolism. Ornithine can be (1) utilized as a component of the urea cycle, (2) decarboxylated to form putrescine, a precursor of the polyamines, spermidine and spermine, and (3) converted metabolically to arginine, proline and glutamate. In prior investigations conducted in our laboratories and others, free endogenous hepatic pools of ornithine were found to be elevated following hydrazine administration. Consequently, time-course alterations of arginase, ornithine \( \delta \)-transaminase, ornithine transcarbamylase, and ornithine decarboxylase activities were investigated regarding the effect of hydrazine treatment on hepatic ornithine metabolism in the rat. As an outgrowth of these investigations, a time-course study of the hepatic concentration of putrescine, spermidine and spermine was warranted.

Male albino rats (Holtzman) were injected with neutralized hydrazine (40 mg/kg, body weight, ip) or isotonic saline (1.0 ml/kg, body weight, ip) and fasted for various times. The effects ascribed to hydrazine treatment were based on the comparison of results from hydrazine-treated animals with those obtained from the saline-injected control animals. Maximal elevations of endogenous hepatic ornithine pool sizes were observed at 12 hr in supernatant preparations (1000% of control) and at 24 hr in homogenate preparations (600% of control). Hepatic arginase activity was found to reach its nadir at 4 hr (70% of control). Thus, arginase did not appear to be responsible for the
increased ornithine levels resulting from hydrazine treatment. Hepatic ornithine \( \delta \)-transaminase activity was decreased (40% of control) at 4 hr and this level of activity was sustained throughout the 24 hr period examined. Hepatic ornithine transcarbamylase activity was shown to be decreased maximally at 12 hr (40% of control). The decreased activities of these latter two enzymes offers a plausible explanation for the increased hepatic ornithine levels following hydrazine treatment. Although a remarkable increase (1500% of control) in ornithine decarboxylase activity was observed at 4 hr, it was not thought to influence significantly the levels of hepatic ornithine since the relative activities of the other enzymes involved in ornithine metabolism have been shown by others to be considerably greater than ornithine decarboxylase. This increase in ornithine decarboxylase activity was followed by sequential elevations in total endogenous hepatic putrescine and then spermidine levels. The total endogenous hepatic spermine levels were not altered except for a slight decrease at 48 hr.

Regenerating liver following partial hepatectomy demonstrated alterations similar in magnitude, but with a different time-course in the changes in these parameters. These similarities along with other biochemical and morphological alterations established by others following either partial hepatectomy or hydrazine treatment suggest that the latter may be tantamount to a "chemical" partial hepatectomy.
HEPATIC ORNITHINE METABOLISM IN
HYDRAZONE-TREATED RATS

by

Van Saxton Hubbard

B.S., Union College, 1967

Thesis
submitted in partial fulfillment of the requirements for the
Degree of Doctor of Philosophy in the Department of
Biochemistry at the Medical College of Virginia
Health Sciences Division, Virginia Commonwealth University
Richmond, Virginia
May, 1974
This thesis by Van Saxton Hubbard is accepted in its present form as satisfying the thesis requirement for the degree of Doctor of Philosophy.

Date:

13 May 1974

Approved:

Advisor, Chairman of Graduate Committee

May 13, 1974

May 13, 1974

May 13, 1974

May 13, 1974

Dean of the School of Basic Sciences and Graduate Studies
ACKNOWLEDGMENTS

I wish to express my deepest appreciation to Dr. W. L. Banks, Jr., my faculty advisor and friend, for his patience, confidence and assistance. To the other members of my committee, Drs. E. S. Kline, E. S. Higgins, J. Andrako and G. H. Bond, thank you for your patience and your helpful criticisms.

Special appreciation is extended to Dr. A. Baich for her generous gift of glutamic acid γ-semialdehyde used in this study.

To all MCV faculty, thank you for the knowledge you have shared with me during the last seven years. Many thanks to all others who have made numerous and varied contributions to the writing of this thesis.
# TABLE OF CONTENTS

CURRICULUM VITAE................................................................. iii

ACKNOWLEDGMENTS............................................................... v

LIST OF FIGURES................................................................. vii

LIST OF TABLES........................................................................... x

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

Hydrazine................................................................. 1

Ornithine Metabolism........................................... 9

Polyamine Metabolism........................................... 20

MATERIALS AND METHODS..................................................... 25

Materials................................................................. 25

General Treatment of Animals................................. 27

Experimental Treatment of Animals........................ 28

Ornithine Decarboxylase Assay............................... 29

Phenylalanine Decarboxylase Assay........................ 30

Ornithine Transcarbamylase Assay........................ 31

Ornithine α-Transaminase Assay............................ 32

Arginase Assay............................................................... 33

Determination of Protein Concentration..................... 35

Determination of Putrescine, Spermidine, and
Spermine in Hepatic Tissue........................................... 35

Determination of Liver Protein, RNA and DNA Concentrations.......................... 37

Determination of Dry Weights.......................................... 39
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determination of Endogenous Concentrations of Free Amino Acids</td>
<td>39</td>
</tr>
<tr>
<td>Statistical Analysis of Data</td>
<td>40</td>
</tr>
<tr>
<td>RESULTS</td>
<td>41</td>
</tr>
<tr>
<td>Endogenous Levels of Ornithine Following Hydrazine Treatment</td>
<td>41</td>
</tr>
<tr>
<td>Ornithine Decarboxylase Studies</td>
<td>48</td>
</tr>
<tr>
<td>Evaluation of ODC Assay Methodology</td>
<td>49</td>
</tr>
<tr>
<td>Time-Course of ODC Activity Following Hydrazine Treatment</td>
<td>57</td>
</tr>
<tr>
<td>Effect of Cycloheximide and Actinomycin D on ODC Activity</td>
<td>61</td>
</tr>
<tr>
<td>Phenylalanine Decarboxylase Activity</td>
<td>65</td>
</tr>
<tr>
<td>Time-course of Hepatic Putrescine, Spermidine and Spermine Levels</td>
<td>67</td>
</tr>
<tr>
<td>Hepatic Levels of γ-Aminobutyric Acid Following Hydrazine Treatment</td>
<td>72</td>
</tr>
<tr>
<td>Other Enzymes Involved in Ornithine Metabolism</td>
<td>74</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>80</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>107</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>109</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metabolic Pathways Involving Ornithine in Mammalian Tissues</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Time-course of Endogenous Hepatic Free Ornithine Pool Size after Experimental Treatment</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>The Proportionality of ODC Activity with Duration of Incubation Time</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>The Proportionality of ODC Activity with Supernatant Volume</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Lineweeaver-Burk Plot for Hepatic ODC from Hydrazine-treated Animals</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>Lineweeaver-Burk Plot for ODC from Regenerating Liver following Partial Hepatectomy</td>
<td>56</td>
</tr>
<tr>
<td>7</td>
<td>Time-course of ODC Activity after Experimental Treatment</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>Time-course of Hepatic Putrescine Concentrations after Experimental Treatment</td>
<td>69</td>
</tr>
<tr>
<td>9</td>
<td>Time-course of Hepatic Spermidine Concentrations after Experimental Treatment</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>Time-course of Hepatic Spermine Concentrations after Experimental Treatment</td>
<td>71</td>
</tr>
<tr>
<td>11</td>
<td>Time-course of OTC Activity after Experimental Treatment</td>
<td>76</td>
</tr>
<tr>
<td>12</td>
<td>Time-course of Arginase Activity after Experimental Treatment</td>
<td>78</td>
</tr>
<tr>
<td>13</td>
<td>Time-course of OTA Activity after Experimental Treatment</td>
<td>79</td>
</tr>
<tr>
<td>14</td>
<td>Lineweeaver-Burk Plot for Hepatic ODC from Hydrazine-treated Animals after Correction for Endogenous Substrate</td>
<td>92</td>
</tr>
<tr>
<td>Figures</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>Lineweaver-Burk Plot for ODC from Regenerating Liver following Partial Hepatectomy after Correction for Endogenous Substrate</td>
<td>93</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Hepatic Pool Size of Endogenous Free Ornithine Following Experimental Treatment</td>
<td>44</td>
</tr>
<tr>
<td>II</td>
<td>Time-course of Endogenous Free Amino Acid Pool Size in Control and Hydrazine-treated Animals</td>
<td>46</td>
</tr>
<tr>
<td>III</td>
<td>Effect of Length of Preincubation Time on ODC Activity</td>
<td>52</td>
</tr>
<tr>
<td>IV</td>
<td>Observed ODC Activity in Boiled Enzyme Blanks</td>
<td>58</td>
</tr>
<tr>
<td>V</td>
<td>Time-course of ODC Activity Following Experimental Treatment</td>
<td>60</td>
</tr>
<tr>
<td>VI</td>
<td>Hepatic ODC Activity in a 1:1 Mixture of Supernatants from Hydrazine-treated and Control Animals</td>
<td>62</td>
</tr>
<tr>
<td>VII</td>
<td>Effects of Cycloheximide and Actinomycin D on Hepatic ODC Activity</td>
<td>63</td>
</tr>
<tr>
<td>VIII</td>
<td>PDC Activity and Phenylalanine Pool Size Following Experimental Treatment</td>
<td>66</td>
</tr>
<tr>
<td>IX</td>
<td>Time-course of Hepatic Polyamine Levels Following Hydrazine Treatment</td>
<td>68</td>
</tr>
<tr>
<td>X</td>
<td>Time-course of Hepatic Protein, RNA and DNA Levels Following Hydrazine Treatment</td>
<td>73</td>
</tr>
<tr>
<td>XI</td>
<td>Hepatic Levels of Endogenous GABA Following Hydrazine Treatment</td>
<td>75</td>
</tr>
</tbody>
</table>
INTRODUCTION

Hydrazine

Hydrazine (H₂N-NH₂) was first identified by Emil Fischer in 1873 and twelve years later was isolated as the sulfate, dihydrochloride, and the aqueous solution of the free base by Curtius (1). In a paper describing the pathological effects of hydrazine poisoning in 1908, Wells (2) described hydrazine as a substance that was not likely to become so commonly used that it would be of general toxicological importance. Hydrazine and its derivatives remained as little used laboratory chemicals mainly involved in the study of the structure of sugars (3) until the period encompassing World War II, when German chemists used hydrazine as part of the propellant for the world's first operational rocket plane, the Messerschmitt 163-B. Hydrazine's explosive power has been found to be one-third greater than that of TNT (1, 4). In 1955, it was stated that "if man ever gets to the moon, it will not be atomic fuels but hydrazine which will power the space ship" (4), a prophesy that was fulfilled when the first lunar entry module used hydrazine as a component of the propellant fuel in its rocket engines (5). In recent years more than two thousand derivatives of hydrazine have been produced, accomplishing tasks familiar to many of the lay public (4). These uses include the manufacturing of nylon-like fabrics, a non-corrosive solder flux, and a preservative for rubber products, paint-drying oils, or photographic developers. Benzene sulfonyl hydrazide mixed with rubber behaves like
yeast in bread dough and is responsible for creating sponge rubber and crepe rubber (4, 6). Other hydrazine derivatives have been found which slow the rate of growth of plants. This characteristic has been used to discourage grass growth and decrease lawn mowing 10-fold by the Connecticut Highway Department along the Merritt Parkway (1, 4, 6). Finally, a whole family of pharmacological agents have been developed from hydrazine derivatives. Among the more widely used of these hydrazine derivative drugs are isonicotinic acid hydrazide (INH) used in the treatment of tuberculosis, hydralazine (1-hydrazinophthalazine, Apresoline) used as an antihypertensive agent, procarbazine (one of the methyl hydrazine derivatives) used as an antitumor agent, and the nitrofurans including Furadantin used in urinary tract infections and Furacin mixed with feed to prevent coccidiosis in poultry.

Associated with the vast variety of biological and pharmacological effects of hydrazine and its derivatives is a definite problem of toxicity. The vapors from hydrazine solutions were recognized to be highly irritating to the nasal and pharyngeal mucous membranes (3). The influence of hydrazine on intermediary metabolism was recognized by Underhill and Kleiner (7) in 1908. In stressing the importance of this observation, Wells (2) stated "the lesions produced by hydrazine are of such a nature that this drug promises to have a field of usefulness in certain metabolic and pathologic investigations."

Underhill and Kleiner (7) described the general effects of hydrazine (100 mg/kg body wt, hydrazine sulfate) upon dogs as vomiting, a period of restlessness, augmentation of the heart rate which later falls below normal and respiratory difficulty accompanied by general
paralysis, coma and death. Other symptoms of hydrazine poisoning (50 mg/kg body wt, hydrazine sulfate) commonly observed in laboratory animals include anorexia, weight loss, decreased body temperature, weakness, and extreme lethargy (3, 8, 9). Higher doses of the compound will cause tonic convulsions in the animals. Krop (3) reported that hydrazine appears to be toxic to all forms of life, and among higher animals there appears to be no marked species difference in sensitivity to the toxic action of hydrazine. Apparently, the toxicity of hydrazine and its derivatives is independent of the route of administration (3, 10). After his histological study of the effects of hydrazine on various organs in the dog, Wells (2) concluded that "hydrazine seems to be a poison with an almost specific effect upon the cytoplasm of the parenchymatous cells of the liver." The most common pathological finding in response to hydrazine is the profound fatty metamorphosis in the liver (2, 11) usually accompanied by hypoglycemia (9, 12). Accumulation of liver triglycerides due to hydrazine treatment was decreased approximately 50% by prior administration of the antioxidant diphenyl p-phenylenediamine even though the hypoglycemic effect of hydrazine was not altered by this antioxidant (13). Other alterations in mammalian metabolism produced by hydrazine include rapid depletion of liver glycogen (11), the accumulation of urea and amino acid in brain, liver and blood (12, 14), and elevation in blood total lipid levels (15). There have been many subsequent studies attempting to delineate the aberrations in metabolism resulting in these effects which will be summarized in the following discussion.
An explanation for the periportal and midzonal fatty infiltration of the liver (2) has been desired for some time. Underhill and Baumann (16) reported increased blood lipid in dogs in 1916. Blood levels of fat were observed to be maximal during the hypoglycemic period produced by hydrazine. Amenta et al. (17) observed that hydrazine elevated liver lipids to the same magnitude as was produced by the hepatotoxin, carbon tetrachloride. Trout (18) reported significant elevation of liver total fatty acid during the first two hours after hydrazine administration to rats. Plasma free fatty acid levels, measured at 3 hr after hydrazine treatment, were elevated about 50%. In addition, when the plasma free fatty acid pool was labeled with $^{14}$C-palmitic or $^{14}$C-oleic acids, there was increased uptake of label into the livers of hydrazine-treated rats compared to their respective controls (19). Other data from this study indicated that the increased plasma free fatty acids were derived from adipose depots. Furthermore, Trout reported that hydrazine did not block the hepatic secretion of triglycerides into the blood (18). Therefore, the elevation of plasma free fatty acids, which in fasting animals are considered to be the main source of fat for the liver, appears to be of importance, especially since "the rate of transport to the liver of free fatty acids is believed usually to be roughly proportional to their plasma concentrations" (18). Thus, accumulation of liver lipid following hydrazine treatment appears to be due to increased hepatic uptake of free fatty acids which have been mobilized from adipose tissue.

Underhill and co-workers investigated the hypoglycemia which accompanied the alterations in lipid metabolism in response to
hydrazine. Their studies in dogs revealed an initial hyperglycemia followed by a persistent hypoglycemia (9, 20). Fortney (21) found that these contradictory reports of hyperglycemia and hypoglycemia were attributable to differences in liver glycogen levels prior to treatment with hydrazine. Animals with glycogen levels greater than one percent of the liver by weight experienced an initial hyperglycemia and eventual hypoglycemia; however, when liver glycogen levels were less than one percent, hypoglycemia was evident within 15 minutes after receiving hydrazine. Additionally, in this study, Fortney noted that glucose infusion did not alter the observed decrease in glycogen levels, the concurrent increase in blood lactate and pyruvate, or the onset of convulsions, thus indicating that these effects were not secondary to the depression in blood glucose levels.

Underhill and Murlin (22) observed an increased respiratory quotient in fasting dogs receiving hydrazine and concluded that the associated hypoglycemia was probably due to increased glucose oxidation. However, Izume and Lewis (12) could not show an increased utilization of glucose in hydrazine-treated animals when compared to appropriate control animals. In another study, these workers demonstrated a decreased ability of the liver to convert glycine and glucose to glycogen in hydrazine-treated rabbits (14). The inability to deaminate glycine, as shown in this study, suggested that normal amino acid deamination does not occur and there is a consequent failure in the formation of α-keto acids needed for gluconeogenesis. In light of these observations, a decrease in gluconeogenesis in response to hydrazine intoxication was proposed to account for the hypoglycemia.
More recently, this inhibition of gluconeogenesis by hydrazine was demonstrated in rats, accompanied by increased blood lactate and pyruvate levels (23).

Since transamination, the initial step for the entry of amino acid into the gluconeogenic pathway, utilizes pyridoxal phosphate as a cofactor, and since hydrazine is known to react with the free aldehyde group of pyridoxal phosphate, the possibility of the inhibition of the transamination reaction by hydrazine was postulated to account for the decreased hepatic gluconeogenesis (23, 24). Besides reacting directly with the B₆ vitamin with the formation of pyridoxal hydrazones (25), hydrazine has been found to inhibit the phosphorylation of pyridoxal by pyridoxal kinase (26). In vitro studies have shown that hydrazine and a number of its derivatives inhibit transamination (15). Killam and Bain (25) showed that inhibition of pyridoxal phosphate-requiring enzymes could be reversed by addition of the B₆ vitamins. In vivo investigations by Fortney et al. (23) revealed strong inhibition by hydrazine of extramitochondrial glutamic-oxalacetate transaminase. Other workers (25) have shown that this in vivo inhibition is not a generalized phenomenon.

Fortney (23) suggested the conversion of pyruvate to phosphoenolpyruvate could be of importance in explaining the decreased gluconeogenesis in response to hydrazine. More recently Ray et al. (27) reported increased levels of liver oxalacetate following hydrazine treatment which they attributed to the in vivo inhibition of the conversion of oxalacetate to phosphoenolpyruvate by phosphoenolpyruvic acid carboxylase. With this knowledge, an explanation for the decrease
in gluconeogenesis from both amino acid and pyruvate was available which would account for the observed hypoglycemia.

Increased levels of blood amino acid nitrogen in rabbits observed by Lewis and Izume (14) suggested that poisoning by hydrazine resulted in a disturbance of protein metabolism. Originally, in 1944, Louis and Lewis (28) found no change in the composition of rabbit liver, muscle and kidney proteins, but their results were based on unaltered percentages of nitrogen and sulfur in protein and not actual protein content. Using the non-essential amino acids 1-\(^{14}\)C-glycine or 1-\(^{14}\)C-alanine, Amenta and Johnston (29) reported increased incorporation of amino acid into protein of liver slices from rats five hours following hydrazine treatment compared to appropriate control animals. Elevation of both liver protein and RNA contents (compared to fasted control animals) occurred 24 hr after treatment with hydrazine (30). Since fasted animals were used in this study, these increases in hepatic protein and RNA could not be ascribed to nutritional factors. Amenta and Johnston (29) suggested that the enhanced incorporation of labeled non-essential amino acid into protein was secondary to the inhibition of transamination by hydrazine as mentioned above. Banks (31) has suggested that the alterations in the levels of amino acids, especially ornithine, citrulline and alanine, as reported by Simonsen and Roberts (32) for mice and Banks (31) and Banks and Petterson (33) for rats, cannot be ascribed exclusively to either a general inhibition of liver transamination or to alterations of hepatic protein biosynthesis.

In order to determine accurately whether increased protein biosynthesis occurred, analysis of hepatic amino acid pool size was used
to correct any fluctuations in the concentration of the labeled amino acid used in the study. Finding that the free endogenous pool size of leucine does not vary with hydrazine treatment, Banks (31) and Smith (34) used $^{14}$C-leucine to show increased uptakes into liver protein at 24 hr as a result of hydrazine administration. The increased protein/DNA and RNA/DNA ratios coincident with the increased uptake of leucine following hydrazine treatment supported the contention that a subconvulsive dose of hydrazine stimulated hepatic protein synthesis in vivo, especially since the protein/RNA ratio did not differ from the controls. In a time-course study of the effects of hydrazine on the uptake of $^{14}$C-leucine into liver protein, an initial decrease in incorporation of the labeled precursor at 4 hr was observed in both the control and hydrazine-treated groups (34). López-Mendoza and Villa-Treviño, (35) using either $^{14}$C-leucine or $^{14}$C-glycine, showed a marked inhibition of the incorporation of amino acid into hepatic protein in vivo within 30 min after giving hydrazine when compared to corresponding control animals. In this study, inhibition reached a maximum at 1 hr and returned to 50% inhibition by 8 hr. Increased protein biosynthesis in other organs has been indicated by greater incorporation of $^{14}$C-glutamate into the protein fraction of liver, kidney and brain 24 hr after hydrazine treatment compared to control animals (36). Thus, it appears that following treatment there is an initial period of decreased synthesis for both hydrazine-treated and control animals followed by a significant stimulation of the machinery for protein synthesis in the animals treated with hydrazine. Banks (31) presented data to suggest that the amino acids required for this increased
protein synthesis are provided by mobilization of protein reserves from skeletal muscle mass.

**Ornithine Metabolism**

The buildup of ornithine in rat liver described by Banks is interesting because of the important role ornithine plays in mammalian biochemistry. Metabolic interconversions between ornithine and arginine, glutamic acid and proline are known to occur. Ornithine, as a component of the urea cycle, is involved in nitrogen metabolism, especially catabolism of amino acid nitrogen. Ornithine also may be decarboxylated to form putrescine (1, 4-diaminobutane), a precursor for polyamine biosynthesis via a pathway currently receiving much attention in investigations surrounding cancer research. It will be the intent of this study to provide an explanation for the elevated hepatic ornithine pool size by studying the various metabolic pathways in which ornithine is a participant in the rat liver (Figure 1). Each of these relationships is discussed in this section and in the following section on polyamines with an attempt made to preserve the chronicity of the events.

The structural similarities of the amino acids proline, hydroxyproline, and glutamic acid with ornithine have been recognized since the time these compounds were identified as constituents of protein (37). Using phlorhizin-induced diabetic dogs, Dakin (38) and Lusk (39) observed that proline, glutamic acid, arginine, and ornithine were all glucogenic; and therefore these amino acids were thought to be related metabolically as well as structurally.
Jaffe was the first to discover the "base", ornithine, and described it as having the formula $C_5H_{12}N_2O_2$ in 1877 (40, 41). Following the discovery of arginine by Schulze and Steiger in the cotyledons of lupine seeds and in the etiols of germinating pumpkin seeds in 1887 (42), Drechsel reported in 1890, that arginine yielded urea and ornithine on decomposition with baryta water (43). In 1898, Schulze and Winterstein also showed that arginine yielded urea and a "base" on treatment with baryta water (42). They then isolated the "base" by forming its benzoyl derivative and found it to have the same composition and properties as the "base" described by Jaffe (42). In the early 1900's both Fischer and Sørensen synthesized ornithine, and Ellinger showed that this synthesized compound yielded putrescine on putrefaction corroborating the structure of Jaffe (41). In 1904, Beebe and Buxton (44) described ornithine in their text as being $\alpha$-dimethylaminovaleric acid which could be acted upon by bacteria to split off the CO$_2$ leaving putrescine. These bases and their oxidative products were then called "ptomaines" by Gautier (45, 46).

In 1940, the conversion of deuterornithine into arginine, proline and glutamic acid was reported using mice fed normal diets (47, 48). Later work from these laboratories showed that both deuterium and isotopic nitrogen ($^{15}$N) from proline fed to rats as part of a regular diet could be found in ornithine, arginine, and glutamic acid (49). Although the metabolic conversion of glutamic acid to proline had been suggested, it was not until 1947 that indirect evidence for the in vivo conversion of glutamic acid to proline or arginine was
reported as part of the nutritional studies done in Rose's laboratory (50). Later, with the use of $^{14}$C-glutamic acid, direct evidence was furnished for its in vivo conversion into proline and arginine (51). Thus, studies delineating the metabolic interconversion of ornithine, arginine, proline and glutamic acid were completed.

Although the occurrence of the biological formation of urea had been known since 1874 (52), the steps involved in its biosynthesis were not established until many years thereafter. Richet was the first to observe the existence of urea-producing ferment in the mammalian liver in 1894 (53). In 1904, Kossel and Dakin showed that the formation of urea from arginine was due to the action of a specific tissue ferment which they named arginase (54). In 1914, Clementi proposed the well verified rule "that arginase occurs in the livers of vertebrates that have ureotelic metabolism, but not in the livers of animals that have a uricotelic metabolism" (53). Mammalian liver has been described as the best source of arginase. The mammary gland has been found to be second in arginase content, followed by the testes and kidney; traces have been found in all body fluids and tissues of mammals (53).

Experiments by Dounce et al. (55, 56) showed that arginase is found in both the nuclei and cytoplasm of mammalian liver cells. Other characterization of arginase has been done in Greenberg's laboratory (57). Krebs and Henseleit included arginase as a component of their ornithine cycle in 1932 (58), thereby showing the physiological significance of its presence in the liver. However, as of 1952, Greenberg wrote "the physiological significance of arginase found in other tissues, e.g., kidney and testes, is as yet unknown" (53). The
presence of arginase in extra-hepatic mammalian tissue would suggest another physiological role besides that in the ornithine cycle. A possible role could well be that of providing ornithine for the conversion to putrescine for polyamine biosynthesis (59). This will be discussed later in this section.

The ornithine cycle proposed by Krebs and Henseleit (58) and mentioned briefly above, was a three reaction sequence which has been expanded and is now more commonly known as the urea cycle. They described the series of reactions as follows (58):

I.  
\[ \text{R-CH}_2\text{NH}_2 + \text{CO}_2 + \text{NH}_3 = \text{R-CH}_2\text{NH-CO-NH}_2 + \text{H}_2\text{O} \]
Ornithin + kohlensäure + Ammoniak = Citrullin + Wasser

II.  
\[ \text{R-CH}_2\text{NH-CO-NH}_2 + \text{NH}_3 = \text{RCH}_2\text{NH.C(NH)NH}_2 + \text{H}_2\text{O} \]
Citrullin + Ammoniak = Arginin + Wasser

III.  
\[ \text{R'CH}_2\text{NH'C(NH)NH}_2 + \text{H}_2\text{O} = \text{R'CH}_2\text{NH}_2 + \text{CO(NH}_2)_2 \]
Arginin + Wasser = Ornithin + Harnstoff

In the experiments leading to their proposed cycle, Krebs and Henseleit used liver slices after the earlier work by Warburg suggested the idea that sliced material might "lend itself to the study of tissue metabolism and its intermediary stages, including synthetic processes" (52). They also reported that in their investigation with rat tissues, the liver was the only organ in which their method of urea synthesis was found. The work of Gornall and Hunter (60) helped to confirm the hypothesis of Krebs and Henseleit. They reported in 1943, (a) that citrulline produces the same kind of catalytic effect as ornithine,
and (b) that when ornithine acts as a catalyst, it actually does form citrulline.

By the late 1940's, enough knowledge had been accumulated to enable urea to be synthesized in liver homogenates (52). Synthesis of citrulline from ornithine in liver homogenates was first observed by Borsook and Dubnoff (61). Later work by Cohen and co-workers (62, 63, 64, 65, 66, 67) attempted to further characterize the conversion of ornithine to citrulline. It was not until after carbamyl phosphate had been synthesized by Jones et al. (68) that Cohen's laboratory identified carbamyl phosphate as an intermediate compound for the synthesis of citrulline (69, 70).

Finally, in 1957, Burnett and Cohen (71) reported the partial purification from beef liver of the enzyme catalyzing the transfer of carbamyl phosphate onto ornithine and named it carbamyl phosphate-ornithine transcarbamylase. At nearly the same time, Reichard (72) purified the same enzyme from rat liver which he named ornithine carbamyl transferase and stated that it was identical to the citrulline phosphorylase described by Krebs (73). Since that time, ornithine transcarbamylase activity has been found in the livers of all ureotelic vertebrates (74), but is absent in the livers of birds and most fish (75). It has not been detected in any mammalian tissue other than liver (74). According to available evidence, this enzyme appears to be mitochondrial in its subcellular location (76). The importance of ornithine transcarbamylase as described in this present study is not its role in the urea cycle but its involvement with the utilization of ornithine as a substrate.
The interconversion of ornithine to glutamic acid and proline as demonstrated by isotope techniques has been mentioned earlier. With the discovery of the transamination reaction by Braunstein and Kitzmann in 1937 (77), a mechanism for the interconversion was made apparent. More specific evidence for the participation of the individual amino acids, including ornithine, in the transamination reaction was furnished by Cammarata and Cohen (78). Utilizing the $^{15}$N isotope, Stetten (79) proposed that ornithine, glutamic acid and proline were metabolically linked through the $\delta$-transamination of ornithine with the possible intermediates of glutamic $\gamma$-semialdehyde and its spontaneous cyclization product, $\Delta^1$-pyrroline-5-carboxylic acid. The presence of ornithine $\delta$-transaminase (OTA) activity in rat liver homogenates was shown by Quastel and Witty (80) in 1951, who suggested that the enzyme "may occupy a significant place in hepatic amino acid metabolism". In 1954, Meister (81) reported further work on OTA activity and provided evidence for the formation of the intermediate glutamic $\gamma$-semialdehyde. In 1963, Peraino and Pitot (82) partially purified the enzyme from various rat tissues and reported some general properties of OTA which was determined to be localized in the mitochondria. In the reaction catalyzed by this enzyme the formation of the products, glutamic $\gamma$-semialdehyde and glutamate is favored, probably because of the spontaneous conversion of the glutamic $\gamma$-semialdehyde to $\Delta^1$-pyrroline-5-carboxylic acid (83). Although Katunuma et al. (84) states that there is a requirement for pyridoxal phosphate, Strecker's (83) work demonstrates that exogenously added pyridoxal phosphate is needed to reactivate the enzyme after periods
of storage, but is not clearly needed to demonstrate enzyme activity in fresh enzyme preparations when assayed immediately after preparation. Since Raina (85) was unable to show any radioactivity in polyamines after administering $^{14}$C-labeled proline in chick embryos and Jänne and Raina (86) had similar results after injecting $^{14}$C-glutamate in normal and partially hepatectomized rats, OTA apparently does not play a direct role in shunting ornithine into the polyamine biosynthetic pathway. Therefore, if OTA were to play a regulatory role in ornithine metabolism, it would act by competing with ornithine transcarbamylase and ornithine decarboxylase for the available ornithine as its substrate (59).

Ornithine decarboxylase is the most recent of the mammalian enzymes utilizing ornithine to be studied. Initial investigations of this enzyme were carried out in bacterial systems. It has been recognized for some time that putrescine could be formed as a product of "putrefaction" of ornithine (41, 44). However, prior to the work of Gale (87) in 1940, little information concerning the actual process, organism or enzymes involved had been reported. Gale (88) studied the activities of the different decarboxylases in _E. coli_ and reported their requirement for pyridoxal phosphate as a coenzyme. The ornithine decarboxylase activity studied by Gale was found to be an induced enzyme, requiring specialized culture medium for its presence (89). In 1965, Morris and Pardee (90) reported the activity of a constitutive ornithine decarboxylase which they refer to as the "biosynthetic enzyme". This "biosynthetic" ornithine decarboxylase activity would account for the production of putrescine normally found in _E. coli_. 
They state that the induced ornithine decarboxylase activity studied by Gale probably served a catabolic function. Morris and Pardee (91) later showed that in *E. coli* there were two routes of synthesizing putrescine. In addition to the decarboxylation of ornithine to form putrescine directly, *E. coli* also has an enzyme system capable of decarboxylating arginine to form agmatine. Then agmatine is acted on by agmatine ureohydrolase to form putrescine and urea.

Although there have been reports of diamines, including putrescine in urine of cystinuric patients from as far back as 1893 (92), most observers considered that the diamines were bacterial degradative products. During the same period, the formation of putrescine and CO₂ from ornithine was reported, but investigators considered it an "alimentary" diaminuria (41, 93) in which the ingested amino acids did not undergo complete oxidative destruction. In 1900, Simon (94) reported on the topic of cystinuria and diaminuria in an article which has apparently been overlooked. He considered that the formation of diamines in body tissues in the absence of putrefactive organisms was very probable. In support of this statement, he observed that intestinal antisepsis did not alter the excretion of the diamines. The significance of Simon's work is dramatized by the fact that in 1965, Meister reported that "the decarboxylation of ornithine to putrescine is catalyzed by bacterial preparations and no evidence has yet been obtained for the occurrence of this reaction in animal tissues" (95).

The earliest report of biosynthesis of diamines and polyamines by animal tissues was an abstract by Tabor *et al.* (96) in 1956, indicating a small incorporation of labeled putrescine into the
polyamines, predominantly spermidine, of minced rat prostate. No incorporation was observed using minced liver, muscle, spleen, or kidney. In a review of the topic of polyamines in 1964, Tabor and Tabor (97) state "no evidence for the biosynthesis of polyamine in mammals has been reported, except for a preliminary report", the latter clause referring to the above abstract (96). They state that the presence of polyamines in most animal tissues is not necessarily indicative of their biosynthesis in those tissues since the compounds could be derived from the diet or intestinal flora.

Although increased incorporation of intraperitoneally injected $^{14}$C-ornithine into hepatic putrescine and spermidine after partial hepatectomy in rats was reported by Jänne (98), the initial assay for ornithine decarboxylase activity from animal systems was done in a suspension of Ehrlich ascites cells utilizing $^{14}$C-1-ornithine as the substrate and trapping the evolved $^{14}$CO$_2$ (99). Putrescine synthesis in a cell-free preparation of mammalian tissue was not described until Pegg and Williams-Ashman (100) reported their work on the activity of rat prostate ornithine decarboxylase (ODC) in 1968. This prostatic ODC was found to be a soluble enzyme with an absolute requirement for pyridoxal phosphate as found in the bacterial systems described above.

Immediately after the report of prostatic ODC activity, Jänne and Raina (101) reported the presence of ODC activity in rat liver and demonstrated an increased activity in regenerating liver following partial hepatectomy of rats. In a later work (102) they partially purified and further characterized ODC from regenerating rat liver, finding a pH optimum of 7.4 and activation by mercaptoethanol,
suggesting the importance of SH groups for activity. More recently, the presence of thiol compounds in both the homogenization and assay media has been used to produce greater ODC activity due to increased stability of the enzyme following purification and storage (103).

Shrock et al. (104) reported that although mercaptoethanol has been shown to stimulate the activity of partially purified ODC preparations, it had no effect on their fresh enzyme preparations. The ODC activity reported in most of the studies in the current literature were determined by assay procedures in which thiol additives to both the homogenization and assay media were not used; although for optimal activity either dithiothreitol or mercaptoethanol should be added (89) and probably will be used in future investigations involving this enzyme.

Russell and Snyder (105), utilizing either puromycin or cycloheximid to inhibit protein synthesis, showed that ornithine decarboxylase had a half-life of about 11 minutes in both normal and regenerating rat liver. This extremely short half-life appears to be the shortest half-life reported for any mammalian enzyme. In 1972, Friedman et al. (106) reported a purification procedure for ODC from regenerating rat liver following partial hepatectomy and stated that they had prepared an antibody against the enzyme which gave a single precipitin line on Ouchterlony plates. If this procedure becomes accepted, it will allow a new approach for investigating the mechanism behind the rapid changes in the level of ODC following various stimuli. The importance of ODC can be inferred from the fact that polyamines have been found in all procaryotic and eucaryotic cells (107). Although its activity is almost non-detectable in static or non-growing tissues, ODC appears
to catalyze the only route for putrescine synthesis in animal tissues (108).

Although arginine-glycine amidotransferase activity is found in primate liver, kidney, and pancreas, it is absent from liver tissue of rats, rabbits and dogs (109) and will not be discussed further in this study.

**Polyamine Metabolism**

Although reference has been made to polyamines during the discussion of ornithine decarboxylase, an additional section has been devoted to this topic because of their importance to the changes observed in ornithine metabolism in the present study.

The polyamines, spermidine and spermine, are non-protein, aliphatic, nitrogenous bases which along with their diamine precursor, putrescine, have been known as normal tissue constituents for many years. It has only been within the last twenty years that the distribution of these compounds has been known to extend to all living organisms (110). In the remaining sections of this study the term "polyamines" will designate all three of these amines, putrescine, spermidine and spermine.

The first report of the occurrence of a polyamine in animal tissue can be found in a letter by Anton van Leeuwenhoek to the Royal Society in 1677 of which a portion of the text is reprinted in a review by Tabor and Tabor (97). Leeuwenhoek described the crystals that he observed in human semen that had been standing for some time. These were presumably spermine phosphate although several names have been applied to them including "Böttcher's crystals," "Charcot-Leyden
crystals," and "Charcot-Robin crystals," until the term "spermine" was first used in 1888 (97). Putrescine was first found during the late 1800's in decomposing animal material (97) and in urine of cystinuric patients (92). Spermidine was discovered and identified by Dudley and co-workers in 1927 (111).

The distribution of spermine in various mammalian organisms was later demonstrated by Dudley and Rosenheim (112). Ox pancreas was found to have 25-30 mg spermine phosphate/100 g fresh tissue while the liver, kidney and spleen had lesser quantities. The richest source of spermine that they noted was human semen (260 mg spermine phosphate/100 g semen), yet none of this base was found in bull semen. Harrison (113) ascertained that the spermine in human semen was derived from the prostate gland which contained 130 mg spermine phosphate/100 g tissue. The wide range of distribution of spermine and spermidine in smaller laboratory animals was studied later by Rosenthal and Tabor (114). Although found in all tissues, putrescine, the main aliphatic amine in bacteria and bacterial viruses, was found to be in very low concentrations in fresh animal tissues when compared to spermidine and spermine levels (98). Elucidation of the intracellular distribution of the polyamines has presented certain technical difficulties because of the high affinity of these polycations for nucleic acids and phospholipids leading to a secondary redistribution during and after tissue homogenization (85).

The physiological importance of the polyamines was first demonstrated by Herbst and Snell (115) who showed that they were required for growth of certain microorganisms. Polyamines are known to exert
a stabilizing effect on certain bacteria, protoplasts and bacterial cell walls and on mitochondrial preparations, probably due to their high affinity for cellular constituents with acidic groups (85). Cohen and Lichtenstein (116) using radioactively labeled polyamines, showed that polyamines were attached to ribosomes prior to cell disruption and not absorbed onto ribosomes in significant amounts after the formation of the extract from E. coli. The complexes formed by polyamines with nucleic acids have been shown to be stable over a wide pH range and to protect the nucleic acids against denaturation and enzymatic destruction (117); spermine, itself, has been shown to inhibit ribonuclease in yeast cells (118). In addition, polyamines are considered to function similarly to the divalent cation, $\text{Mg}^{++}$, in vitro and in vivo and have been shown to stimulate in vitro incorporation of amino acids into ribonucleoprotein particles (119) and adenine nucleotides into RNA (120).

The association of the polyamines with growth processes is an interesting feature of their presumed cellular roles. Polyamine concentration patterns were shown to parallel the concentration patterns of nucleic acids at each step of the development of the chick embryo (121). Jänne et al. (122), who studied the concentration of polyamines in various rat tissues in relation to age, generalized that levels of polyamines decrease with age after attaining maximal levels during fetal development. In recent years, changes in the concentrations of polyamines and of RNA, as well as the activation of the biosynthesis of these compounds have been shown to parallel one another in a variety of physiologic and experimental states (110).
The metabolism of the polyamines in animal tissues is currently being investigated in several laboratories. Raina (85, 123) demonstrated the incorporation of putrescine, ornithine and methionine into spermidine and spermine in the developing chick embryo; similar results were found for bacteria. Putrescine and methionine have been shown to be precursors of spermidine and spermine in rat tissues (124). The conversion of spermine to spermidine has been reported also (125). Besides being a precursor for spermidine and spermine, putrescine has demonstrable regulatory effects upon the polyamine biosynthetic pathway (108). Its increased synthesis is the first indication that a tissue will undergo renewed growth processes. Although some workers have reported that three separate enzyme activities can be isolated (S-adenosyl-L-methionine decarboxylase, spermidine and spermine synthetases), the general consensus is that in mammalian systems, a single enzyme may be responsible for the metabolic steps of decarboxylation of S-adenosyl-methionine, transfer of the propylamino moiety to putrescine to form spermidine and the transfer of a second propylamino group to spermidine to form the symmetrical molecule, spermine (108).

With the newer and more rapid automatic technique for polyamine determination using elution chromatography coupled with the report that patients with diagnosed cancer excrete elevated levels of polyamines in urine (126), it is hoped that the question of whether polyamines may be useful biochemical markers of undetected neoplasms will be answered soon. Furthermore, monitoring the urinary polyamine levels in cancer patients may provide objective criteria to indicate the value of chemotherapeutic and other treatment modalities being employed in the control
of cancer in the specific patient (108).
MATERIALS AND METHODS

Materials

Hydrazine (95\%\,\textsuperscript{+}) was purchased from Eastman Kodak Co., Rochester, N. Y., and 0.4 ml was diluted to 10.0 ml with distilled water on each day of use. Cycloheximide was purchased from Sigma Chemical Co., St. Louis, Mo., and dissolved in 0.9\% saline to a final concentration of 16.7 mg/ml. Actinomycin D, obtained as a gift of Merck Sharp and Dohme Research Labs, Rahway, N. J., was dissolved in a minimal amount of ethanol and diluted to a final concentration of 0.4 mg/ml with 0.9\% saline.

D, L-(\textsuperscript{14}C) ornithine hydrochloride (specific activity 2.74 mCi/mmole), D, L-(\textsuperscript{14}C) phenylalanine hydrochloride (specific activity 2.07 mCi/mmole), putrescine-1, 4-\textsuperscript{14}C dihydrochloride (specific activity 20.94 mCi/mmole), spermidine-\textsuperscript{14}C [(aminopropyl)-tetramethylene -1,4-\textsuperscript{14}C diamine] trihydrochloride (specific activity 9.82 mCi/mmole) and spermine-\textsuperscript{14}C (tetramethylene -1,4-\textsuperscript{14}C) tetrahydrochloride (specific activity 18.75 mCi/mmole were purchased from New England Nuclear Corporation, Boston, Mass. D, L-(\textsuperscript{14}C) ornithine hydrochloride (specific activity 15-37 mCi/mmole) was obtained from Amersham/Searle Corporation, Arlington Heights, Ill. Ornithine and putrescine were tested for radiochemical purity by ascending paper chromatography on Whatman No. 1 paper using a n-butanol: pyridine: acetic acid: water (15:11:3:7 v/v/v/v) solvent system. The radiochemical purity of the phenylalanine was checked by ascending paper chromatography on Whatman No. 1 paper using a n-butanol: acetic acid: water
(25:4:10 v/v/v) solvent system. L-ornithine, L-phenylalanine, putrescine, spermidine and spermine, purchased from Calbiochem, La Jolla, Calif., were used in preparation of the final radioisotopic solutions used in the experiments described below. The radioisotopic solutions used in the ornithine and phenylalanine decarboxylase experiments were made with 0.001 N HCl, while all others were made with deionized distilled water.

Ethylene glycol monomethyl ether and monoethanolamine were obtained from Fisher Scientific Company, Fair Lawn, N. J.

Crystalline bovine serum albumin, L-arginine, L-citrulline, L-ornithine (for ornithine transcarbamylase and ornithine keto-transaminase studies), α-ketoglutarate, pyridoxal phosphate, dilitium carbamyl phosphate, glycylglycine (free base), diacetylmonoxime, α-isonitrosopropiophenone, and o-aminobenzaldehyde were purchased from Sigma Chemical Co., St. Louis, Mo. The bovine serum albumin was made up to 10 mg/ml with distilled water and used as the standard in the determination of protein concentrations. A 500 ml stock solution of α-ketoglutarate (0.2 M) was prepared and approximately 20 ml aliquots were frozen until used for the ornithine keto-transaminase assay. Pyridoxal phosphate solutions were freshly prepared each week and stored in a container protected from light. Dilitium carbamyl phosphate (0.1 M) was prepared immediately before beginning the ornithine transcarbamylase assay. α-Isonitrosopropiophenone (0.3 g/10 ml 95% ethanol) was freshly made on each day that arginase activity was determined. The o-aminobenzaldehyde, stored at -70°C, was diluted to a concentration of 25 mg/ml with 40% ethanol and swirled in warm
water (37°C) for 30 min to aid in the dissolution of the compound.

The glutamic acid γ-semialdehyde used as a standard in the ornithine keto-transaminase assay was the generous gift of Dr. Annette Baich, Southern Illinois University at Edwardsville, Edwardsville, Ill.

Urea (ultra, ultra pure), yeast RNA and salmon sperm DNA were obtained from Mann Research Laboratories, New York, N. Y. An accurately weighed sample of approximately 20 mg of yeast RNA was dissolved and diluted to 100.0 ml with deionized distilled water and used as the RNA standard. The DNA standard was prepared from salmon sperm DNA by dissolving a weighed sample of approximately 20 mg and diluting to 100.0 ml with 0.01 N NaOH.

The scintillation cocktail used in the experiments was either Bray's scintillation fluid (127) or a toluene phosphor as reported by Russell and Snyder (128). Each liter of Bray's scintillation fluid contained 4.0 g of PPO (2, 5-diphenyloxazole), 0.2 g POPOP (p-bis [2-(5-phenyloxazolyl)]-benzene), 60.0 g naphthalene, 100 ml absolute methanol and 20 ml ethylene glycol (all purchased from Baker Chemical Co., Phillipsburg, N. J.) made up to volume with p-dioxane (purchased from Fisher Scientific Co., Fair Lawn, N. J.). The toluene phosphor was prepared by using 40 ml of toluene liquifluor (containing 4.0 g PPO and 0.05 g POPOP per 40 ml toluene solution) and 200 ml absolute ethanol and diluting to 1 liter with toluene (purchased from Fisher Scientific Co., Fair Lawn, N. J.).

General Treatment of Animals

For each experiment described below, adult male albino rats
(Holtzman Co., Madison, Wisconsin) were paired by weight, kept in a temperature controlled room (68-82°F) with alternating periods of illumination (twelve hours on, 7:00 a.m. - 7:00 p.m.; twelve hours off, 7:00 p.m. - 7:00 a.m.) and fed Purina Laboratory Chow and water ad libitum until the start of the experiments.

**Experimental Treatment of Animals**

a.) Administration of hydrazine

Freshly prepared hydrazine solution (40 mg/ml), brought to a pH of 7.4 by 100% CO₂, was injected intraperitoneally in a dose (1 ml/kg) equivalent to approximately 64% of the LD₅₀ (129). Control animals were given injections of 0.9% saline (1.0 ml/kg) intraperitoneally. All animals were fasted from the time of injection until sacrifice. Water was available ad libitum at all times.

b.) Administration of inhibitors of protein and nucleic acid synthesis

Some of the animals, after receiving either hydrazine or saline, were given a second intraperitoneal injection on the opposite side of the abdomen of either cycloheximide (50 mg/kg) (105) or Actinomycin D (0.8 mg/kg) (130).

c.) Partial Hepatectomy

Approximately 67% partial hepatectomy was performed under ether anesthesia by the method of Higgins and Anderson (131), leaving the caudate and right lateral lobes of the liver intact. Sham-operated controls underwent a similar procedure except for excision of tissue. All animals recovered from anesthesia within minutes of closing the abdominal wound. They were allowed food and water ad libitum until
sacrificed.

The livers from the animals receiving either hydrazine or saline were used in all the procedures described below. The livers from the animals which underwent partial hepatectomy and their sham-operated controls were used only in the determination of ornithine decarboxylase activity and endogenous free amino acid levels. All time-course experiments were begun at the same time of day.

**Ornithine Decarboxylase Assay**

Using carboxyl-labeled ornithine as the substrate, ornithine decarboxylase activity was assayed by a modification of the method of Russell and Snyder (128) in which liberated $^{14}\text{CO}_2$ was trapped and quantitated. Animals were sacrificed by decapitation at designated times after the start of the experiment, and their livers were excised and placed in iced 0.05 M sodium-potassium phosphate buffer, pH 7.2. Portions of the right lateral lobe of the liver were homogenized in 5.0 volumes of the cold phosphate buffer containing 1 mM mercaptoethanol and 50 μM pyridoxal phosphate, using a Teflon-glass Potter Elvehjem type homogenizer at 0-4°C. The homogenates were centrifuged at a relative centrifugal force of 20,000 x g for 20 min at 0-4°C using a Sorval RC-2B refrigerated centrifuge equipped with a # SS-34 head. The supernatant was separated using a Pasteur pipet and kept on ice until the start of the assay. Incubations were carried out in 25 ml Erlenmeyer flasks, each equipped with a rubber stopper supporting a polyethylene center well containing 0.20 ml of a 2:1 mixture of monoethanolamine and ethylene glycol monomethyl ether. Incubation mixtures contained 0.15 μmoles pyridoxal phosphate,
0.10 μmole L-[1-\textsuperscript{14}C] ornithine (0.543 μCi of D, L-ornithine), enzyme preparation as indicated in the respective experiments, and 9.75 mmol sodium-potassium phosphate buffer (pH 7.2) in a final volume of 2.0 ml. The reaction was started by the addition of substrate following a preincubation for 10 min at 37° C of all other reaction mixture components in a Dubnoff metabolic shaker set at approximately 100 cycles per min. After 4 or 8 min, depending on the experiment, the reaction was stopped by the injection of 1.0 ml of 2.0 M citric acid into the reaction mixture, making sure the acid mixed adequately with the contents of the flask. The reaction vessels were then agitated for 30 min at room temperature to allow complete absorption of the liberated \textsuperscript{14}CO\textsubscript{2} from the acidic medium. The center wells were removed and placed directly into vials containing 10 ml of the toluene scintillation cocktail. Each vial was counted for 10 min in a liquid scintillation system (Nuclear Chicago, Mark I) and the radioactivity measurements were corrected for quenching by using an external standard. Duplicate assays were run in all cases. Blank samples were run by including reaction flasks containing either boiled enzyme preparations (placed in boiling water bath for 15 min) or water in place of the active enzyme preparation.

**Phenylalanine Decarboxylase Assay**

Phenylalanine decarboxylase activity was assayed by the same procedure used for the ornithine decarboxylase determination except for the substitution of 0.10 μmole L-[\textsuperscript{14}C] phenylalanine (0.5 μCi D, L-phenylalanine) as substrate.
Ornithine Transcarbamylase Assay

Ornithine transcarbamylase activity was assayed by following the formation of the end-product, citrulline, as described by Weber, et al. (132). In these experiments, the animals were sacrificed by decapitation at various times after experimental treatment, and the livers were excised and placed on ice. A 10% (w/v) homogenate was prepared in 0.9% KCl and the ornithine transcarbamylase was assayed using a 1:50 dilution of this homogenate. The assay conditions, as described by Jones, et al. (133), were followed. The assay mixture contained 0.15 ml of a 1:1:1: mixture of 0.05 M glycglycine buffer (pH 7.4), 0.10 M dilithium carbamyl phosphate and 0.10 M L-ornithine. The reaction vessels (12 ml conical centrifuge tubes) were allowed to equilibrate at 37°C in a Dubnoff metabolic shaker set at approximately 120 cycles per min. The reactions were initiated by the addition of 0.35 ml of the 1:50 dilution of homogenate. Reactions were stopped after the desired length of incubation by adding 1.0 ml of 5% trichloroacetic acid and placing the tubes on ice. Subsequently, the supernatant fluid from the reaction mixtures was separated by centrifugation at a relative centrifugal force of 1500 x g (3000 rpm) in a refrigerated International PR-2 centrifuge equipped with a # 811a head; 0.5 ml samples were pipetted into culture tubes (16 x 125 mm). The citrulline present was determined by a modification (132) of the method of Archibald (134). To each culture tube 1.5 ml water, 1.0 ml sulfuric acid-potassium phosphate solution (conc. sulfuric acid: 1.0 M potassium phosphate (monobasic) mixed in a ratio of 1:3) and 0.125 ml of a 3% aqueous solution of diacetylmonoxime were added and then mixed well. The tubes
were capped with marbles and heated in the dark in a boiling water bath for 10 min. Then, they were cooled to room temperature and kept dark until the absorbance of the clear red colored solutions was read at 490 nm in a Beckman DU spectrophotometer. The results obtained in each reaction were within the range of the standard curve prepared at the time of each assay using L-citrulline as a standard.

All determinations were done in triplicate. Blank samples were run by replacing either substrate or enzyme with water. Zero times were determined by the addition of the 1.0 ml of trichloroacetic acid before the addition of the enzyme preparation.

**Ornithine δ-Transaminase Assay**

Ornithine δ-transaminase was assayed according to the method of Peraino and Pitot (82). The animals were sacrificed by decapitation. Their livers were excised, placed on crushed ice, and weighed. Portions of the median lobe of the liver were homogenized in 4.0 volumes of 0.10 M potassium phosphate buffer (pH 8.0) containing 0.01 M mercaptoethanol using a Teflon-glass Potter Elvehjem type homogenizer surrounded with crushed ice. The reaction mixture contained 2.0 ml of 0.375 M potassium phosphate buffer (pH 7.4), 0.3 ml of 1.0 M L-ornithine, 0.3 ml of 0.2 M α-ketoglutarate, 0.20 ml of 20% (w/v) homogenate, 0.1 ml of 0.01 M o-aminobenzaldehyde in 40% ethanol, and 0.10 ml distilled water. The reaction vessels (16 x 125 mm culture tubes) containing the buffer, ornithine and distilled water were preincubated at 37°C for 5 min in a Dubnoff metabolic shaker set at approximately 120 cycles per min. Then, the reaction was started by adding the
enzyme preparation followed by the o-aminobenzaldehyde, and
\( \alpha \)-ketoglutarate at 20 sec intervals. Incubations were carried out
for 30 min at 37°C, after which the reactions were stopped by the
addition of 2.0 ml of 10% trichloroacetic acid. The reaction vessels
were allowed to stand at room temperature for 30 min before centrifuging
at 1500 x g for 10 min in a refrigerated International PR-2 centrifuge
equipped with a # 811a head. The absorbance of the clear yellow
supernatant fluid was determined at 440 nm against a distilled water
blank in a Beckman DU spectrophotometer. Blank samples were run by
substituting water for either the homogenate, ornithine, \( \alpha \)-ketogluta-
rate or both ornithine and \( \alpha \)-ketoglutarate. Zero times were determined
by the addition of the trichloroacetic acid before the above listed
components of the reaction mixture.

**Arginase Assay**

Arginase activity was assayed by following the formation of urea
as reported by Van Slyke and Archibald (135) and modified by Bond (136).
Animals were sacrificed by decapitation at the desired time after experi-
mental treatment. Their livers were perfused with cold isotonic saline
through cannulation of the abdominal aorta followed by the severing of
the inferior vena cava. Livers were excised, placed on crushed ice,
blotted, and rapidly weighed. Portions of the median lobe of the liver
were minced with scissors and 25% (w/v) homogenates were prepared in
0.15 M KCl with a Potter Elvehjem type tissue grinder. To remove cell
debri s, homogenates were centrifuged at a relative centrifugal force
of 750 x g for 10 min at 0-4°C using the Sorvall RC-2B refrigerated
centrifuge equipped with a # SS-34 head. Supernatants were decanted and recentrifuged at 48000 x g for 80 min. The supernatant fluid was separated using Pasteur pipets, was adjusted to pH 7.0 with 0.5 N NaOH, and was either frozen for subsequent assay or kept on ice until the time of assay (if used the same day). The reaction was carried out at room temperature (about 20°C) and consisted of the addition of 0.08 ml of the crude arginase preparations to 1.5 ml of 0.283 M arginine, pH 9.5 (adjusted with 50% NaOH). Usually a 0.2 ml sample of the reaction mixture was taken at 3.0 min and added to 4.80 ml of a sulfuric acid-phosphoric acid solution (90 ml conc. H₂SO₄ and 270 ml conc. H₃PO₄ diluted to 1000 ml with water) and mixed well using a Vortex mixer. Determination of the quantity of urea formed was accomplished by the addition of 0.25 ml of α-isonitroso-propiophenone (3% in 95% ethanol) to the solution containing the aliquot from the reaction mixture. The solutions were mixed well, incubated for 60 min at 99± 1°C in a forced-draft oven, cooled to room temperature by placing in a water bath, and the clear red colored solution read at 540 nm on a Beckman DU spectrophotometer. A stock solution of 4.0 mM urea was kept refrigerated. On the day of the experiment, one volume was diluted with nine volumes of the sulfuric acid-phosphoric acid solution and was used to make the standard curve. All experimental values fell within the linear range of the standard curve. Blank samples were run by replacing either the active enzyme preparation or the substrate with water. All determinations were done in duplicate.
**Determination of Protein Concentration**

The protein concentration of the different enzyme preparations was determined by a biuret method (137). A sample of the final enzyme preparation was diluted to a final volume of 1.0 ml with distilled water; 4.0 ml of biuret reagent was added. The contents of the tubes were mixed; the color was allowed to develop for 30 min at room temperature; and the clear blue color was read against a distilled water blank at 550 nm in a Spectronic 20 colorimeter. The protein concentration was determined from a standard curve constructed at the time of each assay, using bovine serum albumin. The values obtained for all samples fell within the linear portion of the standard curve.

**Determination of Putrescine, Spermidine, and Spermine in Hepatic Tissue**

Solutions for polyamine determinations were prepared essentially as described by Kremzner (138). The animals were sacrificed by decapitation and the livers were perfused with cold isotonic saline as described in the method for arginase determination. Portions of the median lobe of the liver were homogenized in one volume of distilled water. Equal volumes of the homogenate and cold 0.8 N perchloric acid were mixed well and set in ice for 30 min with several additional periods of mixing. The mixtures were centrifuged at a relative centrifugal force of 3000 x g for 15 min in a Sorvall RC-2B refrigerated centrifuge equipped with a # SS-34 head. The supernatant fluid was removed with a Pasteur pipet and used directly for polyamine determination by elution chromatography on a Beckman model 116 automatic amino acid analyzer. A slight modification, described below, of the method of Marton et al.
(139, 140) was used for the analysis. A 0.9 x 23 cm column was packed to a height of 5 cm with Beckman PA 35 custom-made spherical resin. A Teflon filter disc was placed on top of the resin bed to act as a filter and to aid in the prevention of channeling and disruption of the column surface following sample application. A 1.0 ml aliquot of the solution prepared for polyamine analysis was applied onto the column and elution was carried out with two buffer systems. Buffer I (pH 5.06, 0.35 M sodium citrate, and 0.7 M with respect to sodium ion) was pumped for 45 min, during which time free amino acids were eluted. Elution of the polyamines was then carried out with Buffer II (pH 4.08, 0.35 M sodium citrate, and 2.35 M with respect to sodium ion) for an additional 160 min. A recording colorimeter reading at 570 and 440 nm was used to determine the absorbance of the product formed by the reaction of ninhydrin with the polyamines. The flow rate for the buffers was set at 50 ml per hr and for the ninhydrin at 25 ml per hr. Putrescine, spermidine, and spermine were eluted at approximately 70, 110, and 140 min respectively. The resin was regenerated with 0.2 N NaOH and reequilibrated with Buffer I to prepare the column for the next run. Quantitation was accomplished by the height-width method for integration of the area under the peaks (141). Putrescine, spermidine and spermine hydrochloride standards were recrystallized three times from ethanol (111, 142, 143, 144, 145). Recovery rates were calculated by adding $^{14}$C-polyamine to the tissue homogenizing media and determining the percent of the labeled polyamine recovered from the polyamine preparation.
Determination of Liver Protein, RNA and DNA Concentrations

A 0.4 ml sample of the 1:1 homogenate of perfused liver described above in the procedure for the determination of putrescine, spermidine and spermine, was placed in a 12 ml conical centrifuge tube and frozen until assayed for protein, RNA and DNA by the Munro and Fleck modification (146) of the procedure described by Wannemacher et al. (147). In the procedure used, centrifugation after each step was at a relative centrifugal force of 1500 x g for 10 min in a refrigerated International PR-2 centrifuge equipped with a # 811a head. Each sample of homogenate was thawed; 1.0 ml of cold 0.6 N perchloric acid (PCA) was added, mixed and allowed to stand in ice for 10 min. The precipitate was separated by centrifugation and was washed twice with 5.0 ml of cold 0.2 N PCA. Lipids were extracted and removed by washing the precipitate first with 5.0 ml of 3:1 ethanol-diethyl ether and, finally, with 5.0 ml of anhydrous diethyl ether. All of the lipid extraction washes were discarded, and the excess ether was removed by placing the tubes in a convection hood. Precautions were taken so that the precipitate did not become totally dry. The precipitate contains all of the protein and the nucleic acids, RNA and DNA. This precipitate was mixed with 4.0 ml of 0.3 N KOH (kept at 37°C), incubated at 37°C for 60 min, cooled in ice for 10 min and the precipitate separated by centrifugation. This type of alkaline treatment caused hydrolysis of the RNA, solubilized the protein, but left the DNA unhydrolyzed in solution. A 0.4 ml aliquot of this alkaline hydrolysate was used for protein determination by the biuret method described earlier. The remaining hydrolysate was acidified by addition of
2.0 ml of cold 1.2 N PCA, was allowed to stand in ice for 10 min and was then centrifuged. This procedure caused precipitation of the unhydrolyzed DNA, leaving the hydrolyzed ribonucleotides in the supernatant fluid. A 0.4 ml aliquot of this supernatant fluid was removed and placed in 10 ml dilution tubes for RNA analysis as described below. The remaining precipitate was washed twice with 5.0 ml of 0.2 N PCA and stored refrigerated (0-4°C) overnight in 5.0 ml of 95% ethanol. The ethanol was removed after centrifugation, 3.0 ml of cold 0.5 N PCA was added and the sample was incubated at 96±1°C for 45 min in a forced-draft oven. Solutions were refrigerated for 15 min, centrifuged and the supernatant fluid collected in 20 ml dilution tubes. The precipitate was washed twice with 2.0 ml of cold 0.5 N PCA and each resulting supernatant fluid was combined in the 20 ml dilution tubes. Distilled water was added to dilute to 20 ml and the DNA determined as described below.

RNA concentrations were determined by diluting the 0.4 ml aliquot from the acid soluble fraction of the alkaline digest of the lipid extracted homogenate to 10 ml with distilled water. A portion of the diluted RNA solution was read at 260 nm against a distilled water blank in a Beckman DU spectrophotometer. RNA concentrations of the homogenates were estimated from standard curves constructed each time, using yeast RNA as the standard. The values obtained for all tissue samples fell within the linear portion of the standard curve.

DNA concentration was determined on a sample of the diluted deoxyribonucleotide solution read at 265 and 290 nm against a distilled water blank in a Beckman DU spectrophotometer. The DNA concentrations
of the homogenates were calculated by taking the differences in absorbance between the two wavelengths and comparing them to those obtained from standard curves prepared each time using salmon sperm DNA as the standard. The values obtained for all samples fell within the linear portion of the standard curve.

**Determination of Dry Weights**

Duplicate 1.0 ml aliquots of the 1:1 liver homogenate, described in the determination of putrescine, spermidine, and spermine above, were pipetted into tin-foil weighing cups. These samples were dried in an oven at 105-115°C until a constant weight was achieved. The general duration of time was 36 hours.

**Determination of Endogenous Concentrations of Free Amino Acids**

Endogenous levels of free amino acids were determined in (a) the 20,000 x g supernatants described above for the ornithine, decarboxylase enzyme preparations, (b) the liver homogenates (16.7% w/v) obtained from the study involving liver regeneration following partial hepatectomy and (c) the liver homogenates obtained from the study determining putrescine, spermidine, and spermine contents. A protein-free filtrate (148) was prepared by placing a 2.0 ml portion of the above supernatant or homogenate in 4.0 ml of cold 9% sulfosalicylic acid and 0.2 ml of an internal standard containing L-norleucine and D, L plus allo-δ-hydroxylysine in 15 ml Corex centrifuge tubes. This mixture was placed in ice for 30 min and then centrifuged at a relative centrifugal force of 3000 x g for 20 min at 0-4°C using a Sorvall RC-2B refrigerated centrifuge equipped with a # SS-34 head.
The supernatant fluid was separated and frozen until the amino acid content was determined by elution chromatography on a Beckman Model 116 amino acid analyzer using a dual column system (149).

**Statistical Analysis of Data**

Arithmetic means of the resulting data were calculated. The Student's t test (150) was chosen to estimate significance probability of the difference between control and hydrazine-treated groups. The unbiased estimate \( s^2 \) of the variance \( \sigma^2 \) was calculated by the equation

\[
s^2 = \frac{\sum x^2 - (\sum x)^2 / N}{N - 1}
\]

where \( x \) = value of sample

\( N \) = number of samples

The standard deviation, \( \sigma \) was then estimated by taking the square root of \( s^2 \). The unbiased estimate of the standard deviation of the mean (standard error of the mean) was calculated using the value of the standard deviation, derived above, in the following equation:

\[
\text{Standard deviation of the mean, } \sigma_R = \frac{s}{\sqrt{N}}
\]

A level of \( p < 0.05 \) was the minimal significance probability accepted as indicating a difference between groups. All experimental curves were drawn by inspection.
RESULTS

Endogenous Levels of Ornithine Following Hydrazine Treatment

The levels of hepatic free ornithine determined in the protein-free filtrates prepared from the 20,000 x g supernatants described for the ornithine decarboxylase enzyme assay procedure are shown in Figure 2 as a function of time after hydrazine treatment. The animals used in this experiment varied in size between 124-162 grams initial body weight, and were comparable in size to those used by Banks (31) and Banks and Petterson (33) who reported increased hepatic ornithine pool sizes* at 24 and at 12 and 24 hr respectively, following the administration of hydrazine to rats, in two separate studies. In the present study, the points represent the mean of two saline or two hydrazine-treated animals at 1, 2, 4, 6, 12 and 24 hr following administration of the compound. The ornithine concentrations are expressed as μmoles per ml of supernatant in order to facilitate their use in estimating the amount of endogenous ornithine that was present in the reaction mixtures used in the assay of ornithine decarboxylase activity. In the present study, endogenous ornithine levels were elevated in the hydrazine-treated rats relative to their saline-injected control animals as early as 1 hr, with the maximally elevated

* The term "pool size" used in this thesis is meant to represent the total free amino acid concentration found in the protein-free filtrate and does not intend to imply a specific determination of the intracellular pool size.
ornithine concentration observed at 12 hr after treatment with the compound. The ornithine levels appear to remain elevated at 24 hr compared to control levels although, they seem to be returning toward control values. Fasting during this 24 hr period did not alter the concentration of ornithine since the levels in the control animals were unchanged throughout the period of the study. Although the data as reported in Figure 2 are the mean of only two animals, amino acid analyses of liver supernatants in subsequent experiments have shown a pattern consistent with this representative experiment.

A statistical comparison of the ornithine concentrations in the supernatants from rat liver at 4 and 24 hr following hydrazine treatment and in homogenates from regenerating liver at 16 hr following partial hepatectomy compared to their corresponding control groups is shown in Table I. From these data, it was apparent that the ornithine pool size was significantly greater (p < .0005) in the hydrazine-treated animal at both 4 and 24 hr time points than in the 4 and 24 hr control animal, respectively, regardless of whether the ornithine concentration is expressed on a gram wet weight of liver or mg of hepatic DNA basis. The ornithine concentrations from the 4 and 24 hr hydrazine-treated animals were significantly (p < .0005) different from each other; however, there was no change between the levels of ornithine in the 4 and 24 hr control groups. Regenerating liver 16 hr after partial hepatectomy showed a similar increase (p < .01) in ornithine levels when compared to those levels of ornithine found in liver from sham-operated animals. Ornithine levels in adult rats (greater than 250 g) have been shown to follow the same general pattern following hydrazine
Table I

Hepatic Pool Size of Endogenous Free Ornithine Following Experimental Treatment

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>Time After Treatment (hr)</th>
<th>Ornithine Concentration</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µmoles g liver (wet wt)</td>
<td>µmoles mg DNA</td>
<td></td>
</tr>
<tr>
<td>Saline Control</td>
<td>4</td>
<td>0.30 ± .01* (12)**</td>
<td>0.050 ± .004 (9)</td>
<td></td>
</tr>
<tr>
<td>Hydrazine</td>
<td>4</td>
<td>0.98 ± .04 (15)</td>
<td>0.219 ± .019 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0005</td>
<td>p &lt; .0005</td>
<td></td>
</tr>
<tr>
<td>Saline Control</td>
<td>24</td>
<td>0.34 ± .03 (7)</td>
<td>0.039 ± .004 (5)</td>
<td></td>
</tr>
<tr>
<td>Hydrazine</td>
<td>24</td>
<td>2.05 ± .22 (7)</td>
<td>0.454 ± .036 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0005</td>
<td>p &lt; .0005</td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>16</td>
<td>0.19 ± .01 (4)</td>
<td>0.056 ± .007 (4)</td>
<td></td>
</tr>
<tr>
<td>Partial Hepatectomy</td>
<td>16</td>
<td>0.41 ± .06 (4)</td>
<td>0.157 ± .031 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .01</td>
<td>p &lt; .01</td>
<td></td>
</tr>
</tbody>
</table>

* Standard error of the mean

** Number of animals

A single determination of the ornithine pool size was done for each animal.
treatment as observed in the young rats (124-162 g) reported here. The results of a representative study of the ornithine levels in adult animals are presented below (Table I).

Expression of hepatic concentration of cellular constituents on a DNA basis has often been used since Boivin et al. (151) demonstrated a constant average amount of DNA per nucleus of various animal tissues; Williams (152) showed that the quantity of DNA in normal liver was a direct function of the number of hepatocytes. Thus, DNA was used as another basis for expression of these results as well as others in order to assess alterations in the cell composition due to hydrazine treatment. As shown by Smith (34) and confirmed by data in a later section of the present study, total liver DNA content did not vary significantly over the 48 hr period following hydrazine treatment. Therefore, it is useful to observe the effect of hydrazine on the liver by comparing the concentrations of cellular constituents on a DNA basis in an attempt to describe the cellular changes produced by the compound.

Alterations of other free amino acid concentrations in liver homogenates from adult rats (370-430 g) are shown in Table II. These data from Table II can only be used to show general patterns of change since they represent a single analysis on pooled samples from four animals in each group. The most pronounced changes resulting from either hydrazine treatment or fasting alone over the 48 hr period investigated were noted for alanine, tyrosine, citrulline and ornithine. Hepatic alanine levels were elevated in the hydrazine-treated animals relative to the controls as early as 2 hr. This elevation was about
Table II

Time-course of Endogenous Free Amino Acid Pool Size

in Control and Hydrazine-treated Animals

A. Control Animals

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>12</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>5.42*</td>
<td>3.94</td>
<td>4.88</td>
<td>2.58</td>
<td>4.06</td>
<td>2.42</td>
</tr>
<tr>
<td>Amides</td>
<td>5.02</td>
<td>4.74</td>
<td>4.74</td>
<td>2.57</td>
<td>5.64</td>
<td>5.34</td>
</tr>
<tr>
<td>Ala</td>
<td>2.78</td>
<td>3.18</td>
<td>3.16</td>
<td>1.96</td>
<td>2.12</td>
<td>2.24</td>
</tr>
<tr>
<td>Gly</td>
<td>2.20</td>
<td>2.24</td>
<td>1.96</td>
<td>2.74</td>
<td>2.86</td>
<td>2.68</td>
</tr>
<tr>
<td>Glu</td>
<td>1.94</td>
<td>2.18</td>
<td>2.24</td>
<td>1.54</td>
<td>1.78</td>
<td>1.90</td>
</tr>
<tr>
<td>His</td>
<td>1.28</td>
<td>1.08</td>
<td>1.12</td>
<td>0.84</td>
<td>0.82</td>
<td>0.78</td>
</tr>
<tr>
<td>Lys</td>
<td>1.00</td>
<td>0.90</td>
<td>1.06</td>
<td>1.40</td>
<td>1.18</td>
<td>1.02</td>
</tr>
<tr>
<td>Ser</td>
<td>0.58</td>
<td>0.56</td>
<td>0.58</td>
<td>0.66</td>
<td>0.62</td>
<td>0.52</td>
</tr>
<tr>
<td>Thr</td>
<td>0.52</td>
<td>0.42</td>
<td>0.66</td>
<td>0.48</td>
<td>0.54</td>
<td>0.48</td>
</tr>
<tr>
<td>Leu</td>
<td>0.32</td>
<td>0.40</td>
<td>0.46</td>
<td>0.48</td>
<td>0.50</td>
<td>0.36</td>
</tr>
<tr>
<td>Val</td>
<td>0.22</td>
<td>0.26</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.28</td>
</tr>
<tr>
<td>Ile</td>
<td>0.18</td>
<td>0.22</td>
<td>0.24</td>
<td>0.24</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>Phe</td>
<td>0.14</td>
<td>0.16</td>
<td>0.20</td>
<td>0.20</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.14</td>
<td>0.16</td>
<td>0.18</td>
<td>0.20</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>Met</td>
<td>0.12</td>
<td>0.14</td>
<td>0.14</td>
<td>0.20</td>
<td>0.22</td>
<td>0.14</td>
</tr>
<tr>
<td>Tau</td>
<td>4.80</td>
<td>2.96</td>
<td>5.66</td>
<td>5.86</td>
<td>5.80</td>
<td>6.90</td>
</tr>
<tr>
<td>Orn</td>
<td>0.86</td>
<td>0.52</td>
<td>0.54</td>
<td>0.84</td>
<td>0.60</td>
<td>0.64</td>
</tr>
<tr>
<td>Cit</td>
<td>0.06</td>
<td>-</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>DNA</td>
<td>3.10**</td>
<td>4.13</td>
<td>3.51</td>
<td>4.86</td>
<td>5.31</td>
<td>5.41</td>
</tr>
</tbody>
</table>

* Concentration expressed as μ moles/g liver (wet wt)
** Concentration expressed as mg/g liver (wet wt)

Each value represents a single determination on a pooled sample from four animals.
Table II

Time-course of Endogenous Free Amino Acid Pool Size in Control and Hydrazine-treated Animals

### B. Hydrazine-treated Animals

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>12</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>5.42*</td>
<td>4.02</td>
<td>3.02</td>
<td>1.22</td>
<td>1.92</td>
<td>2.54</td>
</tr>
<tr>
<td>Amides</td>
<td>5.02</td>
<td>4.36</td>
<td>4.30</td>
<td>2.02</td>
<td>4.34</td>
<td>4.88</td>
</tr>
<tr>
<td>Ala</td>
<td>2.78</td>
<td>5.18</td>
<td>6.22</td>
<td>12.28</td>
<td>12.58</td>
<td>1.76</td>
</tr>
<tr>
<td>Gly</td>
<td>2.20</td>
<td>5.02</td>
<td>3.44</td>
<td>2.72</td>
<td>1.92</td>
<td>2.36</td>
</tr>
<tr>
<td>Glu</td>
<td>1.94</td>
<td>3.86</td>
<td>3.04</td>
<td>1.70</td>
<td>2.08</td>
<td>2.72</td>
</tr>
<tr>
<td>His</td>
<td>1.28</td>
<td>0.96</td>
<td>1.10</td>
<td>0.98</td>
<td>1.34</td>
<td>0.78</td>
</tr>
<tr>
<td>Lys</td>
<td>1.00</td>
<td>2.00</td>
<td>1.74</td>
<td>1.34</td>
<td>1.40</td>
<td>0.92</td>
</tr>
<tr>
<td>Ser</td>
<td>0.58</td>
<td>2.40</td>
<td>1.20</td>
<td>0.82</td>
<td>0.54</td>
<td>0.46</td>
</tr>
<tr>
<td>Thr</td>
<td>0.52</td>
<td>1.34</td>
<td>1.08</td>
<td>0.48</td>
<td>0.42</td>
<td>0.44</td>
</tr>
<tr>
<td>Leu</td>
<td>0.32</td>
<td>0.48</td>
<td>0.48</td>
<td>0.44</td>
<td>0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>Val</td>
<td>0.22</td>
<td>0.42</td>
<td>0.38</td>
<td>0.38</td>
<td>0.38</td>
<td>0.62</td>
</tr>
<tr>
<td>Ile</td>
<td>0.18</td>
<td>0.22</td>
<td>0.22</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Phe</td>
<td>0.14</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.14</td>
<td>0.30</td>
<td>0.68</td>
<td>0.84</td>
<td>0.60</td>
<td>0.26</td>
</tr>
<tr>
<td>Met</td>
<td>0.12</td>
<td>0.28</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
<td>0.20</td>
</tr>
<tr>
<td>Tau</td>
<td>4.80</td>
<td>5.12</td>
<td>2.42</td>
<td>1.46</td>
<td>2.82</td>
<td>6.16</td>
</tr>
<tr>
<td>Orn</td>
<td>0.86</td>
<td>1.92</td>
<td>1.72</td>
<td>3.36</td>
<td>5.38</td>
<td>1.12</td>
</tr>
<tr>
<td>Cit</td>
<td>0.06</td>
<td>3.30</td>
<td>4.00</td>
<td>5.04</td>
<td>3.30</td>
<td>0.36</td>
</tr>
<tr>
<td>DNA</td>
<td>3.10**</td>
<td>3.72</td>
<td>3.50</td>
<td>3.77</td>
<td>3.60</td>
<td>5.03</td>
</tr>
</tbody>
</table>

* Concentration expressed as μ moles/g liver (wet wt)

** Concentration expressed as mg/g liver (wet wt)

Each value represents a single determination on a pooled sample from four animals.
six-fold at 12 hr through 24 hr before returning to the level of the fasted control group at 48 hr. Tyrosine was increased about four-fold at 4 hr in the liver from the hydrazine-treated rats compared to the appropriate control animal, and then gradually decreased until at 48 hr it was approaching the value of the 48 hr control animal. Citrulline, which was found in very small concentration if at all in the analysis of the control liver during this 48 hr period, showed an early increase at 2 hr in the liver of hydrazine-treated animals. This increased level of citrulline was maintained through the 24 hr point, after which the value in the hydrazine-treated animal almost returned to that found in the liver of the controls at 48 hr. Similarly, ornithine was elevated at 2 hr in the hydrazine-treated rat relative to the level in the control animals and peaked at 24 hr in this study rather than at 12 hr as shown in Figure 2. This difference might be due to the high concentration of ornithine within the mitochondria which have been shown to concentrate ornithine several-fold (153). In the preparation of the 20,000 x g supernatants used for the analysis reported in Figure 2, the mitochondria are discarded as part of the sediment.

Ornithine Decarboxylase Studies

Since the changes in ornithine pool size as noted earlier occurred so rapidly, it appeared that there should be some demonstrable alterations in ornithine metabolism within the liver due to hydrazine treatment. In a preliminary study (154), increased hepatic ornithine decarboxylase (ODC) activity was observed at 4 hr after administration of
hydrazine in comparison to the saline-injected control animals. The level of the liver ODC activity was comparable in magnitude to that reported by others in 16 hr regenerating liver following partial hepatectomy (155). This initial work on hepatic ODC in the hydrazine-treated animal was completed using a homogenizing medium lacking the addition of mercaptoethanol and pyridoxal phosphate as described in the methods section. The addition of these two compounds to the homogenizing medium, as reported by Jänne and Williams-Ashman (156) was to aid in maintaining the stability and to prevent polymerization of the enzyme. In our hands, slightly higher observed ODC activity was found when livers were homogenized using the buffer with mercaptoethanol and pyridoxal phosphate than when these two compounds were not added. These preliminary studies also indicated that adult animals should be used in order to lessen observed animal variation in hepatic ODC activity since this enzyme seems to be altered during rapid growth.

**Evaluation of ODC Assay Methodology**

In an experiment designed to ascertain the efficiency of the release of liberated $^{14}$CO$_2$ from the reaction mixture and the absorption of the $^{14}$CO$_2$ into the trapping agent present in the center well, radioactive carbonate (as Na$_2^{14}$CO$_3$) was added along with the other components of the normal reaction mixture to reaction vessels with and without enzyme preparation present. All of the radioactivity added in this acid labile form was accounted for in the CO$_2$ trap after $^{14}$CO$_2$ was liberated by injection of acid into the reaction vessels. Furthermore, aliquots of the reaction mixture were counted and found to contain no radioactivity. The fact that no label remained in the reaction mixture
of vessels containing the active enzyme preparation, indicates that
the labeled carbonate was not incorporated into other non-acid labile
metabolites during the incubation period and remained in an acid
labile form.

Figure 3 demonstrates that the ODC activity was linear for the
8 min incubation period used for the remainder of this study. The
use of non-saturating substrate concentration was a probable factor
for the observed deviation from linearity starting at about 10 min.
As can be seen from endogenous levels of ornithine determined for
similar enzyme preparations (Figure 2), the amount of substrate present
in the enzyme preparation (80 μM) was within the same range as the
exogenous ornithine added for the assay (50 μM). In addition, data
presented in Table III indicated that exposure of the enzyme prepara-
tion to the temperature (37°C) at which the activity was determined
could be a second factor causing the decrease in observed activity
after about 10 min. Except for the length of the preincubation periods,
all conditions of the assay procedure were as described in the methods.
Reaction mixtures were preincubated 10, 20 or 30 min before starting
the assay with the addition of 14C-l-ornithine. The observed ODC
activity from the liver of a hydrazine-treated rat decreased about
50% with each additional 10 min of preincubation. The boiled enzyme
blanks appear not to change with the variation of the preincubation
times. The less than saturating ornithine concentration also could
have caused a loss of any protective effect that the substrate may
have had on preventing the inactivation of the enzyme. In another
preliminary experiment to study the assay method, ODC activity was
## Table III

Effect of Length of Preincubation Time on ODC Activity

<table>
<thead>
<tr>
<th>Nature of Enzyme Preparation</th>
<th>Preincubation time (min)</th>
<th>ODC Activity* (dpm/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>10</td>
<td>1469</td>
</tr>
<tr>
<td>Boiled</td>
<td>10</td>
<td>206</td>
</tr>
<tr>
<td>Active</td>
<td>20</td>
<td>794</td>
</tr>
<tr>
<td>Boiled</td>
<td>20</td>
<td>185</td>
</tr>
<tr>
<td>Active</td>
<td>30</td>
<td>425</td>
</tr>
<tr>
<td>Boiled</td>
<td>30</td>
<td>181</td>
</tr>
</tbody>
</table>

* Values are the average of duplicates using 0.4 ml supernatant

Activity was determined on a single ODC preparation from a 4 hr hydrazine-treated animal.
found to be proportional to the enzyme concentration over the range 0.0 to 0.8 ml (0-3.3 mg supernatant protein) of the enzyme preparation used (Figure 4).

In a series of experiments Km's were determined for hepatic ODC from animals following either hydrazine treatment (4 hr) or partial hepatectomy (16 hr). Since there was significant animal variation in levels of ODC activity, the data for each individual animal is illustrated graphically. Figures 5 and 6 are Lineweaver-Burk plots of ODC activity in the hydrazine-treated and regenerating liver supernatants, respectively. The Km's for ODC in the hydrazine-treated and partially hepatectomized animals were between 0.05 and 0.1 mM, values comparable to the 0.1 mM reported for regenerating liver (157). No Km values were determined for control rat liver ODC since its activity was essentially nil.

As reported by others (104, 158), reaction blanks have been a problem in this assay procedure. In most cases in the present study, the reaction blanks were in the same range as the values obtained in enzyme preparations from control animals. Therefore, in all experiments comparing ODC activity from control and hydrazine-treated animals, reaction blanks have not been subtracted from the results unless stated otherwise. In so doing, the pattern of changes in observed ODC activity due to hydrazine treatment was not altered; in fact, the relative difference between the control and experimental group was actually lessened since the correction for blanks would have caused a greater proportional reduction in the data from the control animals than the hydrazine-treated animals. This would be especially true
since boiled enzyme blanks from the enzyme preparations from the control animals were higher \( (p < .02) \) than for the hydrazine-treated animals (Table IV). The difference between these two groups became more significant when larger volumes of boiled enzyme preparations were added to the reaction mixtures. Pyridoxal phosphate is known to aid in the non-enzymatic decarboxylation of amino acids. This role for pyridoxal phosphate present in these reaction mixtures could be offered as a plausible explanation for the data reported in Table IV. Since hydrazine is known to inhibit pyridoxal phosphate-requiring enzymes (15), a reduction in the ability of pyridoxal phosphate to aid in the non-enzymatic decarboxylation of \(^{14}\)C-1-ornithine might be expected. The inverse relationship between the amount of boiled enzyme and the observed radioactivity in the blanks is similarly explained. If exogenous pyridoxal phosphate were complexed with the denatured protein present in the reaction mixture, greater amounts of boiled enzyme would cause less pyridoxal phosphate to be free to act on the carboxy-labeled substrate.

**Time-course of ODC Activity Following Hydrazine Treatment**

Figure 7 and Table V depict the changes in the levels of hepatic ODC activity as a function of time after the administration of hydrazine for a period of 12 hr. Preliminary studies have shown that there was no difference in observable ODC activity from the livers of fasting controls and hydrazine-treated animals at several time points over the 12 to 64 hr interval. In comparison to the values obtained from control livers, ODC activity was enhanced \( (p < .0125) \) within 2 hr
Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC Activity</th>
<th>ODC Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.3 ml boiled enzyme)</td>
<td>(0.6 ml boiled enzyme)</td>
</tr>
<tr>
<td>Control</td>
<td>185 ± 7*</td>
<td>160 ± 7</td>
</tr>
<tr>
<td></td>
<td>p &lt; .05</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>153 ± 9</td>
<td>120 ± 8</td>
</tr>
<tr>
<td></td>
<td>p &lt; .02</td>
<td>p &lt; .005</td>
</tr>
</tbody>
</table>

* Standard error of the mean of 10 animals for each group with activity expressed as dpm/reaction vessel/8 min

Preparations from 4 hr control and hydrazine-treated animals were used for these determinations.
Table V

Time-course of ODC Activity Following Experimental Treatment

<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>Enzyme Activity (p moles (^{14}\text{CO}_2) formed/mg prot/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>98 ± 7* (3)**</td>
</tr>
<tr>
<td></td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>2</td>
<td>85 ± 25 (3)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .0125</td>
</tr>
<tr>
<td>3</td>
<td>134 ± 30 (3)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .005</td>
</tr>
<tr>
<td>4</td>
<td>81 ± 15 (3)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .0025</td>
</tr>
<tr>
<td>5</td>
<td>191 ± 126 (3)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>6</td>
<td>85 ± 4 (3)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .025</td>
</tr>
<tr>
<td>8</td>
<td>99 ± 1 (2)</td>
</tr>
<tr>
<td></td>
<td>p &lt; .025</td>
</tr>
<tr>
<td>12</td>
<td>85 ± 12 (3)</td>
</tr>
<tr>
<td></td>
<td>N.S.D.</td>
</tr>
</tbody>
</table>

* Standard error of the mean

** Number of animals

Enzyme activities were determined in duplicate for each animal.
following the administration of hydrazine. The enzyme activity reached a maximum at 4 hr and returned to levels comparable to control liver ODC activity by 12 hr after hydrazine treatment. There was essentially no change in the observed level of ODC activity determined for the control animals at any point over this period of time.

These results have not been corrected for the increased ornithine pool size noted for the hydrazine-treated animals. Correction for this difference in pool size would magnify the relative enhancement in ODC activity due to hydrazine treatment.

Using the 4 hr time point, a 1:1 mixture of ODC enzyme preparations was assayed to examine the possibility that an activator might be present in the liver supernatant from the hydrazine-treated animal or an inhibitor in the preparation from the control liver. As shown in Table VI, the ODC activity in the 1:1 mixture was nearly midway between the activity in the control and hydrazine-treated preparations. These results tend to eliminate the possible presence of dissociable activators or inhibitors in the supernatants used for the assay procedure.

Effect of Cycloheximide and Actinomycin D on ODC Activity

The effects of two inhibitors of nucleic acid and protein biosynthesis were studied in an attempt to suggest a possible mechanism for the elevated ODC activity observed following hydrazine treatment (Table VII). Since the change in ODC activity reached a maximum at about 4 hr after experimental treatment, this time point was selected to study the effects of prior exposure of the animals to either cycloheximide or Actinomycin D.
# Table VI

Hepatic ODC Activity in a 1:1 Mixture of Supernatants from Hydrazine-treated and Control Animals

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>ODC Activity (pmoles $^{14}$CO$_2$ formed/8 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56 ± 24*</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>940 ± 124</td>
</tr>
<tr>
<td>1:1 Mixture</td>
<td>576 ± 60</td>
</tr>
</tbody>
</table>

* Standard error of the mean from 4 animals (400-480 g)

Preparations from 4 hr control and hydrazine-treated animals were used for these determinations.
Table VII

Effects of Cycloheximide and Actinomycin D on Hepatic ODC Activity

<table>
<thead>
<tr>
<th>Additional Treatment</th>
<th>Control (Fasted)</th>
<th>Hydrazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None 4 hr</td>
<td>150 ± 17 (8)</td>
<td>980 ± 89 (8)</td>
</tr>
<tr>
<td>Cycloheximide 4 hr</td>
<td>62 ± 10 (4)</td>
<td>60 ± 9 (4)</td>
</tr>
<tr>
<td>Cycloheximide 1 hr</td>
<td>83 ± 8 (4)</td>
<td>261 ± 73 (4)</td>
</tr>
<tr>
<td>Actinomycin D 4 hr</td>
<td>102 ± 13 (10)</td>
<td>161 ± 25 (9)</td>
</tr>
<tr>
<td>Actinomycin D 1 hr</td>
<td>373 ± 9 (4)</td>
<td>1178 ± 62 (4)</td>
</tr>
</tbody>
</table>

* Standard error of the mean

** Number of animals

# Animals in this study were either 4 hr control or hydrazine-treated rats. Animals given either cycloheximide or Actinomycin D at the onset of the experiment are indicated by the 4 hr notation below the name of the drug. Animals receiving these drugs three hours after the onset of the experiment are indicated by the 1 hr notation below the name of the drug.
In this experiment cycloheximide (50 mg/kg), an inhibitor of protein synthesis at the translational level, not only completely prevented the increased ODC activity when administered at the same time as hydrazine (4 hr), but produced a significant decrease in activity in both the control (p < .0025) and hydrazine-treated animals (p < .0005) compared to the 4 hr fasted controls. When cycloheximide was injected three hours after the hydrazine (indicated in the table as 1 hr before sacrifice of the animals), hepatic ODC activity was significantly less (p < .0005) than the activity observed in the 4 hr hydrazine-treated animal without inhibitor. Although the ODC activity in this latter group of cycloheximide and hydrazine-treated animals was greater (p < .05) than in the respective control animals, it was not significantly higher than the 4 hr fasted control animals. These control animals receiving cycloheximide 1 hr before sacrifice had lower (p < .005) hepatic ODC activity than in the 4 hr fasted controls, but were not different from the control group receiving cycloheximide 4 hr before sacrifice.

Actinomycin D (0.8 mg/kg), which interferes with protein synthesis at the transcriptional level (159), prevented the increase in ODC activity produced by hydrazine treatment when it was administered at the same time as hydrazine. However, the hydrazine-treated animals demonstrated slightly higher (p < .05) ODC activities than the corresponding fasted controls. When Actinomycin D was injected 3 hrs after the administration of either hydrazine or saline (1 hr before sacrifice), it did not interfere with the observed elevation in ODC activity due to hydrazine. Indeed, in addition to a significant difference (p < .0005)
between the hydrazine and saline-injected animals, both showed an actual increase in the ODC activity (p < .0005) when compared to the 4 hr fasted control animals. The level of ODC activity in this hydrazine-treated group of animals was even slightly greater (p < .05) than that group receiving only hydrazine.

**Phenylalanine Decarboxylase Activity**

By determining hepatic phenylalanine decarboxylase (PDC, 3, 4-dihydroxy-L-phenylalanine carboxy-lyase, E.C.4.1.1.26) activity, we were able to ascertain that the observed changes in ODC activity did not appear to be due to a non-specific effect of hydrazine treatment with respect to pyridoxal phosphate-requiring decarboxylases. In fact, Table VIII A contains data showing an actual decrease (p < .005) in PDC activity at 4 hr after hydrazine treatment when compared to the activity in the 4 hr control liver supernatant. However, there was an increase (p < .001) in the PDC activity observed at 24 hr in the hydrazine-treated animal when compared to either the 4 or 24 hr values for PDC activity in control animal preparations. The 24 hr control animal also showed a significant decrease (p < .01) in PDC activity when compared to the activity found in the 4 hr control liver supernatant. In contrast to ornithine levels, there was no significant difference in free endogenous phenylalanine pools as the result of hydrazine treatment or fasting over the time period studied (Table VIII B). Thus, the changes in observed PDC activity were neither the result of a difference in phenylalanine pool size nor the cause of alterations of liver phenylalanine concentration.
Table VIII

PDC Activity and Phenylalanine Pool Size Following Experimental Treatment

A. PDC Activity
(p moles $^{14}$CO$_2$/8 min/g liver)

<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>Control</th>
<th>Hydrazine</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4309 ± 261*</td>
<td>3171 ± 187</td>
<td>(p &lt; .005)</td>
</tr>
<tr>
<td>(9)**</td>
<td></td>
<td>(9)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>3099 ± 258</td>
<td>6901 ± 285</td>
<td>(p &lt; .001)</td>
</tr>
<tr>
<td>p &lt; .01</td>
<td></td>
<td>(5)</td>
<td></td>
</tr>
</tbody>
</table>

B. Free Endogenous Phenylalanine Pools
(\(\mu\) moles/g liver)

<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>Control</th>
<th>Hydrazine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.11 ± .02*</td>
<td>0.168 ± .03</td>
<td>N.S.D.</td>
</tr>
<tr>
<td>(8)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.11 ± .03</td>
<td>0.12 ± .03</td>
<td>N.S.D.</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td>(4)</td>
<td></td>
</tr>
</tbody>
</table>

* Standard error of the mean

** Number of animals
Time-course of Hepatic Putrescine, Spermidine and Spermine Levels

After the increase in liver ODC activity due to hydrazine treatment was observed, a time-course study (0, 2, 4, 12, 24, 48 hr time points) was designed to determine whether this change was reflected in alterations in hepatic putrescine, spermidine and spermine levels. The results from 4 animals at each point are summarized in Table IX. The time-course of changes in putrescine, spermidine and spermine levels reported on a DNA basis from zero time to 48 hr are illustrated in Figures 8, 9, and 10, respectively. Hepatic putrescine levels were elevated significantly \( p < .005 \) 2 hr after hydrazine treatment relative to the 2 hr control animals and reached a maximum concentration about 12 hr after the animals were injected with the compound. At the 48 hr time point, putrescine levels appear to be returning toward, but were still greater \( p < .025 \) than the levels found in the livers of the control animals fasted for the same period of time. The putrescine levels in the livers of control animals were not affected significantly as the result of fasting up to 48 hr. The spermidine levels of the 2 hr control, fasted groups decreased \( p < .025 \) relative to the zero hour controls and the 2 hr hydrazine-treated animals. At 4 hr, there was no significant difference between the two experimental groups, or relative to the zero time levels. By 12 hr spermidine levels from the livers of the hydrazine-treated group were elevated \( p < .0005 \) compared to the levels in the liver of the 12 hr control rats. Spermidine levels in the hydrazine-treated group reached their maximum at about 24 hr and started to return toward the levels observed in the liver from the 48 hr fasted control rats. The level at 48 hr in the
<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>Group</th>
<th>Putrescine Concentration (μmoles/g dry wt)</th>
<th>Spermidine Concentration (μmoles/g dry wt)</th>
<th>Spermine Concentration (μmoles/g dry wt)</th>
<th>Liver Weight Ratio (g dry wt/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>27 ± 4</td>
<td>4997 ± 146</td>
<td>5029 ± 144</td>
<td>0.260 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>18 ± 4</td>
<td>4707 ± 207</td>
<td>5348 ± 43</td>
<td>0.260 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>185 ± 20</td>
<td>5328 ± 121</td>
<td>5757 ± 73</td>
<td>0.242 ± 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0005</td>
<td>p &lt; .025</td>
<td>p &lt; .0025</td>
<td>p &lt; .01</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>31 ± 3</td>
<td>5047 ± 147</td>
<td>5045 ± 27</td>
<td>0.252 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>314 ± 25</td>
<td>6258 ± 177</td>
<td>5608 ± 130</td>
<td>0.220 ± 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0005</td>
<td>p &lt; .0025</td>
<td>p &lt; .005</td>
<td>p &lt; .0005</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>54 ± 5</td>
<td>5373 ± 373</td>
<td>6960 ± 56</td>
<td>0.242 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>667 ± 112</td>
<td>7105 ± 170</td>
<td>5423 ± 48</td>
<td>0.238 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0025</td>
<td>p &lt; .005</td>
<td>p &lt; .0005</td>
<td>N.S.D.</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>30 ± 3</td>
<td>4845 ± 372</td>
<td>7077 ± 231</td>
<td>0.252 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>445 ± 104</td>
<td>9402 ± 125</td>
<td>4961 ± 206</td>
<td>0.244 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .005</td>
<td>p &lt; .0005</td>
<td>p &lt; .0005</td>
<td>N.S.D.</td>
</tr>
<tr>
<td>48</td>
<td>Control</td>
<td>27 ± 2</td>
<td>4487 ± 282</td>
<td>7329 ± 132</td>
<td>0.240 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>94 ± 26</td>
<td>7485 ± 576</td>
<td>5117 ± 100</td>
<td>0.268 ± 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .025</td>
<td>p &lt; .0025</td>
<td>p &lt; .0005</td>
<td>p &lt; .01</td>
</tr>
</tbody>
</table>
hydrazine-treated animals was the same as that found in the zero hour controls. In the case of spermidine, fasting did produce a decrease ($p < .005$ at 48 hr) in observed hepatic concentrations compared to the levels found in the fed control (zero time point) and these were still declining after 48 hr at the conclusion of the study. There was a slight decrease ($p < .025$) of spermine levels in the livers of the 2 hr control group compared to the 2 hr hydrazine-treated group, no significant difference at the points measured between 2 and 24 hr, and a decrease ($p < .0025$) in the hydrazine-treated group compared to the levels in the control group at the 48 hr time point. The level found at 48 hr in the hydrazine-treated group was also decreased ($p < .005$) from both of the 24 hr groups. Except for the 2 hr point the levels of spermine in control animals were not decreased relative to the zero time controls.

Hepatic protein, RNA and DNA levels are included in Table X to facilitate additional calculations in order to examine the results on a different basis than that presented above. However, there is no change in the interpretation of the data when these calculations are done.

**Hepatic Levels of $\gamma$-Aminobutyric Acid Following Hydrazine Treatment**

Seiler et al. (160) reported the incorporation of radioactive label into $\gamma$-aminobutyric acid (GABA) in rat liver as early as 1 hr after an intraperitoneal injection of $^{14}$C-putrescine. These investigators suggested that GABA may be a catabolic metabolite from putrescine and related polyamines in vivo. As part of the amino acid
<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>Group</th>
<th>Liver Weight (g)</th>
<th>Protein (mg protein/g wet wt)</th>
<th>RNA (mg RNA/g wet wt)</th>
<th>DNA (mg DNA/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>14.612 ± 0.247</td>
<td>199 ± 14</td>
<td>8.68 ± .06</td>
<td>3.10 ± .27</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>14.211 ± 0.310</td>
<td>219 ± 11</td>
<td>8.82 ± .07</td>
<td>4.13 ± .12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>p &lt; .005</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>14.095 ± 0.430</td>
<td>216 ± 18</td>
<td>8.55 ± .00</td>
<td>3.72 ± .15</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>15.837 ± 0.430</td>
<td>215 ± 6</td>
<td>8.51 ± .14</td>
<td>3.50 ± .05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>p &lt; .025</td>
<td>N.S.D.</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>16.090 ± 0.373</td>
<td>216 ± 12</td>
<td>7.98 ± .13</td>
<td>3.50 ± .05</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>16.095 ± 0.430</td>
<td>214 ± 13</td>
<td>7.85 ± .19</td>
<td>3.77 ± .15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>p &lt; .005</td>
<td>p &lt; .0025</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>10.726 ± 0.662</td>
<td>214 ± 13</td>
<td>9.58 ± .12</td>
<td>3.50 ± .15</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>14.008 ± 0.363</td>
<td>214 ± 13</td>
<td>7.85 ± .19</td>
<td>3.77 ± .15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0025</td>
<td>p &lt; .0025</td>
<td>p &lt; .0005</td>
<td>p &lt; .0025</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>9.621 ± 0.253</td>
<td>168 ± 4</td>
<td>9.31 ± .26</td>
<td>3.50 ± .15</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>14.261 ± 0.230</td>
<td>149 ± 8</td>
<td>8.84 ± .07</td>
<td>5.31 ± .28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0005</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>p &lt; .0005</td>
</tr>
<tr>
<td>48</td>
<td>Control</td>
<td>9.630 ± 0.207</td>
<td>238 ± 11</td>
<td>8.13 ± .81</td>
<td>5.41 ± .20</td>
</tr>
<tr>
<td></td>
<td>Hydrazine</td>
<td>11.921 ± 0.341</td>
<td>213 ± 17</td>
<td>9.75 ± .11</td>
<td>5.03 ± .08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .0025</td>
<td>N.S.D.</td>
<td>p &lt; .05</td>
<td>N.S.D.</td>
</tr>
</tbody>
</table>
analysis of the 20,000 x g liver supernatants in the present study, measurable levels of GABA were noted as early as 1 hr following hydrazine treatment, whereas, GABA was not detected in significant quantity in the liver supernatants from the zero time control animals or animals fasted up to 24 hr. The limits of the assay procedure are such that approximately 1 nmole of GABA would be needed for detection. As shown in Table XI, there is essentially a constant level of GABA present for at least 24 hr following hydrazine treatment.

Other Enzymes Involved in Ornithine Metabolism

Since ornithine is known to play a central role in the catabolism of amino acid nitrogen as part of the urea cycle, the activities of the urea cycle-related enzymes directly involved in ornithine metabolism, ornithine transcarbamylase (OTC) and arginase, were studied.

The results of a time-course study of hepatic OTC activity in control and hydrazine-treated animals is illustrated in Figure 11. The OTC activity from the livers of 4, 12 and 24 hr fasted control animals did not vary significantly from the level of activity in livers from zero hour control animals. In the hydrazine-treated animal, hepatic OTC activity was significantly lower (p < .0005) at 12 hr when compared to the 12 hr fasted control animals. This value was also decreased (p < .025) in relation to the activity observed in the 0 hr controls and the 24 hr hydrazine-treated group (p < .005). The observed activities from the 24 hr control and hydrazine-treated groups were no longer statistically different from one another or from the zero hour controls.

A similar time-course study depicting hepatic arginase activity in
Table XI

Hepatic Levels of Endogenous GABA Following Hydrazine Treatment

<table>
<thead>
<tr>
<th>Time After Treatment (hr)</th>
<th>$\mu$ moles GABA $g$ liver</th>
<th>$\mu$ moles GABA $mg$ DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33 (2)*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.22 (2)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.22 (12)</td>
<td>0.04 (9)</td>
</tr>
<tr>
<td>6</td>
<td>0.43 (2)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.31 (2)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.26 (7)</td>
<td>0.04 (5)</td>
</tr>
</tbody>
</table>

* Number of animals
control and hydrazine-treated animals is shown in Figure 12. Arginase activity in livers from the 4 hr hydrazine-treated rats was less (p < .05) than that found in the 4 hr fasted saline-injected rats. At 4 hr, both the hydrazine-treated (p < .0005) and control animals (p < .025) had lower arginase activities than the zero hour controls. Arginase activity in the livers from the hydrazine-treated animals seemed to be returning toward zero hour values by 12 hr and was slightly greater (p < .05) than the zero hour control levels at 24 hr. Arginase activity in the control animals also returned toward normal values and was no longer different from either the zero hour controls or the 24 hr hydrazine-treated groups at 24 hr.

Ornithine Ə-transaminase (OTA), the remaining enzyme involved directly in hepatic ornithine metabolism in the rat, has been described as playing a regulatory role connecting both the urea and tricarboxylic acid cycle functions (161). A time-course study following OTA activity for the 24 hr period after hydrazine treatment is illustrated in Figure 13. The observed OTA activity in the hydrazine-treated rat was decreased significantly (p < .0005) at 4 hr when compared to the activity from the 4 hr control group. The observed levels of OTA activity stayed constant through the remaining time period studied. OTA activity from the control animals did not vary significantly from the zero hour control animals at any point in the study.
DISCUSSION

The elevation of endogenous hepatic free ornithine pool size due to hydrazine treatment observed in the present study and reported previously by our laboratory (31, 33) and others (32) appears to be the result of alterations in the activities of the hepatic enzymes involved in ornithine metabolism. These changes revolve around alterations in the four enzymes that participate directly in the hepatic metabolism of ornithine described in the introduction. The relative ratios of the activities of these enzymes in rat liver have been established to be in the order of magnitude of $7.5 \times 10^6 : 1 \times 10^6 : 2.5 \times 10^3 : 1$ for arginase, OTC, OTA and ODC, respectively (157, 162). In reviewing the data presented, consideration as to the significance of each separate experiment is needed before correlative discussion of the whole study can become meaningful.

The anorexia produced following hydrazine treatment results in the failure of the rats to consume food for several days (11). In the experiments involving hydrazine-treated animals, a comparison of fasted control rats with fed control animals was made in order to ascertain the effect of starvation alone on the parameters being examined. The effects produced by hydrazine treatment were determined by comparison between these fasted control animals and the hydrazine-treated animals.

Hydrazine treatment produced approximately a 45% decrease in observed arginase activity at the 4 hr time point (Figure 12). This decrease in activity was not as remarkable as it first might appear
since the activity in the control animals also decreased approximately 30% at the same time point. Following this decline in activity, arginase activity began to increase. The rate of increase in activity was noted to be slightly greater in the hydrazine-treated animals than the corresponding control group. It is possible that this latter increase in levels of arginase activities in both control and hydrazine-treated animals was due to fasting. If this increase were to continue, it would be consistent with observations reported by Schimke (163). His study showed a slow, two-fold elevation in hepatic arginase activities over a six day fasting period. In his study, the trend towards increased arginase activities had developed by one day, but there too, the level of enzyme activity was not significantly different from the zero hour control animals' level.

In addition, Schimke (164) has shown that the activities of all of the hepatic urea cycle enzymes are enhanced in rats fasted for either four or seven days. Knox and Greengard (165) explained the elevated hepatic urea cycle activity in fasting rats as being a response to the increased breakdown of tissue proteins. The initial decrease in arginase activity in the control animals is perhaps due to a diurnal variation of the enzyme activity. Furthermore, Katunuma et al. (166) have noted increased hepatic urea cycle activity with increased hepatic ornithine concentrations. This interdependency between ornithine levels and urea cycle activity might account for the observed rapid rate of increase in hepatic arginase activity noted due to the hydrazine treatment between 4 and 24 hr since this period of time encompassed the time of the maximal ornithine levels.
However, no increase in hepatic arginase activity was observed by Roberge et al. (167) after four days of fasting. In this study of the effects of a chronic treatment with hydrazine on hepatic urea cycle enzyme activities compared to levels from fasted controls, these workers administered a daily subconvulsive dose of hydrazine (1.0 mmole/kg/day, neutralized hydrazine sulfate) intraperitoneally for four consecutive days. (The single dose of hydrazine used in the present study was 1.25 mmole/kg.) They reported activities for the hepatic urea cycle enzymes from fed control animals, four day hydrazine-treated and four day fasted control animals with no examination of intermediate time points. From this model, they concluded that hydrazine treatment did not produce alterations in arginase activity in rat liver since they obtained the same results from all three groups of animals.

The data presented in the present study tend to support the trend noted by Katunuma et al., Knox and Greengard, and Schimke, rather than the observations reported by Roberge et al. However, the experimental model of the present study was not similar enough to that of Roberge et al. for exact comparisons of results and conclusions to be made.

Alterations similar to those noted for arginase activity were observed for hepatic OTC activity in the present study. OTC is also a component of the urea cycle but utilizes ornithine as a substrate rather than catalyzing its formation. Except for the significantly decreased hepatic OTC activity (40%) noted in the liver of hydrazine-treated animals at the 12 hr time point (Figure 11), no differences in the activities of this enzyme were observed between the control and hydrazine-treated groups at the other points studied when compared
with the zero hour control animals. OTC activity developed an apparent trend, similar to the temporal trend described for arginase activity above, indicating the possibility that a further increase in activity could be anticipated as would be predicted from the above general discussion of the urea cycle enzymes. Although there was only one time point at which a difference in OTC activity was observed between the hydrazine-treated and control animals in the present study, that decrease in activity correlated with the increase in hepatic ornithine concentrations and could explain, in part, the alteration in the levels of the latter (Figure 2).

In the study by Roberge et al., no difference in the level of OTC activity was observed after four consecutive days of hydrazine administration when compared to untreated, fed controls. However, fasted control animals showed significant increases in hepatic OTC activities relative to either the hydrazine-treated or the untreated, fed groups. These results were in agreement with predicted increases in the enzymes of the urea cycle after periods of starvation as would be derived from the results reported by others and discussed above. A possible explanation for their observation that hydrazine treatment (which involves fasting) did not increase OTC activity may be that the daily dose of hydrazine caused a reduction in OTC activity similar to the results of the present study shown in Figure 11.

Although the remaining urea cycle enzymes were not examined in the present study, the effects produced by hydrazine have been studied by Roberge et al. (167) and others (32). Of these remaining enzymes, the activity of argininosuccinate synthetase was singled out as the most
influential in affecting the alterations in ornithine metabolism via the urea cycle. Simonsen and Roberts (32) noted increased liver citrulline contents with no indication of increased argininosuccinate accumulation following hydrazine treatment in mice. Their data led them to suggest that hydrazine caused the condensation of citrulline with aspartic acid by argininosuccinate synthetase to be the rate-limiting step in urea production and thus explained the increased levels of citrulline observed following hydrazine treatment. Roberge et al. (167) studied the argininosuccinate synthetase system from rat liver and their results supported the same conclusion. This mechanism would explain the increased citrulline levels noted (Table 2) after hydrazine treatment in the present study.

As similarly shown for the enzymes of the urea cycle, studies have revealed enhancement of OTA activities under circumstances, such as starvation, in which protein is mobilized via catabolism (165, 168), thus increasing the hepatic levels of amino acids (31). These increased OTA activities in the livers of both the fasted controls as well as the hydrazine-treated rats would be expected, yet OTA activity was clearly decreased within 4 hr due to hydrazine treatment (Figure 13). This depression in hepatic OTA activity due to hydrazine treatment remained at approximately 40% of the activity observed in the fasting control animals during the course (24 hr) of the study. Since the reaction catalyzed by OTA is reversible, the question could be raised as to what effect this decrease in OTA activity would have on the ornithine pool size. The equilibrium constant for this reaction strongly favors the conversion of ornithine to glutamic γ-semialdehyde (59). Moreover,
most of the glutamic γ-semialdehyde derived from ornithine was converted to glutamate in a rat liver mitochondrial system (169).

In another study, Katunuma et al. (166) have reported quantitative formation of glutamate from ornithine in isolated mitochondria from rat liver. Thus, it appears that this decrease in OTA activity could be a primary cause for the initial elevation in ornithine pool size as well as the sustained increased levels present at the conclusion of the time-course study (Table II).

Roberge et al. (167) also included OTA as one of the enzyme activities they studied following hydrazine treatment. These workers reported a marked inhibition of OTA activity (15% of the control activity) after four consecutive days of hydrazine administration. These workers postulated that this inhibition provoked the accumulation of hepatic ornithine (as reported by Banks (31) 24 hr after a single dose of hydrazine) and implied that this decreased OTA activity was the only explanation of the increased hepatic ornithine levels resulting from hydrazine treatment. As will become apparent later in this discussion, the decreased OTA activity observed in the present study following a single subconvulsive dose of hydrazine can only partially explain the resultant time-course of alterations in hepatic ornithine pool size.

In addition to influencing the levels of hepatic ornithine concentration, Katunuma and co-workers (166, 169) believe that OTA plays an important role in the liver by interrelating the urea and tricarboxylic acid cycles. This role would explain the dependency of the urea cycle
activity on the ornithine content of the liver as mentioned earlier. In their view, the rate at which OTA helps to catalyze the reaction between ornithine and $\alpha$-ketoglutarate to form glutamic $\gamma$-semialdehyde is a controlling factor on the concentration of ornithine available for the urea cycle. Since $\alpha$-ketoglutarate is formed in the tricarboxylic acid cycle, it affords the connection between these two cycles associated with the name of Sir Hans Krebs.

The remaining enzyme involved in ornithine metabolism in rat liver, ODC, showed marked changes in activity in the hydrazine-treated animal when compared to control animals. The rapidity of the rise and decline of ODC activity after hydrazine treatment is a characteristic behavior for this enzyme and has been related to its extremely short half-life of 10-15 min (105). The alterations in ODC activities probably had a negligible effect on the observed fluctuations in the endogenous ornithine pool sizes because of the relative activities previously mentioned for the enzymes involved in hepatic ornithine metabolism.

The increased ODC activity (Figure 7) at 4 hr resulting from hydrazine treatment was remarkable in face of the observed concurrent decreased PDC activity (Table VIII) at 4 hr since hydrazine has been observed to cause inhibition of enzymes utilizing pyridoxal phosphate as a cofactor in both in vitro and in vivo studies. In this regard, Fortney et al. (23) reported an inhibition of extramitochondrial glutamic-oxalacetate transaminase in vivo. Simonsen and Roberts (32) accounted for increased hepatic concentrations of alanine, glutamic
acid, aspartic acid, ornithine and glycine which they observed after hydrazine treatment in mice by inhibition of the Vitamin B₆-requiring enzymes by hydrazine. Killam and Bain (25) observed that in vitro inhibition by hydrazine and its derivatives could be reversed by the addition of the Vitamin B₆ complex. These workers assayed three rat enzyme systems requiring pyridoxal phosphate as a cofactor after the animals received convulsive doses of the hydrazide, thiosemicarbazide or semicarbazide. Of the enzymes studied, brain L-glutamic acid decarboxylase, brain L-glutamic-aspartate transaminase, and liver L-cysteine desulfhydrase, the in vivo effects of the hydrazides produced inhibition of only the L-glutamic acid decarboxylase. Thus, it still remains unclear whether hydrazine inhibits most pyridoxal-requiring enzymes in vivo and if so, whether the inhibition is by the same mechanism as that observed in vitro.

The experiments utilizing the metabolic inhibitors, Actinomycin D and cycloheximide attempted to establish the mechanism responsible for the hydrazine-induced increase in hepatic ODC activity (Table III). It should be recognized that the dosages of Actinomycin D and cycloheximide commonly used in in vivo experimentation cause the animals to become extremely lethargic. In the present study, the dose used, which was the lowest dose reported in the literature for similar studies, occasionally resulted in the death of the animal. Therefore, one should consider that the effects of these inhibitors on the observed enzyme activities might be explained by other events than inhibition of the protein synthesis machinery.

Actinomycin D was orginally reported to act at the level of
transcription, inhibiting DNA-dependent RNA synthesis (170). Later investigations have shown that there is a subsequent increased rate of breakdown of cellular RNA following administration of Actinomycin D (2.5 mg/kg, body weight) to rats (193). Recently, evidence using mouse L-cells has indicated that the observed inhibition of protein biosynthesis by Actinomycin D was due to an inhibition in the rate of initiation of protein biosynthesis (194). Thus, it appears that a combination of the above factors may be responsible for the inhibition of protein biosynthesis by Actinomycin D. In the present study, the observed increase in ODC activity following hydrazine treatment was prevented when Actinomycin D was given concurrently with hydrazine. This observation could be explained by inhibition of DNA-dependent RNA synthesis, increased breakdown of cellular RNA, inhibition of the rate of initiation of protein biosynthesis, or a combination of these mechanisms ascribed to Actinomycin D. However, when Actinomycin D was given three hours after hydrazine administration (1 hr before sacrifice), it did not interfere with the normally observed increased hepatic ODC activity resulting from hydrazine treatment. One might postulate that during the 3 hr period before Actinomycin D was administered to the hydrazine-treated rats, the messenger RNA (mRNA) that contains the message for the synthesis of ODC had sufficient time to become part of the translational complex. Once a part of the translational complex, the mRNA could code for the biosynthesis of new enzyme needed to replenish the ODC levels due to its short half-life. The half-life of this specific mRNA has been estimated from studies with regenerating rat liver following partial hepatectomy to be four to six hours (105).
However, these studies were based on data resulting from treatment of the animals with Actinomycin D. Since Actinomycin D has been shown to cause increased breakdown of cellular RNA, this estimate is believed to be too short and the mRNA should be expected to be longer-lived than previously reported (194). Increased hepatic ODC activity, associated with the synthesis of a new species of short-lived RNA, has been observed in mice during the first hour after partial hepatectomy (173). In addition, Fausto (174) found increased incorporation of $^{14}$C-orotic acid into a fraction of RNA from rat liver following partial hepatectomy which was in synchrony with the rise in ODC activity. Though not a proof in and of itself, these data add support to the hypothesis that new mRNA is being formed in the rat liver which in turn will lead to increased levels of ODC activity.

Alternatively the enhancement of ODC activity by Actinomycin D (when given 1 hr before sacrifice) in both the control and hydrazine-treated animals might be the result of decreased degradation of the enzyme resulting from decreased levels of enzymes catalyzing the catabolism of ODC and the mRNA coding for ODC in the presence of this metabolic inhibitor. A similar enhancement has been observed using the same dose of Actinomycin D one hour after the intraperitoneal injection of histidine in rats which also produces an elevated hepatic ODC activity of similar magnitude to that noted in this present study (130).

Cycloheximide, a known inhibitor of protein biosynthesis at the translational level (175), was capable of preventing the increased ODC activity due to hydrazine treatment whether the inhibitor was given
concurrently with or three hours after the administration of the compound. In the hydrazine-treated animal, ODC activity would be expected to increase during the three hour period before receiving cycloheximide. However, this increased level of activity was prevented when the animals received cycloheximide one hour prior to sacrifice. This observation could be ascribed to a combination of factors including a decreased synthesis of enzyme \textit{de novo} as a result of the block at the translational level of protein biosynthesis by cycloheximide, and a significant loss of preexisting enzyme activity as a result of the extremely short half-life of the enzyme.

The combined results of these studies with the metabolic inhibitors suggested that the increased ODC activity resulting from hydrazine treatment appears to be due to \textit{de novo} synthesis of the enzyme. However, the actual stimulus activating this increase in the synthesis cannot be explained at present. In an attempt to determine if the factor stimulating the apparent increase of ODC was mediated through a hormonal mechanism, various hormones including hydrocortisone, growth hormone, insulin, glucagon and thyroxin were shown to produce an increase in hepatic ODC activity. None of these hormones were able to maintain the elevated levels of the ODC activity upon repeated treatment (176). Although these hormones stimulated ODC activity, the question as to the mechanism by which this stimulation takes place was not answered.

The property of having such a short half-life, in combination with the fact that ODC catalyzes the rate-limiting step in the biosynthesis of the polyamines, might suggest a possible regulatory role for this enzyme. There was some evidence from the present study to add
additional support to this possibility. The Lineweaver-Burk plots for ODC from both the liver of the hydrazine-treated animals (Figure 5) and the regenerating liver after partial hepatectomy (Figure 6) could be redrawn if one corrects for the endogenous concentrations of ornithine contained in the crude enzyme preparations. These new reciprocal plots are non-linear with upward curvatures at the lower substrate concentrations, as noted in Figures 14 and 15. This kinetic behavior is similar to that reported for substrates which were also activators (177). No mention of this effect of endogenous substrate on the apparent activity of ODC in supernatants prepared from various tissues is found in the literature. Presumably, the endogenous ornithine is either discounted as a non-influential factor or the other investigators dialyzed their enzyme preparations without stating so in their text. However, Raina et al. (102) did dialyze the preparation of ODC from regenerating rat liver following partial hepatectomy as part of the purification procedure in order to characterize ODC. Kinetic studies by these workers provided a Km value of 0.2 mM for the enzyme with respect to ornithine. This value was slightly higher than the 0.1 mM they determined on earlier crude preparations. Extrapolation of the curves from Figures 14 and 15 yields Km's of about 0.33 mM and 0.20 mM for ODC from hydrazine-treated and partially hepatectomized animals, respectively. These various Km values should not be considered significantly different from one another. However, these can be used to show the meaningful influence of endogenous ornithine levels on the kinetics of the enzyme which might suggest an allosteric effect. The possibility of substrate activation of ODC as suggested by the kinetic
plots might be offered as an explanation for the increased ODC activity. However, preliminary experiments, using higher exogenously-added concentrations in the assay medium, indicated that the differences in final substrate concentrations in the reaction mixtures due to the ornithine concentration in the enzyme preparations from control and hydrazine-treated animals would not explain the increase in observed ODC activity produced by hydrazine treatment.

The alterations in observed hepatic ODC activity discussed above due to hydrazine treatment are reflected in subsequent changes in the endogenous polyamine concentrations. The early and rapid elevation of putrescine levels (Figure 8) following hydrazine administration closely parallels, but follows the rise in hepatic ODC activity. Putrescine content continues to increase as long as there is greater ODC activity in the hydrazine-treated group compared to the control group. By 48 hr, putrescine levels seemed to have returned almost to the levels found for the control animals. At this time the liver appears histologically nearly to have recovered from the toxic effects of hydrazine (11).

The increased spermidine levels (Figure 9) parallel and follow the build up of putrescine. Spermidine concentrations peaked at 24 hr which might imply that the levels of this polyamine were increased as a consequence of the prior rise in putrescine content. The fact that spermine levels did not show any significant changes except when expressed on a gram of liver weight basis (wet or dry) tends to support the greater importance of increased spermidine levels to RNA and protein synthesis (178). The observation that the total spermine levels did not decrease in conjunction with the rise in spermidine demonstrates
that the increased spermidine levels in the present study were not at
the expense of the spermine already present in the liver as suggested
by Russell et al. (178) for similar changes in regenerating liver
following partial hepatectomy.

The expression of spermidine and putrescine on a per gram of liver
weight (wet or dry) basis did not change the pattern of the observed
alterations in their concentrations compared to the results obtained
when they were expressed on a per mg DNA basis. Expression of the data
on a per gram of tissue basis does not take into consideration differential changes in liver size nor cellular changes between the control and
hydrazine-treated animals in a unit weight of tissue. Fasting itself
resulted in an initial decline in total liver weight which appeared to
level off by 48 hr (163). In the hydrazine-treated, fasted animals,
this effect of fasting did not produce a decreased liver weight which
one might presumably attribute to the known liver lipid accumulation and
increased protein contents discussed in the introduction (11, 30, 31,
34, 179). Therefore, in the present study, comparing various parameters
on a per gram of tissue basis does not permit comparison between the
same number of liver cells from the control and from the hydrazine-
treated animals. The quantity of DNA is generally considered to
approximate the number of hepatocytes (152). Consequently, it seems
useful to view the data in the present study on a DNA basis. However,
since other workers have calculated their results on a unit weight of
tissue basis, the results of the present study have been presented on
both a gram tissue weight and a mg DNA basis in order that comparisons
to other findings could be made.
Although the specific time-course of changes are different, alterations in hepatic putrescine, spermidine and spermine levels in regenerating rat liver following partial hepatectomy reported by Russell et al. (178) were remarkably similar in pattern to those observed in the present study as a response to hydrazine treatment. In comparison to sham-operated controls, Russell et al. (178) showed that putrescine concentrations rose five-fold within four hours after partial hepatectomy, but thereafter showed a much more gradual decline than was noted following hydrazine treatment in the present study. The return of putrescine concentration to basal levels did not occur until seven days after the partial hepatectomy. According to Bucher (180), seven days after partial hepatectomy, a rat's liver has essentially returned to "normal", showing the same size, growth pattern, and histological and biological characteristics as the normal, unoperated control liver. Thus, the return of putrescine to basal levels seems to occur when the liver has returned to its "resting" state in the hydrazine-treated as well as the partially hepatectomized animals. Spermidine levels began to increase about ten hours after partial hepatectomy and remained elevated for at least fourteen days (178). A portion of the increased spermidine level was attributed by Russell et al. (178) to conversion of spermine to spermidine. This conclusion was based on a coincidental decrease in spermine levels relative to those found for the sham-operated control animals. Siimes (125) had previously demonstrated the in vivo conversion of spermine to spermidine in rat liver. This decrease in spermine levels reported by Russell et al. (178) was comparable in magnitude to that observed in the present study after
hydrazine treatment (Table IX) when the data were expressed on a per gram of tissue basis (as reported by Russell et al.). However, from the data presented in Tables IX and X, it is apparent that total spermine content of the liver was not altered by hydrazine treatment. Therefore, it would seem less likely that the increase in spermidine in the liver of the hydrazine-treated rat was the result of a conversion from spermine than due to synthesis de novo from putrescine. However, the hydrazine-treated animals showed a small decrease in their hepatic spermine levels at the 48 hr time point as compared to the control animals when the results were expressed on a mg DNA basis. By 48 hr after hydrazine treatment, putrescine levels were returning to the levels observed for the control animals; therefore, at the 48 hr point and perhaps at slightly later time points, some conversion of spermine to spermidine may have been occurring which would account for the elevated levels of spermidine.

It has been suggested that GABA is a product of the catabolism of the polyamines in rat liver and brain (160). In bacteria, during the oxidation of these amines, the segment of these polyamines derived from putrescine is oxidized to γ-aminobutyraldehyde followed by dehydrogenation to form GABA (181). In the present study, the appearance of GABA in the liver within one hour after hydrazine treatment (Table XI) adds further support for the existence of such a pathway in rat liver. The other known alternative pathway of forming GABA is via the decarboxylation reaction catalyzed by glutamic acid decarboxylase, an enzyme known to be present in extremely low concentration in mammalian liver (166). Furthermore, this enzyme has been shown to
be inhibited by hydrazine in vivo when assayed in brain tissue (25) and might be expected to be inhibited in rat liver. Even though GABA has been known principally as a neuro-inhibitory transmitter in mammals, Seiler et al. (160) have demonstrated the incorporation of radioactive carbon atoms from putrescine into GABA in various rat tissues including the liver. These workers observed the formation of \(^{14}C\)-GABA within one hour after injecting \(^{14}C\)-putrescine intraperitoneally into rats. In their study a greater proportion of the exogenously labeled putrescine injected was found in the liver as GABA rather than as spermidine. No clear explanation for their findings can be offered at the present time, but one might speculate that possibly the exogenous putrescine might not have entered the endogenous cellular pools of putrescine, and therefore was directed preferentially into the catabolic pathway leading to GABA instead of the anabolic pathway leading to spermidine.

Russell et al. (178) reported a biological half-life of about 90 min for intraperitoneally injected \(^{14}C\)-putrescine as assayed in the rat liver. The rapidity of the loss of the labeled putrescine is somewhat surprising in view of the results reported by Williams-Ashman et al. (157). These workers determined the hepatic polyamine level after allowing tissues to autolyze for four hours at room temperature and found no differences in their levels when compared to those obtained from livers removed by a freeze-clamp technique. Although these workers did not measure turnover per se, their results would suggest that endogenous polyamines had a relatively slow rate of turnover in vivo.

As mentioned in the introduction, the intent of this study was to delineate the course of metabolic alterations resulting in the increased
liver ornithine content in response to hydrazine treatment. In the present study, hydrazine produced peak elevations in hepatic ornithine levels that were nearly ten-fold greater when determined in the supernatant fraction prepared for the ODC assays (Figure 2) and were six-fold greater in whole liver homogenates (Table II) than the corresponding control levels. The difference in the time-course of peak elevation observed between these two types of preparations already has been ascribed to the separation and subsequent discarding of the mitochondrial fraction during centrifugation of the homogenates.

The initial elevation of ornithine levels coincided with decreased OTA activity due to hydrazine treatment. As previously mentioned, this decreased OTA activity was sustained throughout the 24 hr period examined and could be responsible for ornithine levels remaining elevated at 24 hr and at later time points. The additional rise of ornithine levels (between 4 and 24 hr) appears to be a reflection of the slight decrease in OTC activity which reached a nadir at 12 hr after hydrazine treatment. A small decrease in OTC would create a greater proportional change in ornithine levels than a much larger decrease in OTA because of the relative rates of activities of the two enzymes indicated above. The later decline of ornithine levels then seems to reflect increased utilization of ornithine in the reaction catalyzed by OTC.

Both Simonsen and Roberts (32) and Roberge et al. (167) have suggested that argininosuccinate synthetase is the rate-limiting step in urea production following hydrazine treatment. This "blockage" of the urea cycle would play a role in the increased ornithine levels and
would help account for the increased levels of citrulline found in the present study in response to hydrazine treatment. However, even though the concentration of ornithine may be elevated as a result of a block in the urea cycle, it should be remembered that de novo biosynthesis of ornithine per se does not occur in the urea cycle. Thus, the alterations responsible for the increased ornithine levels must ultimately be due to factors outside of the urea cycle.

Increased catabolism of skeletal muscle protein could be partially responsible for the observed increase in hepatic ornithine levels. Banks (31) has suggested a "small mobilization of skeletal muscle amino acids through protein catabolic pathways summed over the large muscle mass" could account for some of the increased amino acid levels observed in hepatic tissues. Although ornithine is not usually found in protein, increased levels of this amino acid were reported in skeletal muscle from rats treated with hydrazine (31). Perhaps this rise in muscle ornithine concentration could be a function of the arginase activity present in skeletal muscle.

The mobilization of amino acids from the muscle to the liver could supply the increased hepatic amino acid levels which then could be used for the apparent stimulation of the hepatic protein synthesis as described in the introduction. This increased incorporation of amino acid into hepatic protein in response to hydrazine reported by Banks (31) and Smith (34) followed an initial period of decreased incorporation of amino acid into hepatic protein in both hydrazine-treated and control animals. In the animals receiving hydrazine treatment, the suggested decrease in protein synthesis during the first several hours
followed by a subsequent increase in protein synthesis can be correlated
with observations from this present study and studies from other labora-
tories which indicate that the hepatic response to hydrazine treatment
may be described as an initial "degenerative phase" followed by a
"regenerative state" with respect to cellular metabolism. It should
be noted that the observed increase in ODC activity occurred during
this "degenerative phase," possibly setting the stage for the liver to
initiate the "regenerative phase" to return the liver to the normal
condition after the insult produced by hydrazine treatment. With the
consideration of these two phases and the apparent role played by ODC,
it is interesting to compare the events following hydrazine treatment
to those found in regenerating liver following partial hepatectomy.
Much of the early work involving ODC and polyamine metabolism utilized
the regenerating rat liver as a model system. It has been shown that
ODC activity increases ten to forty-fold during the first few hours
following partial hepatectomy (101, 128) in comparison to the fifteen-
fold increase in response to hydrazine treatment observed in the present
study. The similarities in the Km's determined for ODC from rat liver
following either hydrazine treatment of partial hepatectomy in the present
study indicates that the enzymes in the two systems are probably the
same.

In regenerating liver, fatty globules begin to appear within the
first few hours until the infiltrates almost fill the parenchymal cells
by eight hours (182, 183, 184). At the same time glycogen stores can
be seen to fall to very low levels by 10 hrs and then reappear gradually.
Soon after partial hepatectomy the mitochondria become swollen, pale
are reduced in number (182, 185). Hydrazine has been shown to produce the same histological changes with the appearance of fat droplets in the periportal and midzonal region and the depletion of glycogen deposits in the same areas within four hours after exposure to the compound with both of these alterations being maximal at 24 hr after hydrazine administration (11). Hydrazine treatment also causes early mitochondrial swelling (within 4 to 6 hr) with the return to a normal profile observed at 24 hr (186). In addition, mitochondrial generation of ATP is stimulated, accompanied by increased state 3 respiration, phosphorylation rate, and respiratory control and ADP:O ratios in mitochondria isolated from the livers of animals 17 hr after partial hepatectomy or 24 hr after hydrazine treatment (187). Finally, the magnitude of increases in protein, RNA and DNA content and protein biosynthesis following hydrazine treatment (31, 34, 188) have been shown to be comparable to those following partial hepatectomy (180, 182, 189).

In regenerating hepatic tissue from rats, there is a long period of over 12 hr before a sudden burst of incorporation of labeled precursor \(^3\text{H-}\) thymidine into DNA is observed which peaks at 24 hr and gradually declines (180). A rise in the rate of RNA synthesis of two to three-fold that of controls is observed within the first three to six hours and then persists (180). A net increase in hepatic protein is not detectable until about twelve hours, with the most rapid rate of synthesis being from 12-36 hr following partial hepatectomy (180). Rates of synthesis for DNA and RNA have not been reported following hydrazine treatment; however, preliminary results in our laboratory indicate that there appears to be a decrease in incorporation of
labeled precursor into RNA in the early stages (4 hr) following hydrazine treatment, after which there is an increased incorporation of precursor into RNA in rat liver (190).

In addition, studies reporting the activities of the enzymes involved in ornithine metabolism revealed changes in activities of similar magnitude, though following a different time-course, as observed in the present study. Arginase and OTC activities were decreased with a maximal change (23% and 52%, respectively) observed four days after partial hepatectomy (191). OTA activity also was reported to be decreased following partial hepatectomy, although the time that this change occurred and the percent of change were not reported (168).

Some additional general observations resulting from the present study could support this division of the response to hydrazine treatment into "degenerative" and "regenerative" phases. The initial decreases in observed PDC and arginase activities might be due, in part, to the generalized catabolic effect soon after the animals received hydrazine. These enzymes returned to at least basal levels at a later stage in the response to hydrazine. The elevation of many of the amino acids, two hours following hydrazine treatment (Table II), tends to add further support to the postulation of the early generalized catabolic effect referred to above. Thus, these observations and the biochemical and morphological events in response to hydrazine treatment together with those related to liver regeneration followed partial hepatectomy, present the possibility that hydrazine might be producing a "chemical" partial hepatectomy which may in turn explain the hydrazine-
induced alterations described in this study.

In the preceding discussion, there have been several explanations for the observed alterations in hepatic ornithine metabolism in response to hydrazine treatment. In reviewing the effects of hydrazine, one could speculate that these effects may be mediated, at least partially, through the action of growth hormone. Stimuli for the secretion of growth hormone by the pituitary gland are present in the hydrazine-treated animal. Although the fasting state is reported to cause a slight increase in plasma growth hormone levels, this increase would occur in both control and hydrazine-treated animals. However, hypoglycemia results in a marked (25 to 50-fold) rise in plasma levels of growth hormone within 30 min in humans (192). Underhill (9) first described the hypoglycemic effect of hydrazine in dogs and rabbits and a similar effect was later shown in the rat (23). Growth hormone has been shown to increase incorporation of amino acids into protein and to increase lipid mobilization (192). Fausto (130) demonstrated that ODC activity was enhanced twenty-fold at three hours after intraperitoneal injection of growth hormone (0.55 unit). In his study, simultaneous injection of puromycin, an inhibitor at the translational step in protein synthesis, blocked the effect of the hormone. However, Actinomycin D (0.8 mg/kg) was without effect on the growth hormone-induced enhancement of ODC activity when given concurrently with the hormone, but was shown to block the increase in activity when given 20 min prior to hormone treatment. If part of the response to hydrazine treatment were due to the release of growth hormone by the pituitary, the administration of Actinomycin D at the same time as hydrazine would be comparable to the injection of
Actinomycin D 20 min before growth hormone in the study cited above. Under these conditions the results reported in Table VII for Actinomycin D treatment of the hydrazine-treated animals are similar to the response observed by Fausto (130) after treatment with growth hormone and Actinomycin D. Thus, the presence of an adequate stimulus for the release of growth hormone and the similarities of the alterations due to growth hormone to those changes induced by hydrazine treatment suggest that this proposed mechanism of action should be considered for hydrazine.

Although the specific mechanism of action for the hydrazine-produced changes in ornithine metabolism is not known, the observed alterations in hepatic ornithine and polyamine concentrations seemed to be a reflection of changes in enzyme activities or enzyme levels. It has "become evident that the mechanism of regulation of metabolism involves an ongoing process of protein turnover to which most proteins are subjects" (193). Ratner (193) proposed that, in the case of arginase, any increase of enzyme levels during starvation or while being fed a high (70%) protein diet is mediated primarily through an increase in biosynthesis since the rate of degradation did not change. However, this is in opposition to a report by Schimke (163) who observed that the rate of biosynthesis of arginase during fasting was relatively constant and the increased arginase levels were due to a cessation of protein degradation. However, one could propose that the observed increase in ODC activity and possibly the activities of other enzymes in the present study might be the end result of the stabilization of the mRNA template coding for the individual enzyme. Russell et al. (105)
showed that there was no difference in the half-life of ODC from normal or regenerating liver following partial hepatectomy. This finding would indicate that more enzyme was being synthesized rather than there being a decreased inactivation of ODC. If each mRNA template coding for ODC were stabilized, a greater number of "ODC enzyme proteins" might be synthesized against each mRNA template through the translational process of protein biosynthesis. The possibility of the polyamines binding to nucleic acids has been shown in vitro, and the implication has been made for their role in the metabolism of RNA (108). A precedent for the presence of mRNA templates for individual enzymes having different half-lives has been reported in a study comparing the half-lives of various mRNA templates in normal liver with those from hepatomas (194).
It was the intent of this study to delineate the alterations in hepatic ornithine metabolism which would explain the increased hepatic ornithine levels after hydrazine treatment previously observed in our laboratory. In attempting to accomplish this task, the activities of hepatic enzymes catalyzing the biosynthesis, interconversion and degradation of ornithine were examined at various times after administration of hydrazine. The extraordinary elevation of ODC in response to hydrazine treatment directed the investigation to include alterations in polyamine levels in response to hydrazine treatment in order to gain greater insight into the role of ODC in hepatic metabolism. The principle findings in this thesis that can be ascribed to hydrazine treatment are described below.

Endogenous hepatic ornithine levels were shown to be elevated at 12 hr in supernatant preparations (1000% of control) and at 24 hr in homogenate preparations (600% of control). This increase seemed to be a result of an imbalance in hepatic ornithine metabolism. Hepatic arginase activity was found to reach its nadir at 4 hr (70% of control). Thus, arginase did not appear to be responsible for the increased ornithine levels. Hepatic ornithine Δ-transaminase activity was decreased (40% of control) at 4 hr and this level of activity was sustained throughout the 24 hr period examined. Hepatic ornithine transcarbamylase activity was shown to be decreased maximally at 12 hr (40% of control). The decreased activities of these two enzymes offers an explanation for the increased hepatic ornithine levels. The increase
in ODC activity which reached a maximum at 4 hr (1500% of control) was not thought to influence significantly the levels of hepatic ornithine due to the greater relative activities of the other enzymes involved. This increase in ODC activity was followed by sequential elevations in total endogenous hepatic putrescine and spermidine levels. The total endogenous hepatic spermine levels were not altered except for a minimal decrease at 48 hr.
REFERENCES


190. W. L. Banks, Jr. and C. Casey, Unpublished Results.


