DIETARY EFFECTS ON THE ENZYME ACTIVITIES OF THE HYPOTHESIZED LYSINE-UREA CYCLE IN RATS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MICHAEL A. ABRUZZO
1973

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ABSTRACT

DIETARY EFFECTS ON THE ENZYME ACTIVITIES OF THE HYPOTHESIZED LYSINE - UREA CYCLE IN RATS

by

Michael A. Abruzzo

Rat livers were analyzed *in vitro* for the purpose of studying the possible conversion of lysine to urea. The data indicate that lysine is converted to homocitrulline in a reaction dependent on carbamyl phosphate. Also, it is indirectly shown that this homocitrulline is then metabolized to homoargininosuccinate. Homoarginine conversion to urea and lysine is also demonstrated. The possible interrelationship of these reactions in a "lysine - urea" cycle is discussed.

Rats were fed high protein and high lysine diets to study the response of the above reactions and the reactions of the Krebs-Henseleit urea cycle to these conditions. It is shown that on the high protein diet the reactions of both urea cycles increased in activity. On the high lysine diet, the activity of ornithine

transcarbamylase and argininosuccinic acid synthetase decreased, while arginase increased. The conversions from homocitrulline to homoargininosuccinate and from homoarginine to urea increased in activity in rats on high lysine diets. The possible *in vivo* stimulation of arginase by lysine, and the possible role of the "lysine - urea" cycle in patients with urea cycle disorders is discussed.

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by

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A Thesis

Submitted to

Michigan State University

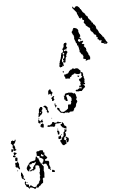
in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1973



To

Linda

Mike and Alison

ACKNOWLEDGMENTS

I wish to thank my professor, Dr. James V. Higgins for his many suggestions and criticisms, not only throughout this study, but during the many years I spent in his laboratory.

Special thanks are due Dr. Arthur F. Kohrman for the many hours he spent with me planning experiments, for making his laboratory available for most of the crucial work, and for his untiring advice and support. I also thank Dr. James Asher and Dr. Herman Slatis for their critical reading of the thesis. The many helpful suggestions, not only for this work but also for future studies, are invaluable.

A very special thanks are due Dr. Ajovi B. Scott-Emuakpor for his friendship and advice in my first years as a graduate student. His academic outlook was an inspiration and a guide in my research and teaching.

I thank Dr. Dorice Narins for her assistance on the development of the diets and feeding procedures. I also thank Habib Fakhrai for his friendship and help in the urea determinations. Thanks are also due Terry Hassold, Larry Yotti, LouBetty Richardson, Diana Smith, and Chris Liebrock for their friendship, help, and encouragement

over the last five years. Life at M.S.U. would not have been the same without them.

Thanks are also due Bruce Corigliono, Carlo Picione, Bob Pandolfi, Dan Mankoff, and Cathy Blight for their assistance throughout this work.

For their friendship, and encouragement, I thank Ron and Carol Wilson, Dan Friderici, Gary Marsiglia, Astrid Mack, Rachel Rich, Frankie Brown, and Michelle Kipp.

This work was supported in part by a grant from the Society of the Sigma Xi.

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INTRODUCTION

It has been known for many years that in individuals with proven enzymatic errors in urea synthesis normal or near normal urea production occurs. The question is how does the biosynthesis of urea occur in these individuals. There is much speculation on the possible mechanisms for this synthesis.

In 1969, studies in our laboratory on rat livers indicated that there may be an alternate urea cycle. The results of these earlier studies led to the hypothesis of a cycle with reactions analagous to the classic Krebs-Henseleit urea cycle. In this, it is believed that lysine is converted to homocitrulline which is subsequently metabolized to homoarginine which is then hydrolyzed to urea and lysine, thus completing the cycle. This lysine - urea cycle was not totally demonstrated because of the inability to detect homocitrulline metabolism. Speculation on the possible adaptive role of this hypothesized cycle in a patient with citrullinemia centered around the mechanisms or environmental stimuli that could cause an increase in

the synthesis of urea by this pathway. It was speculated that in patients with an enzymatic error in the Krebs-Henseleit urea cycle, the activity of the lysine - urea cycle increases and thus takes over the function of urea production.

The purposes of this study are:

(1) to test for the ability of rat liver to catalyze the postulated reactions of the lysine - urea cycle, with emphasis on the metabolism of homocitrulline which is the only step in the cycle which has not been demonstrated

(2) to study the response of the reactions of the lysine - urea cycle to dietary differences in rats

It is hoped that these studies will yield further information on the basic question: how is urea produced in individuals with inborn errors in the Krebs-Henseleit urea cycle.

LITERATURE REVIEW

INTRODUCTION

The first report of a metabolic error affecting the urea cycle was in 1958. Since that time the relationships of urea synthesis, ammonia toxicity, and ammonia detoxication have been studied in detail. The possibility of alternate mechanisms for urea synthesis arose because of the finding of normal urea synthesis in individuals with urea cycle errors. The metabolic basis of ammonia toxicity and the regulatory mechanisms for the maintenance of normal blood ammonia levels have now been brought to light, mainly due to studies of urea cycle disorders.

AMMONIA FORMATION

The major source of ammonia nitrogen is dietary protein. Ammonia is formed in many metabolic reactions within the tissues and also by bacterial enzyme activity within the intestine. Whatever the source of ammonia, it is rapidly metabolized to maintain a very low level in blood.

There are two bacterial enzymes present in the intestine of mammals which will form ammonia. The most important one

is urease which will split urea into ammonia and CO_2 (Calloway et al., 1966). The urea is present in the intestine because of its free diffusion from the blood. Ammonia is formed at a much greater rate than urea diffusion and the formed ammonia is reabsorbed along the entire length of the intestine at a high rate (Bourke et al., 1966; Summerskill, 1966). Most authors believe that there is no endogenous urease in the intestine (Levenson et al., 1959) but in a more recent study activity was detected in the intestine after treatment with antibiotics to eliminate the bacterial flora. If there is any endogenous urease activity, it is minor when compared to the bacterial enzyme (Summerskill and Wolpert, 1970). The other bacterial enzyme present in the intestine is glutaminase which splits glutamine (liberated by protein hydrolysis) into glutamic acid and ammonia.

The ammonia formed by these two reactions then enters the portal circulation and undergoes detoxication in the liver. Bacterial activity accounts for 20 - 25% of the daily ammonia production.

Many intracellular reactions in mammals form ammonia as one of the products, the most important of which are glutamate dehydrogenase and glutamine degredation. The other reactions are only minor contributors to the ammonia pool but when considered in totality they can be important as possible causative factors in ammonia toxicity.

In the glutamate dehydrogenase system, amino acids are first transaminated with α-ketoglutarate to form glutamic acid which is then converted back to α-ketoglutarate with the liberation of ammonia. NAD+ and NADP+ serve as coenzymes. ADP and amino acids stimulate the reaction and NADH and NADPH inhibit it (Frieden, 1963, Hershko and Kinder, 1966).

Oxidation deamination of amino acids can also lead to ammonia formation (Levin, 1971) but most of the amino acid oxidase activity is due to transamination and glutamate dehydrogenase. More important than oxidative deaminations are amine oxidases which utilize dopamine, histamine, tyramine etc., as substrates (Colombo, 1971). There are also non-oxidative deamination systems for certain amino acids but these are very minor.

Nucleotide and Nucleoside deaminases also give rise to ammonia. This source of ammonia is most important in muscle. It has been shown that after muscular work, ammonia production doubles and is due mainly to the deamination of 5' adenosine monophosphate (Parnas et al., 1927, Schmidt, 1928). The amount of ammonia released depends on the type and adaptive characteristics of the muscle that is exercised (Gerez and Kirsten, 1965).

Folk and Cole (1965) have shown a transglutaminase which splits ammonia from protein bound glutamine molecules.

Ammonia is also liberated in the synthesis of heme (Levin, 1971).

The production of ammonia in the kidney deserves special attention because it is a major source of arterial and urinary ammonia. Glutamine serves as the major precursor of renal ammonia under conditions of both normal acid base balance and mild ammonium chloride acidosis. The resulting glutamic acid may also serve as a potential source of renal ammonia. Other amino acids do not contribute appreciably to renal ammonia production at normal plasma amino acid levels (Owen and Robinson, 1963, Stone and Pitts, 1967).

From all of the above reactions, it is quite clear that the body has the capability to produce large quantities of ammonia. It is also evident however, that blood ammonia levels are very slight, usually less than 45 ug/100 ml even though 70 grams or more of protein are ingested daily by an adult (Levin, 1971). These two facts indicate that ammonia once formed is rapidly detoxified.

AMMONIA DETOXICATION MECHANISMS

Ammonia is normally detoxified by several reactions, the most important of which is mitochondrial carbamyl phosphate synthetase, the first reaction in the formation of urea. Acetylglutamate activates this reaction and the formation of acetylglutamate is stimulated by L - arginine

(Kim et al., 1972). Under high arginine conditions this mechanism is capable of detoxifying a potentially lethal dose of ammonium acetate and therefore points to the importance of urea formation as an ammonia detoxication mechanism.

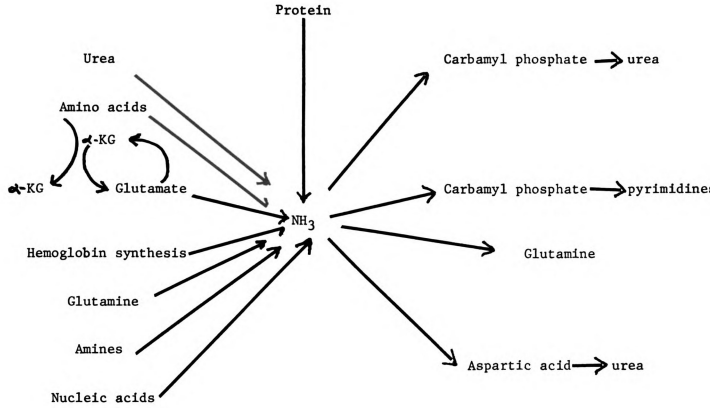
Carbamyl phosphate synthetase in the cytosol, is also a mechanism for ammonia detoxication but unlike mitochondrial carbamyl phosphate synthetase, the ammonia so fixed by this enzyme does not enter into the structure of urea but is incorporated into pyrimidines. This reaction system functions only when pyrimidine synthesis is required and therefore ammonia concentrations do not appreciably stimulate this mechanism (Bresnick, 1963).

Glutamine synthetase serves as a detoxication reaction in the liver and the brain. The reaction is:

$$\text{Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{glutamine} + \text{ADP} + P_i$$

The resulting glutamine can then be split by glutaminase I which functions in the kidney and the liver. In the kidney, the liberated ammonia is utilized as a urinary buffer and in the liver it enters the urea cycle by way of mitochondrial carbamyl phosphate synthetase (Colombo et al., 1967). The formation of glutamine in the brain is the major detoxication mechanism in this tissue. Figure I summarizes the ammonia formation and detoxication reactions found in mammals.

AMMONIA FORMATION AND DETOXICATION



AMMONIA TOXICITY

The toxic effect of ammonia was first shown by Kirk and Sumner (1931, 1932). They injected urease into rabbits and noted convulsions and death in less than one hour. Since that time many studies have shown that high levels of ammonia are toxic (Clark and Eiseman, 1958, Bessman, 1958, Hutchinson *et al.*, 1964, 1965, Katanuma *et al.*, 1966, Schenker *et al.*, 1967, Roberge and Charbonneau, 1969, Walker and Schenker, 1970, Kim *et al.*, 1972). The actual mechanism of this toxicity is not clearly understood but evidence seems to indicate that the ammonia affects the energy metabolism of the brain.

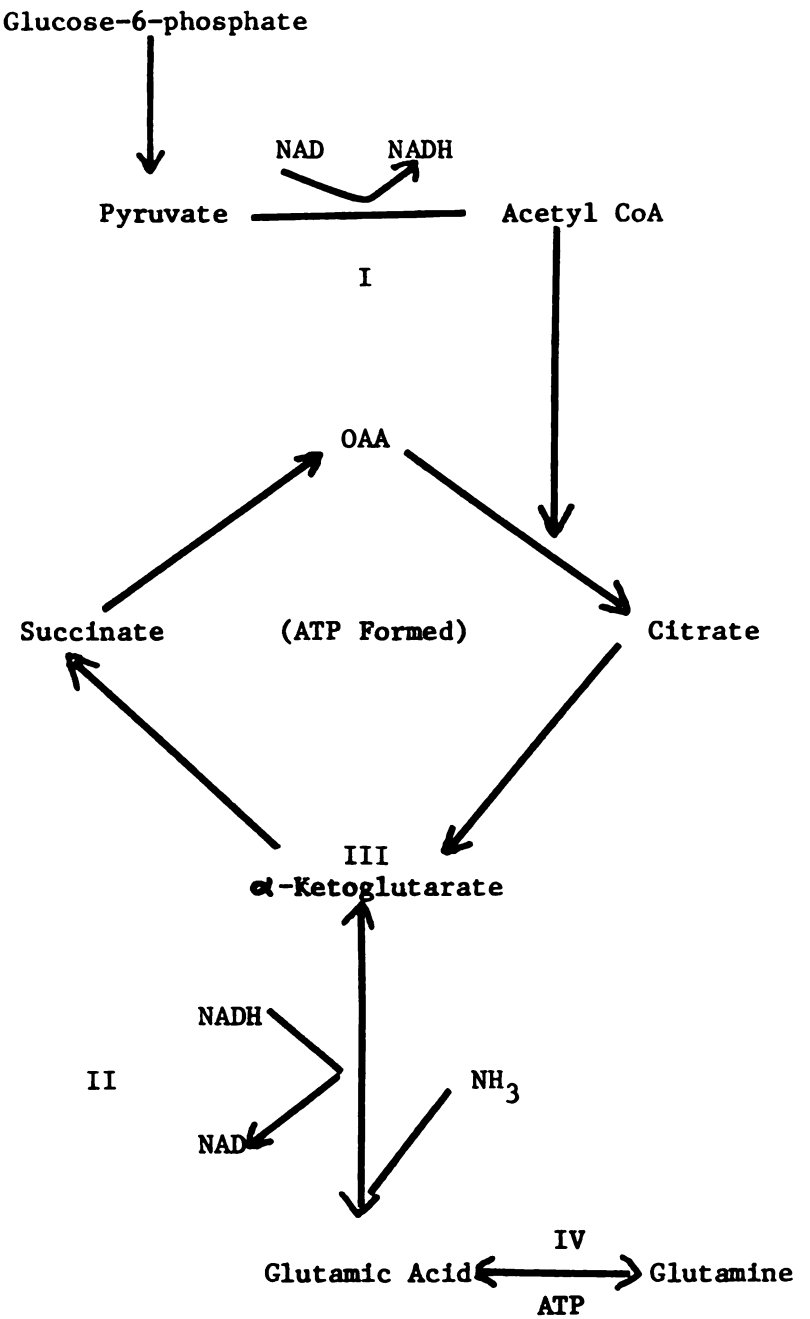
Bessman (1958) formulated the hypothesis that the toxicity of ammonia was due to a depletion of essential cerebral metabolites. The first direct evidence that toxic doses of ammonia *in vivo* acutely affects cerebral energy metabolism came from Schenker *et al.*(1967) who found that ATP and phosphocreatinine levels dropped significantly in the base of the brain in rats treated with ammonium-acetate. There are many possible reasons for reduced ATP. Studies with cat cortex mitochondria show that ammonia may interfere with the entry of pyruvate into the Krebs cycle and thus slow the cycle down (McKhann and Tower, 1961). However, that theory is disputed by

Walker and Schenker (1970) who could detect no affect of ammonia on pyruvate decarboxylase in the brain. Another theory, which is based on *in vitro* studies in rats, suggests that during detoxication of cerebral ammonia with the formation of glutamic acid from α-ketoglutarate the supply of available NADH, necessary for mitochondrial generation of cerebral energy, is depleted (Worcel and Erecinska, 1962). The hypothesis which gains the widest acceptance stipulates that ammonia toxicity depends on the depletion of cerebral α-ketoglutarate resulting in impairment of the Krebs cycle and a subsequent decrease in ATP synthesis (Walker and Schenker, 1970). Decreased α-ketoglutarate has been demonstrated in the brain of dogs and rats treated with ammonia (Bessman, 1961).

After the administration of ammonia chloride in rats, brain glutamine increased (Nakazawa and Quastel, 1968). Because of this the authors feel that the low ATP levels are due to increased consumption through the glutamine synthesis reaction. It has also been noted that ATPase activity is stimulated *in vitro* by ammonia but this even occurs at physiological concentrations of ammonia and therefore is probably not significant. Whatever the actual mechanism for depletion of cerebral energy, it appears that toxic levels of ammonia affect the brain by disrupting energy metabolism. Figure 2 summarizes the speculated sites of

Figure 2

POSSIBLE SITES OF AMMONIA TOXICITY



I. Impaired oxidative decarboxylation of pyruvate
II. Depletion in supply of NADH
III. Depletion of α-Ketoglutarate ⟶ decreased ATP synthesis by Krebs cycle
IV. Increased consumption of ATP through glutamine synthesis

ammonia toxicity in the brain.

POSSIBLE MECHANISMS FOR UREA PRODUCTION

Urea, the form of nitrogen waste excreted by mammals has classically been considered to be synthesized exclusively by the Krebs-Henseliet urea cycle (Figure 3). Ammonia may enter the cycle in two places: through carbamyl phosphate and through aspartate. This is believed to be the major mechanism for the synthesis of urea and subsequent ammonia detoxication. However, there are many observations and some evidence which point to the possibility of alternate mechanisms for urea synthesis.

In many individuals with inborn errors of the urea cycle, urea production is normal. Such normal production occurs even when direct assay of the hepatic enzyme in question shows zero activity (for a review of urea cycle errors, see Levin, 1971). When α-D, L-methyl aspartic acid is added to a liver homogenate, it completely inhibits argininosuccinic acid synthetase. However, the injection of this compound into rats did not inhibit *in vivo* urea synthesis (Cedrangolo *et al.*, 1962, Rowe and Miller, 1971). What is interesting is that these authors also noted that in the presence of glutamine as nitrogenous substrate the conversion of citrulline to urea is inhibited by methyl aspartate but there is no inhibition of urea synthesis. Because of these two findings; urea production in individuals

THE KREBS-HENSELEIT UREA CYCLE

$$NH_3 + HCO_3^- + 2ATP \xrightarrow[\text{acetyl glutamate}]{1 \quad Mg^{++}} \text{carbamyl phosphate} + 2ADP + P_i$$

$$\text{Carbamyl phosphate} + \text{ornithine} \xrightarrow{2} \text{citrulline} + P_i$$

$$\text{Citrulline} + \text{aspartate} + ATP \xrightarrow[Mg^{++}]{3} \text{argininosuccinate} + AMP + P_i$$

$$\text{Argininosuccinate} \xrightarrow{4} \text{arginine} + \text{fumarate}$$

$$\text{Arginine} \xrightarrow[Mn^{++}]{5} \text{ornithine} + \text{urea}$$

1. Carbamyl phosphate synthetase I
2. Ornithine transcarbamylase
3. Argininosuccinate synthetase
4. Argininosuccinate lyase
5. Arginase

Figure 3

with urea cycle errors, and continued urea production in the presence of a urea cycle inhibitor, many studies have been undertaken in a search for alternate mechanisms for the synthesis of urea.

One possible explanation for urea synthesis in individuals with urea cycle errors centers around the belief that the enzymes involved are not totally inactive and that the level of *in vivo* activity is sufficient for normal or near normal urea synthesis. This level of activity is not, however, believed to be sufficient for ammonia detoxication, thus leading to the usual hyperammonemia. Levin (1967) believes that if this theory is correct it can not be used to explain urea production in the patient he studied. His patient routinely produced 5 grams of urea per day and given a maximum *in vivo* activity of 2%, it would be impossible to explain this quantity of urea (by way of the urea cycle). Another theory based on *in vivo* activity of urea cycle enzymes being different than *in vitro* determinations is discussed by Scriver (1969). Necessary assumptions are that the patient has a mutant form of the enzyme and that this mutant has a much higher Km. In this way, the increased concentrations of urea cycle substrates found in these patients would allow normal urea production because the maximum rate of reaction would be reached at

the much increased substrate level. This theory gains support from the study of Tedesco and Mellman (1967) on skin fibroblasts of a citrullinemic patient. They found that normal urea production occurred when citrulline concentrations were raised above what was believed to be the maximum substrate concentration. An apparent Km shift could therefore explain normal urea production in patients with urea cycle errors. A problem with this hypothesis is that the patient studied by Tedesco and Mellman does not have normal urea production. Another problem with this theory is that the substrate accumulations noted are probably due to the defective enzyme and therefore can hardly be considered an adaptation to a Km shift (Levin, 1971). Under normal circumstances, urea cycle enzymes believed to be defective are assayed with excess substrate and are still found to have a very low, if not zero, activity and therefore refute or cast doubt on the Km shift theory.

Another possible theory is that the enzymes of the urea cycle exist as isozymes and although the liver isozyme is defective, urea synthesis will occur in the other tissues of the body. This theory is supported in part by the study of Vidailhet *et al*. (1971). They studied the tissues of a patient with citrullinemia (died at 7 1/2 months) and found that argininosuccinic acid

synthetase was completely absent in the liver while the kidney assay showed normal activity. The problem with this case is that the kidney arginase activity was zero and therefore normal urea production could not occur in this tissue. The authors could not, therefore, use the isozyme theory to explain their patients normal urea production without assuming a transport between tissues.

Some authors believe that urea production in the presence of a defective Krebs-Henseleit cycle is due to alternate metabolic cycle(s). This theory was first suggested by Bach (1939). He believed that glutamine could combine with ammonia and carbon dioxide to form glutamic acid and urea. That observation was later shown to be in error (Archibald, 1945). The urea production was due to arginine contamination. In 1961, Levin et al. suggested that an alternate cycle may exist but did not speculate on the possible mechanisms. Cedrangolo et al. (1962, 1963) suggested that alternate pathways of urea synthesis exist. This is based upon their work with methyl aspartate. They, like Levin, did not speculate on the possible pathways.

Cohen et al. (1968) suggested that urea could be produced from guanidinosuccinic acid. They postulated two possible pathways:

a) aspartate + arginine ⟶ ornithine + guanidinosuccinate

guanidinosuccinate ⟶ urea + aspartate

b) arginosuccinate + NH_3 ⟶ ornithine + carbamyl aspartate

carbamyl aspartate + NH_3 ⟶ guanidinosuccinate

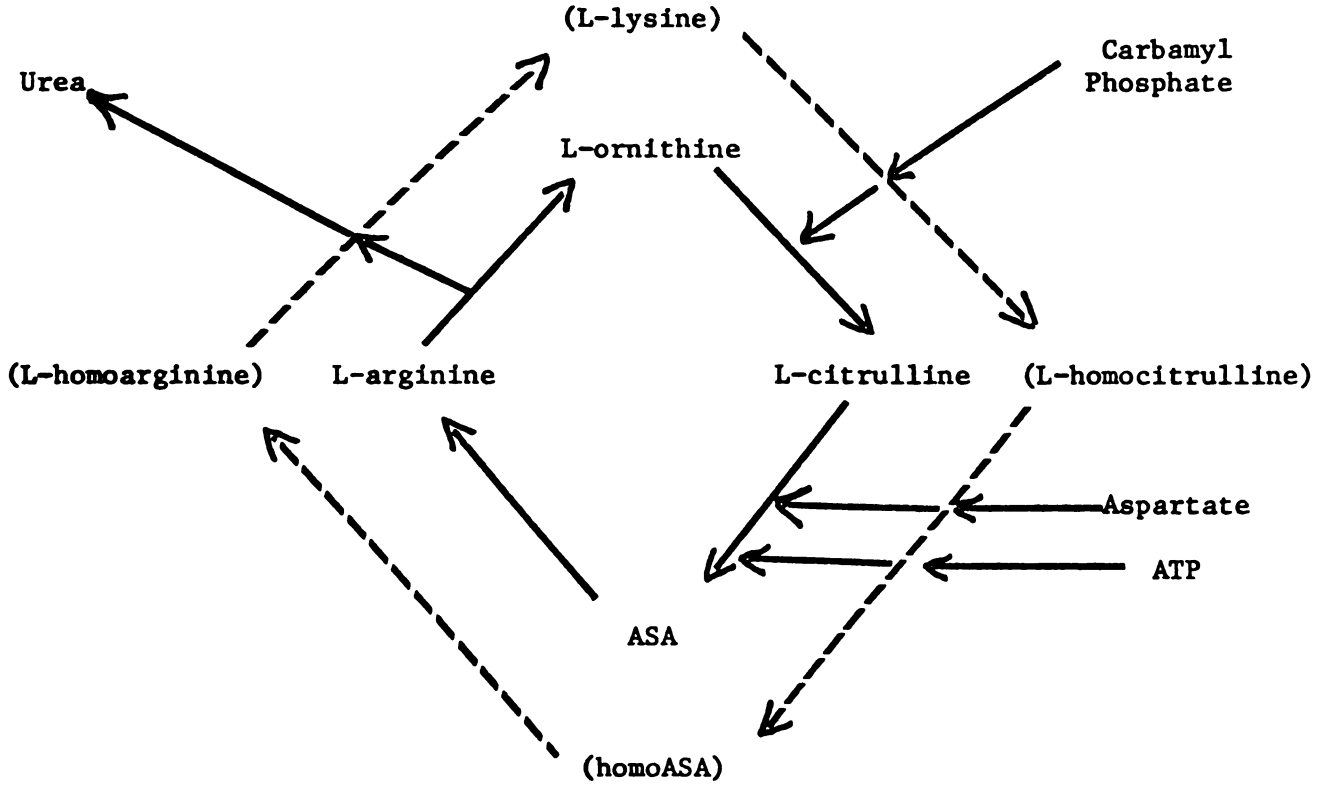
guanidinosuccinate ⟶ urea + aspartate

The evidence for these pathways comes from their finding of guanidinosuccinate in the urine of patients with uremia. The reactions are only speculative and further work by Stein et al. (1969) shows that guanidinosuccinate production requires an intact urea cycle, the evidence being the finding of no guanidinosuccinate in the urine of individuals with urea cycle disorders. If this speculated pathway exists, it would appear as though it is not responsible for normal urea production in disorders of the urea cycle.

Another pathway for urea biosynthesis was postulated by Scott-Emuakpor (1970). This pathway (Figure 4) parallels the classic ornithine urea cycle utilizing the homocompounds (one additional methyl group in the carbon skeleton) of the intermediates of the ornithine urea cycle as substrates. There are many pieces of direct and indirect evidence which support this hypothesis.

Vidailhet et al. (1971) described a patient with elevated homocitrulline, homoarginine, lysine, ornithine, and citrulline. Liver biopsy showed that there was zero activity for argininosuccinic acid synthetase. Likewise, these same amino acids were found to be elevated in a

A SPECULATED PATHWAY FOR UREA SYNTHESIS



------- The hypothesized Lysine-Urea Cycle

——— The Ornithine-Urea Cycle

ASA = argininosuccinic acid

L-lysine = homoornithine

Figure 4

patient described by Scott-Emuakpor et al. (1972). The patient has no detectable activity of argininosuccinic acid synthetase in either his skin fibroblasts (Scott-Emuakpor et al., 1972) or lymphoblasts (Spector and Bloom, 1973). This patient, like most with urea cycle disorders, has normal urea production but unlike most also has normal blood ammonia levels.

Homoarginine and homocitrulline have been detected in the urine of patients with hyperlysinemia and in normals after lysine loading (Woody, 1967, Armstrong and Rabinow, 1967, Ghadimi et al., 1965, Colombo, 1971, Ryan and Wells, 1964). In 1964, Ryan and Wells showed that lysine could be converted to homocitrulline and homoarginine. They injected C^{14} - lysine into rats and isolated C^{14} - homocitrulline and C^{14} - homoarginine. The reaction mechanisms were not known. In 1968, Ryal et al. showed that homocitrulline production from lysine was enzymatic. They, however, could not show any production of homoarginine, nor could they detect any further metabolism of homocitrulline. They were also able to show that homoarginine could be cleaved by arginase into lysine and urea. This was not confirmed by Colombo(1971). Scott-Emuakpor (1970), however, was able to demonstrate homoarginine conversion to urea and lysine by both rat liver homogenate and commercial arginase. The conversion of lysine to homoarginine was shown by Ryan et al. (1969) to occur in rat kidney by

transamidination.

The reaction mechanism for the conversion of lysine to homocitrulline was demonstrated by Scott-Emuakpor (1970). Lysine was converted to homocitrulline by rat liver homogenate in a reaction that was linear in relation to protein, carbamyl phosphate, and lysine concentrations, and time of reaction. The transcarbamylation of lysine was not found to occur when human ornithine transcarbamylase was the enzyme source (Colombo, 1971). Strandholm et al. (1971) were able to show the enzymatic conversion of homoargininosuccinic acid to homoarginine and fumarate in pig kidney. This conversion is one of the speculated reactions of the lysine - urea cycle.

In summary, the finding of elevated levels of the homocompounds and normal urea cycle substrates in patients with citrullinemia and hyperlysinemia is indirect evidence for the existence of the lysine - urea cycle. Lysine loading tests also support this hypothesized cycle. Specific reactions of the lysine - urea cycle have been shown to occur by three independent researchers but two of these reactions have not been confirmed by one researcher and the third reaction has not been subjected to complete kinetic studies. The metabolism of homocitrulline, a necessary prerequisite of the postulated cycle has not been demonstrated. No evidence as to the nature of the enzymes which catalyze the speculated reactions has been

reported.

This cycle, although not confirmed, has substantial evidence in its favor. It is because of this evidence and because of the conflicts in the literature concerning lysine metabolism that the following experiments were performed. It is hoped that these experiments will illustrate the mechanism or mechanisms which allow normal urea synthesis in patients with inborn errors of the Krebs-Henseliet urea cycle.

MATERIALS AND METHODS

ENZYME STUDIES

The animals used in these experiments were from colonies of adult Sprague-Dawley rats obtained from Spartan Research Animals. They were routinely maintained on a Wayne laboratory block diet containing 24% protein. A rat was selected at random, killed by decapitation, and the liver was quickly removed and placed on ice. A one gram slice of liver was homogenized in either 19 ml or 9.0 ml of ice cold water depending on the assay to be run. The remaining liver was frozen at -80°C. The activities of the enzymes assayed from the frozen livers were not significantly different from those for fresh liver. This is in agreement with Hutchinson et al. (1964).

The assays were based on the colorimetric determination of urea, citrulline and homocitrulline, by a modification of the method of Archibald (1944). The color reagent used for all determinations was diacetyl monoxime (0.75% in water). For color development, an aliquot of the reaction

tube was mixed with 1 ml of the color reagent and 5 ml of acid reagent prepared as follows: commercial grade sulfuric acid (concentrated), phosphoric acid (85%), and water in a ratio of 1:3:6. The tubes were boiled for 15 minutes and cooled in a room temperature water bath for 10 minutes. The color was read at 490 nm for citrulline and homocitrulline and at 480 nm for urea. The color was stable for 2 hours when tubes were kept in the dark.

The methods used for the assays of ornithine transcarbamylase and arginase are patterned after those of Schimke (1962). The unit of activity for these two enzymes is expressed as uMoles of product formed per hour per gram wet weight of liver. The method used for argininosuccinic acid synthetase is a modification of the method of Wixom et al. (1972). The unit of activity for this enzyme is expressed as uMoles of substrate depleted per hour per gram wet weight of liver. Protein was determined by the method of Lowry et al. (1951).

Because the assays were performed on rat liver homogenates the actual enzymes that catalyze the lysine to homocitrulline, homocitrulline to homoargininosuccinate, and homoarginine to urea reactions are not known. The methods for these assays are described under the methods for ornithine transcarbamylase, argininosuccinic acid

synthetase and arginase for simplicity. It is not to be inferred that these enzymes are catalyzing the reactions of the lysine - urea cycle. The methods described only test the ability of a rat liver homogenate to catalyze the lysine - urea cycle reactions.

Ornithine Transcarbamylase

This enzyme activity was determined as the rate of citrulline formation. The assay medium contained 50mM glycyl-glycine buffer, 20mM dilithium carbamyl phosphate, 15mM L-ornithine, and 50 ul of a 1:20 liver homogenate. The final volume of the medium was 1.0 ml and the pH 8.3. The medium was prepared immediately before the assay was performed and kept on ice because of the instability of the carbamyl phosphate. To start the reaction, the tubes were removed from the ice bath and incubated for 15 minutes at 37.5° C. The reaction was stopped with the addition of 1.0 ml of 30% perchloric acid, and centrifuged for 10 - 20 minutes at 2000 rpm. 50 ul of the supernatant were used for color development. When homocitrulline production was assayed L-lysine was substituted for ornithine in the same concentration, but the liver homogenate volume was 500 ul. The final volume of the medium was 1.0 ml and the pH 8.3. 1000 ul of the supernatant was used for the color reaction.

Argininosuccinic acid synthetase

The activity of this enzyme was measured as the depletion of citrulline or homocitrulline, depending on the substrate used in the assay. The assays were performed by two different methods; one with an ATP regenerating system and one without. The assay medium with an ATP regenerating system contained 200mM tris buffer, 5mM aspartate, 5mM magnesium sulfate, 2mM L-citrulline or L-homocitrulline, 1mM ATP, 2mM phospheonolpyruvate (PEP), 2 ug pyruvate kinase, 0.5 mg urease, and 500 ul of 1:20 liver homogenate for citrulline as substrate or 500 ul of 1:10 liver homogenate for homocitrulline as substrate. The final volume was 1.0 ml at a pH of 7.6. The reaction medium without an ATP regenerating system was the same except that ATP was increased to a 5mM concentration and neither PEP nor pyruvate kinase added. The mediums were prepared shortly before the assays and kept on ice. The reaction was started by removing the tubes from the ice bath and incubating at 37.5° C for 30 minutes (citrulline as substrate) or for 120 minutes (homocitrulline as substrate). The reaction was stopped by boiling for five minutes and the tubes were cooled and centrifuged for 30 minutes at 2000 rpm. For color development, 50 ul of the supernatant was used for either homocitrulline or citrulline determinations.

Arginase

This reaction was measured as the production of urea. The assay medium contained L-arginine or L-homoarginine in a concentration of 0.250 M, pH 9.7, and 0.001 M $MnSO_4$. For the arginine medium 20 ul of a pre-treated liver homogenate was added and for the homoarginine medium 100 ul of this homogenate was added. The final volume of the reaction medium was 0.5 ml. To prepare the treated liver homogenate a 1:10 homogenate was incubated with an equal volume of 0.1 M $MnSO_4$ for 5 minutes at 55° C. The tubes were then centrifuged for five minutes at 2000 rpm and the supernatant used as the enzyme source. The reaction tubes were kept in an ice bath until the reaction was started by incubating the tubes at 37.5° C for 15 minutes. The reaction was stopped by the addition of 1.0 ml of 30% perchloric acid. The tubes were centrifuged for 10 - 20 minutes at 2000 rpm and 50 ul (arginine as substrate) or 500 ul (homoarginine as substrate) are used for color development.

Radio - Isotope Assay

In an attempt to show the conversion of L-lysine to urea, a radio-isotope assay using C^{14} - lysine was developed. The reaction medium contained 0.05 M potassium phosphate ($KH_2 PO_4$) buffer, 0.003 M $MgSO_4$, 0.01 M L-aspartate, 0.03 M L-lysine, 0.004 M ATP, 0.002 M PEP, 0.5 ug pyruvate kinase, arginase (1 mg/ml),

dilithium carbamyl phosphate (in one of four concentrations: 0.0, 0.04 M, 0.08 M or 0.120 M), 50 ul of 1:20 liver homogenate, and 1 uC of L-lysine - C^{14} (U.L.) in a final volume of 300 ul at a pH of 7.5. All reaction tubes were prepared in an ice bath and the reaction was started by incubating in a water bath at 37.5° C. Tubes were incubated for one hour and the reaction was stopped by boiling for five minutes. The tubes were centrifuged for 30 minutes at 2000 rpm and the supernatant was used for the isolation of homocitrulline and homoarginine.

Homocitrulline and homoarginine were isolated by paper chromatography on Whatman 3MM paper. 50 ul of the supernatant was spotted (3 ul portions). Lysine, homocitrulline, and homoarginine standards were spotted for identification purposes. The chromatograms were run in a descending direction for 24 hours in a solvent of butanol, acetic acid, and water (12:3:5). The chromatograms were then dried in a fume hood and the standards selectively stained with ninhydrin reagent. Those areas corresponding to homocitrulline and homoarginine, utilizing the stained standards as a guide, were then cut out and placed in counting vials. 20 ml of dioxane counting fluid was added to each vial and allowed to stabilize in the dark for 24 hours before counting. Counting was performed on a Beckman room temperature liquid scintillation counter.

ANIMAL FEEDING EXPERIMENTS

To investigate the possible activation of the hypothesized alternate urea cycle and the enzymes involved in the reactions, an experiment was designed in which controlled groups of animals were fed three different diets. These animals were then killed and their blood and liver analyzed for specific enzyme activities and blood substances that could be considered related to the overall problem of the biosynthesis of urea.

Animals

The animals used in this experiment were taken from six litters of highly inbred colonies of Sprague-Dawley strain rats. They were raised from the day of weaning until the start of the experiment on Wayne Lab Blox (crude protein content, 24%). The animals were separated by sex before reaching maturity and were placed in similar cages and housed together in an animal care facility. All animals were the same age on the day of killing.

Diets

Table 1 shows the ingredients of the control and experimental diets. The casein, dextrine, salt mix, vitamin mix and agar were purchased from Nutritional Biochemicals, Cleveland, Ohio. The corn oil used was

Table 1

RAT CASEIN DIETS

	22% Protein	60% Protein	22% Protein + 5% Lysine
Casein (g)	660	1800	660
Dextrose (g)	555	150	555
Sucrose (g)	441	147	441
Dextrine (g)	591	150	591
Corn Oil (g)	450	450	450
Harper Salt Mix (g)	120	120	120
Vitamin Mix (g)	66	66	66
Lysine (g)	---	---	150
Water (ml)	3000	3000	3000
Agar (g)	105	105	105

Mazola brand. It should be noted that the 60% protein diet replaces carbohydrates with protein and that the high Lysine diet is exactly the same as the 22% protein diet except lysine is added. The diets were developed in this manner so that they would be isocaloric. The diet mixes were cut into blocks, weighed and placed directly into the cages. To determine intake, the uneaten portion of the diets was removed daily and weighed again. Because of this technique, the food consumption is only an approximation to the actual consumption.

Experimental Design

The experiment was structured as a completely randomized block design. There were nine blocks in the experiment formed as follows: blocks 1 through 6 contained males, three males from the same litter per block. The males were selected at random from each litter. Blocks 7, 8 and 9 were formed in the same manner except females were used. There was no blocking for weight. The weight differences were blocked as a secondary result of blocking for sex. The three treatments were the diets; 22% Protein (control), 60% protein, and 22% protein + 5% lysine. The treatments were assigned at random to the animals in a block. Table 2 is a summary of the blocks. The results from all of the determinations made on these

TABLE 2

EXPERIMENTAL DESIGN

THE BLOCKING

Blocks	Animals
1	3 males from litter 1
2	3 males from litter 2
3	3 males from litter 3
4	3 males from litter 4
5	3 males from litter 5
6	3 males from litter 6
7	3 females from litter 1
8	3 females from litter 2
9	3 females from litter 3

THE FEEDING

Diets	Animal Cages			
22% Protein	1,* 2	3,4	5,6	7,8,9
60% Protein	1,2	3,4	5,6	7,8,9
22% Protein + 5% Lysine	1,2	3,4	5,6	7,8,9

*--numbers represent the block from which animals were taken

rats were analyzed by an Analysis of Variance for completely randomized blocks design. The contrasts tested were: 22% protein diet vs 60% Protein and 22% protein diet vs 22% protein + 5% lysine. These contrasts were non orthogonal and were therefore tested using Bonferroni's t-test (Dunn, 1961).

All the animals were fed the 22% protein diet for seven days (time required to stabilize consumption rate) before the start of the experiment. At the end of the seven days, each treatment group (9 rats/treatment) was placed on the diet for that group for an additional ten days. During that period, each rat was weighed every two days and food consumption was determined daily. At the end of the ten days the rats were anaesthesized with ether and 5.0 to 10.0 ml of blood was collected from the vena cava. (There was no difference in the enzyme activity between the decapitated or ether killed rats). The livers of each animal were rapidly removed and frozen at -80° C.

Liver Enzymes

The activities of ornithine transcarbamylase, argininosuccinic acid synthetase and arginase were determined for each liver. In addition the ability of each liver homogenate to metabolize lysine, homocitrulline, and homoarginine was determined. These enzymes were

assayed as previously described except that each liver homogenate was prepared from 0.5 g in 9.5 ml of ice cold water. Each assay was run in duplicate along with appropriate blanks. All assays were run within three days from the date of killing of the animal.

Serum Ammonia Nitrogen Determination

The method for ammonia determination is a modification of that described by Chaney and Marback (1962). The freshly collected blood was refrigerated for fifteen minutes to allow clotting to occur. It was then centrifuged for ten minutes at 2000 rpm to separate the serum. The reagents for the determination are:

1) phenylnitroprusside solution - 50 grams of reagent grade phenol and 0.25 grams of sodium nitroprusside are dissolved in distilled deionized water.
2) alkaline hypochlorite solution - in 600 ml of distilled deionized water dissolve 25 grams of NaOH pellets, let cool, then add 40 ml of NaOCl (Clorox) and bring up to 1000 ml with distilled deionized water.

For the reaction add 0.5 ml of serum and 1.0 ml of reagent and mix. Then add 1.0 ml of reagent 2 and mix; stopper the test tube and incubate for five minutes at 37.5° C.

To each tube add 3.5 ml of distilled deionized water and read at 630 nm. Each determination was completed within thirty minutes from the time of death. With each set of determinations, ammonia standards were run.

Blood Urea Nitrogen

Blood urea nitrogen was determined using an automated technique. The determinations were done on a technicon 2 channel auto analyzer by the method of Marsh et al. (1965).

RESULTS

ENZYME STUDIES

Lysine to Homocitrulline

Table 3 gives the result of the radioisotope assay using C^{14} - Lysine. This table shows that the conversions of lysine to homocitrulline only occurs in the presence of carbamyl phosphate. In this experiment, lysine concentration was held constant at 0.030 M.

Because the radioisotope experiments consistently showed a carbamyl phosphate dependence, this reaction (Figure 5) was assayed colorimetrically in an attempt to demonstrate linear response to protein and time. Figure 6 shows the results of the reaction when lysine (15mM) and carbamyl phosphate (20mM) concentrations were held constant and protein was increased. Figure 7 gives the results of the same reaction with ornithine as substrate. When the two figures are compared, it is seen that approximately 35 times as much protein is needed for the lysine reaction. Figure 8 shows that the conversion

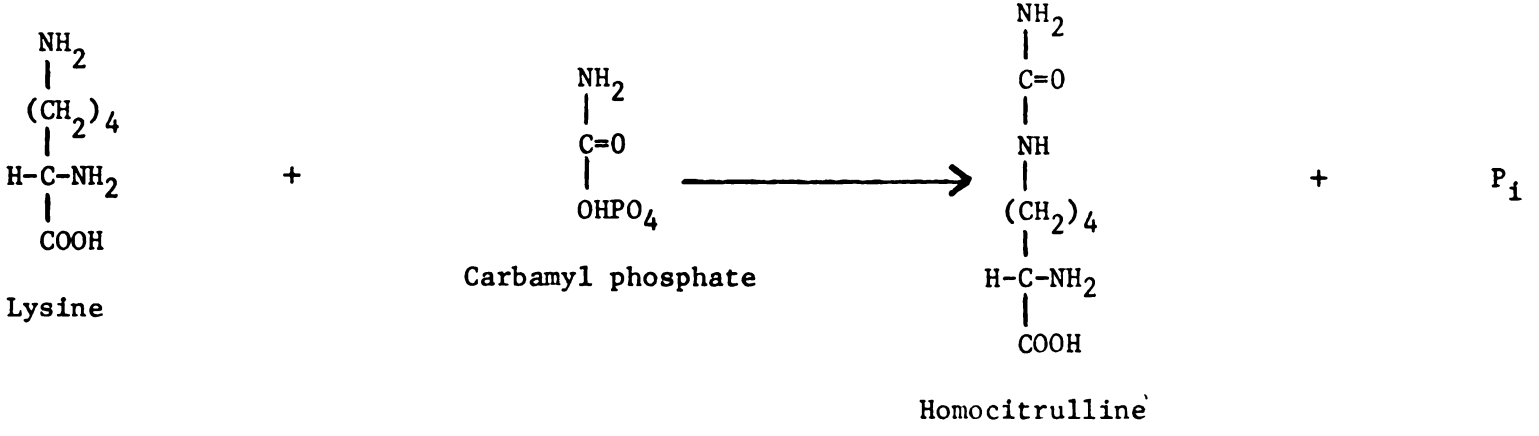
TABLE 3

Conversion of C^{14}-Lysine to C^{14}-Homocitrulline and C^{14}-Homoarginine

Carbamyl Phosphate Concentration	Homocitrulline	Homoarginine
	counts per minute	
0.0	2,612	1,399
0.040 M	48,026	18,207
0.080 M	55,544	38,757
0.120 M	69,748	30,656

THE INITIAL REACTIONS OF THE LYSINE-UREA CYCLE

AND THE ORNITHINE-UREA CYCLE



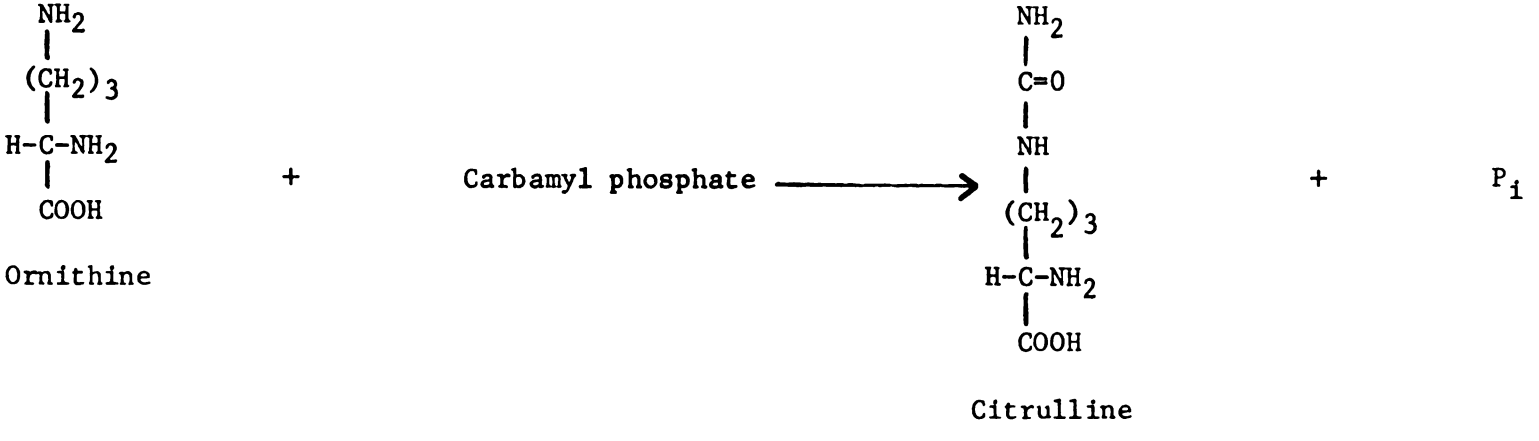


Figure 5

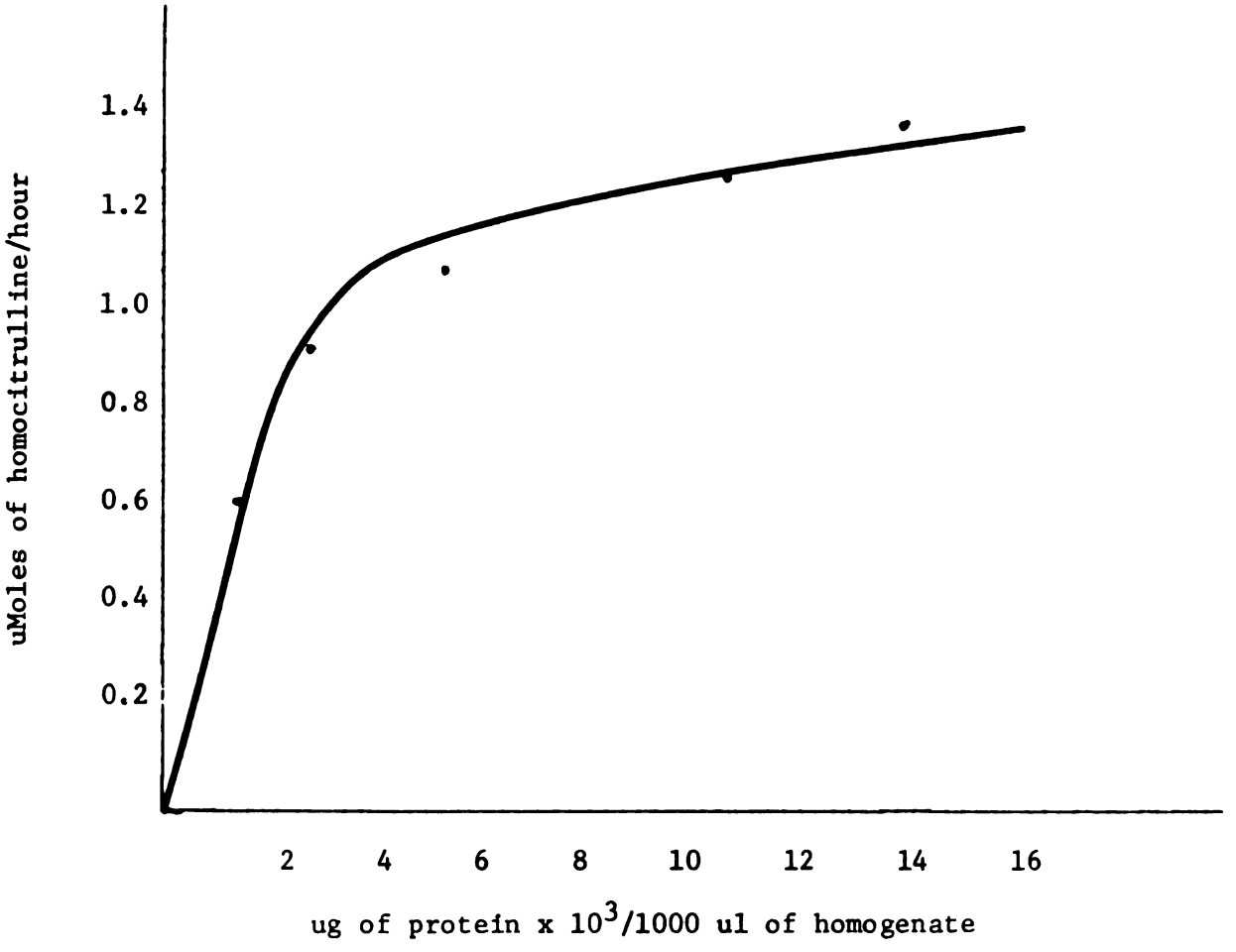


Figure 6: Lysine conversion to homocitrulline as a function of enzyme concentration

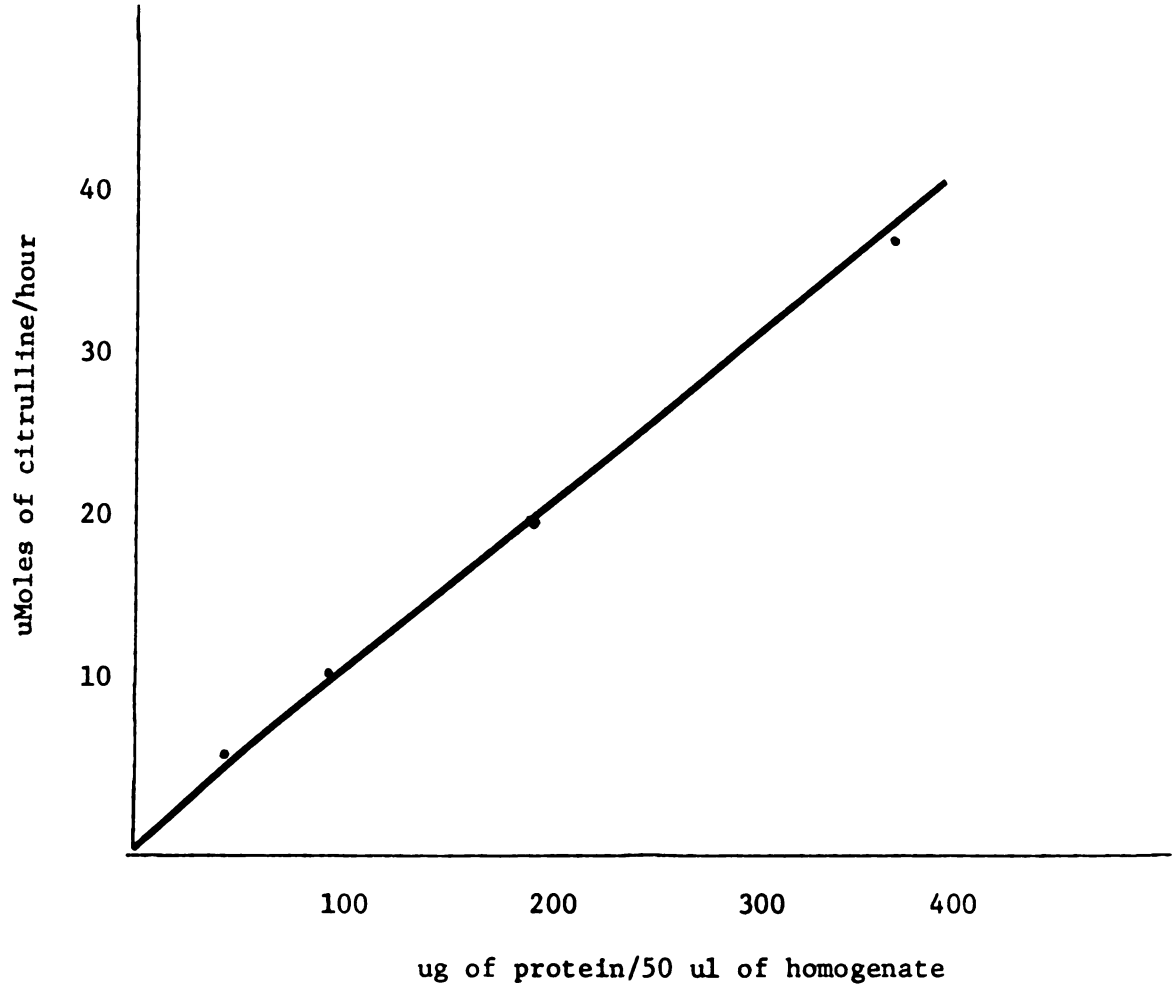


Figure 7: Ornithine conversion to citrulline as a function of enzyme concentration

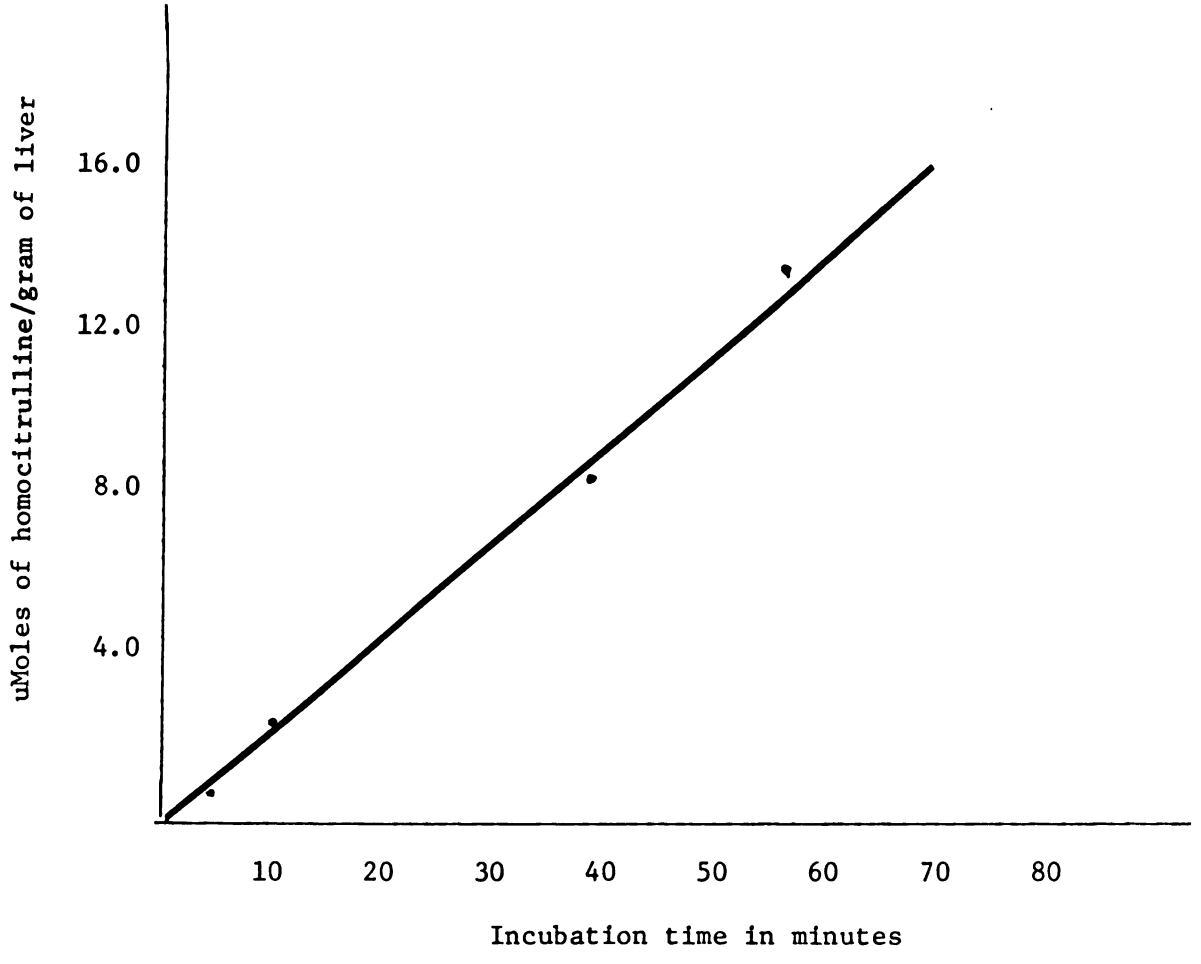


Figure 8: Lysine conversion to homocitrulline as a function of time

of lysine to homocitrulline increases linearly with time. The lysine concentration was 15mM and the carbamyl phosphate concentration was 20mM. When ornithine is used as substrate (Figure 9) it is seen that maximum activity is reached at 40 minutes. Figure 10 is a graph of the results of an experiment with increasing concentrations of lysine. The carbamyl phosphate concentration was 20 mM. The reaction ran for 30 minutes with 400 ul of 1:20 liver homogenate. Figure 11 shows the results of the same experiment when ornithine is substrate. Again, as with time and protein, it is evident that the rate of conversion of lysine to homocitrulline is very low compared to the ornithine to citrulline reaction. The uMoles of citrulline produced per hour with ornithine as substrate is 21.38; with lysine as substrate 0.223 uMoles of homocitrulline are produced. The rate of activity, therefore, when lysine is substrate is approximately 1/100 that of ornithine.

Lysine to Homoarginine

Table 3 shows the results of an experiment where the conversion of C^{14} - Lysine to C^{14} Homoarginine was measured. It can be seen from the data that this conversion is dependent on carbamyl phosphate concentration. It, however,

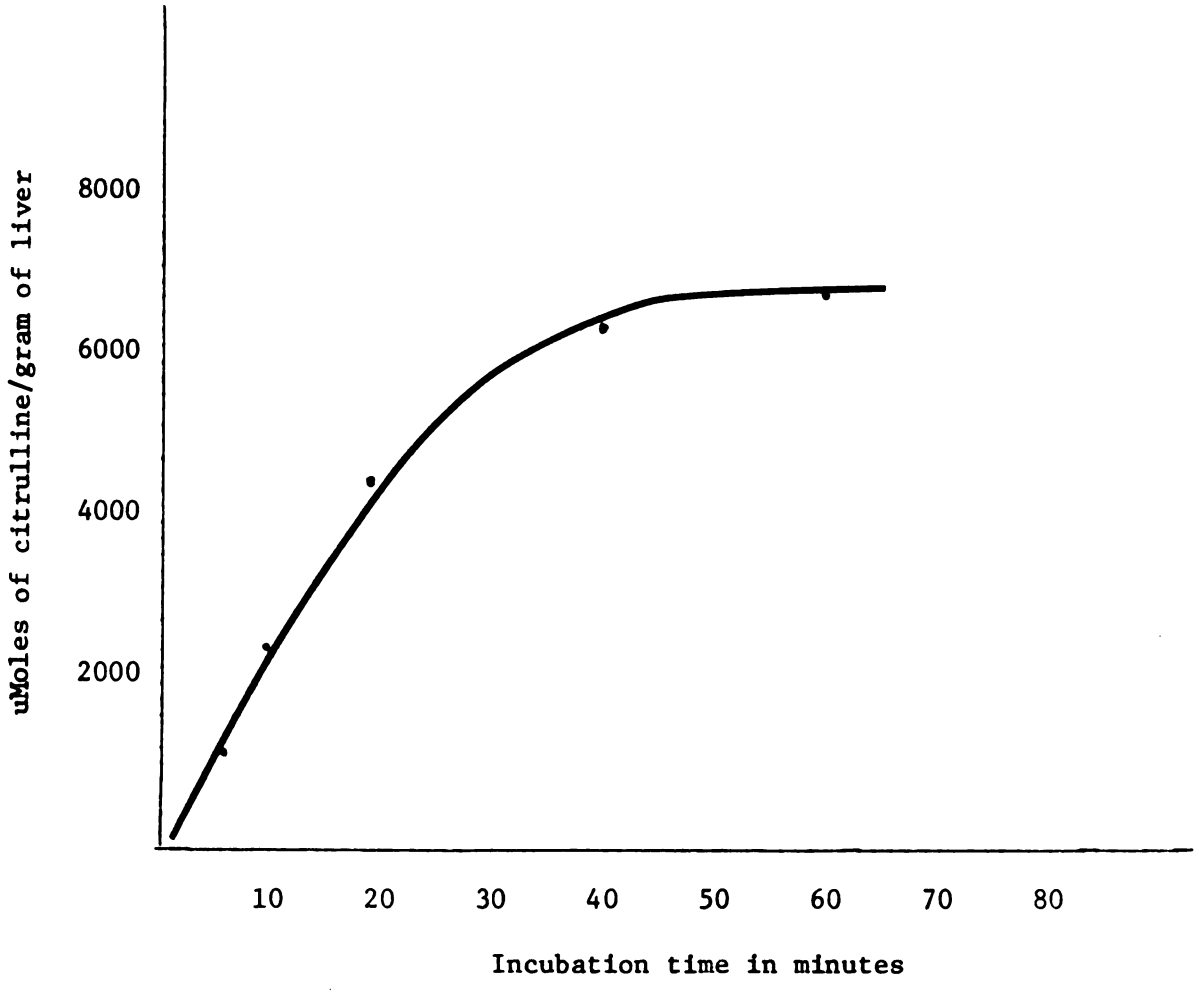


Figure 9: Ornithine conversion to citrulline as a function of time

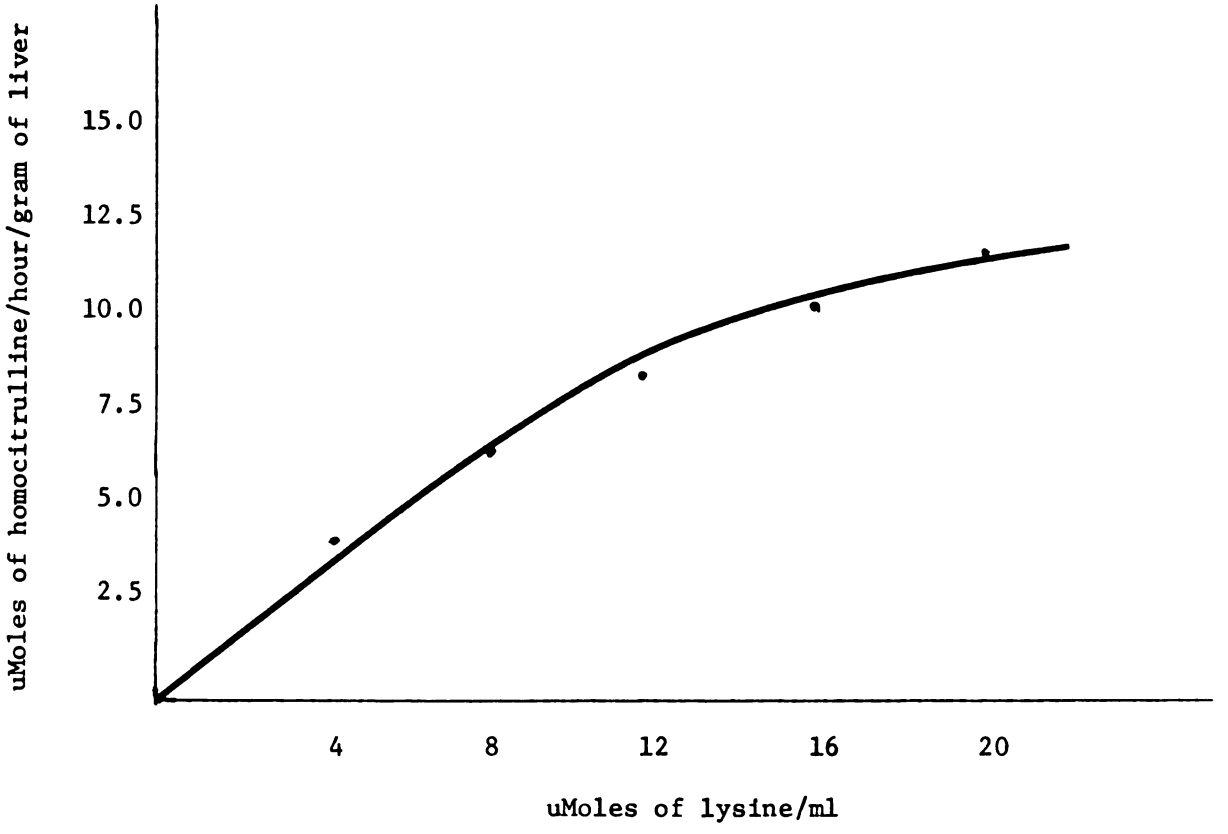


Figure 10: Homocitrulline formation as a function of lysine concentration

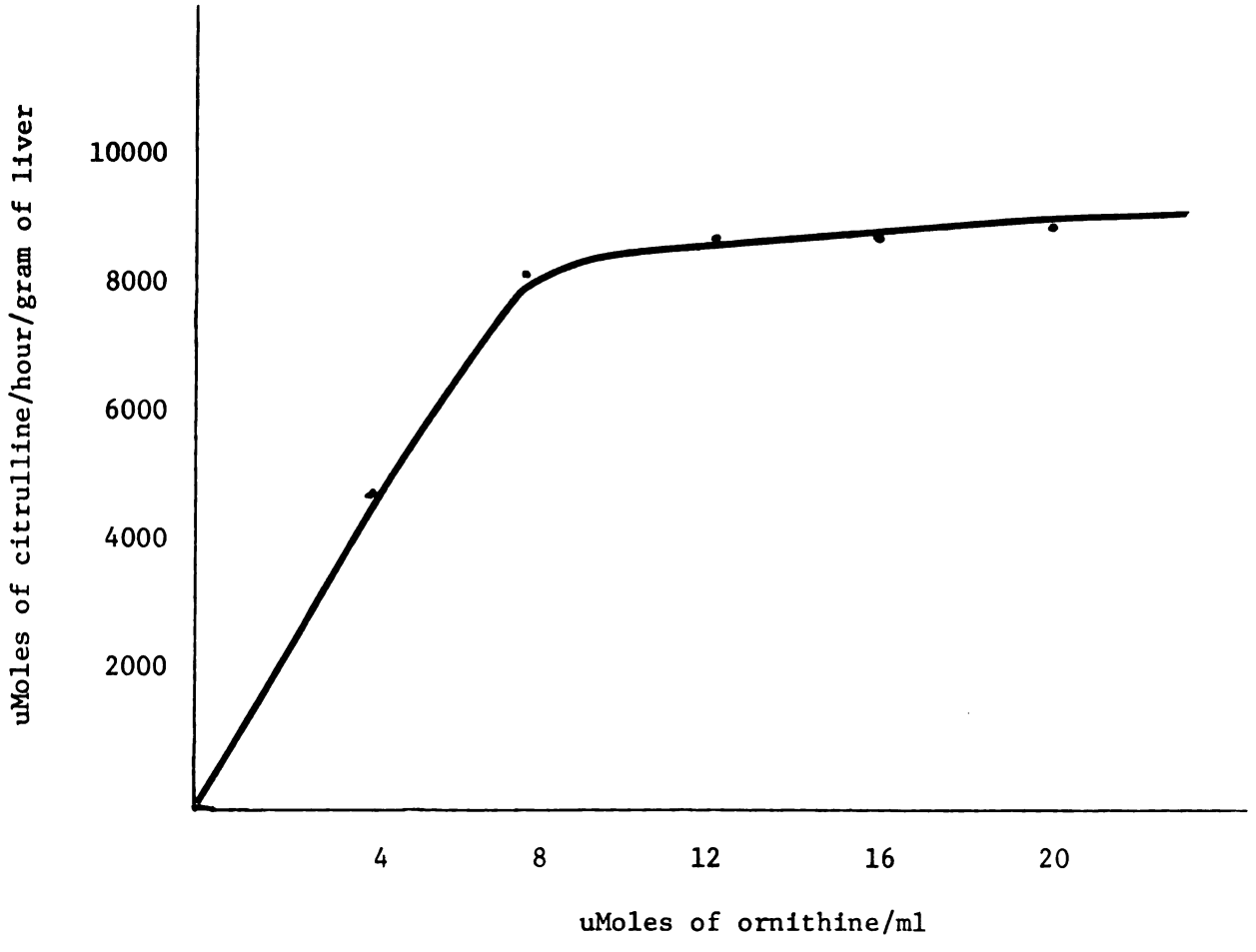


Figure 11: Citrulline formation as a function of ornithine concentration

was not possible to demonstrate a dependence on protein concentration. Because there is no ureido carbon label in lysine the complete cycle from lysine to urea cannot be demonstrated by this assay method. Therefore, assays using colorimetric methods were established in an attempt to demonstrate an enzymatic conversion of homoarginine to urea (Figure 12).

Homoarginine to Urea

Figure 13 shows the results of an experiment in which homoarginine concentration was held constant (250mM) and protein was increased. The results indicate a linear dependence of this conversion on protein. When these results are compared with the results in Figure 14, it is seen that the rate of conversion of homoarginine to urea is approximately 1/30 of that when arginine is substrate. Figure 15 shows that the conversion of homoarginine to urea increases linearly with time. The concentration of homoarginine was 0.250 M and each reaction tube contained 100 ul of a 1:20 liver homogenate. Figure 16 is the same reaction with arginine as substrate (0.250 M). The reaction tubes contained 20 ul of a 1:20 liver homogenate. These results again show that the conversion of homoarginine to urea is very slight when compared to the conversion of arginine to urea. Figure 17 is a graph of the results obtained when protein and time were held constant and urea

THE FINAL REACTIONS IN THE LYSINE-UREA CYCLE

AND THE ORNITHINE-UREA CYCLE

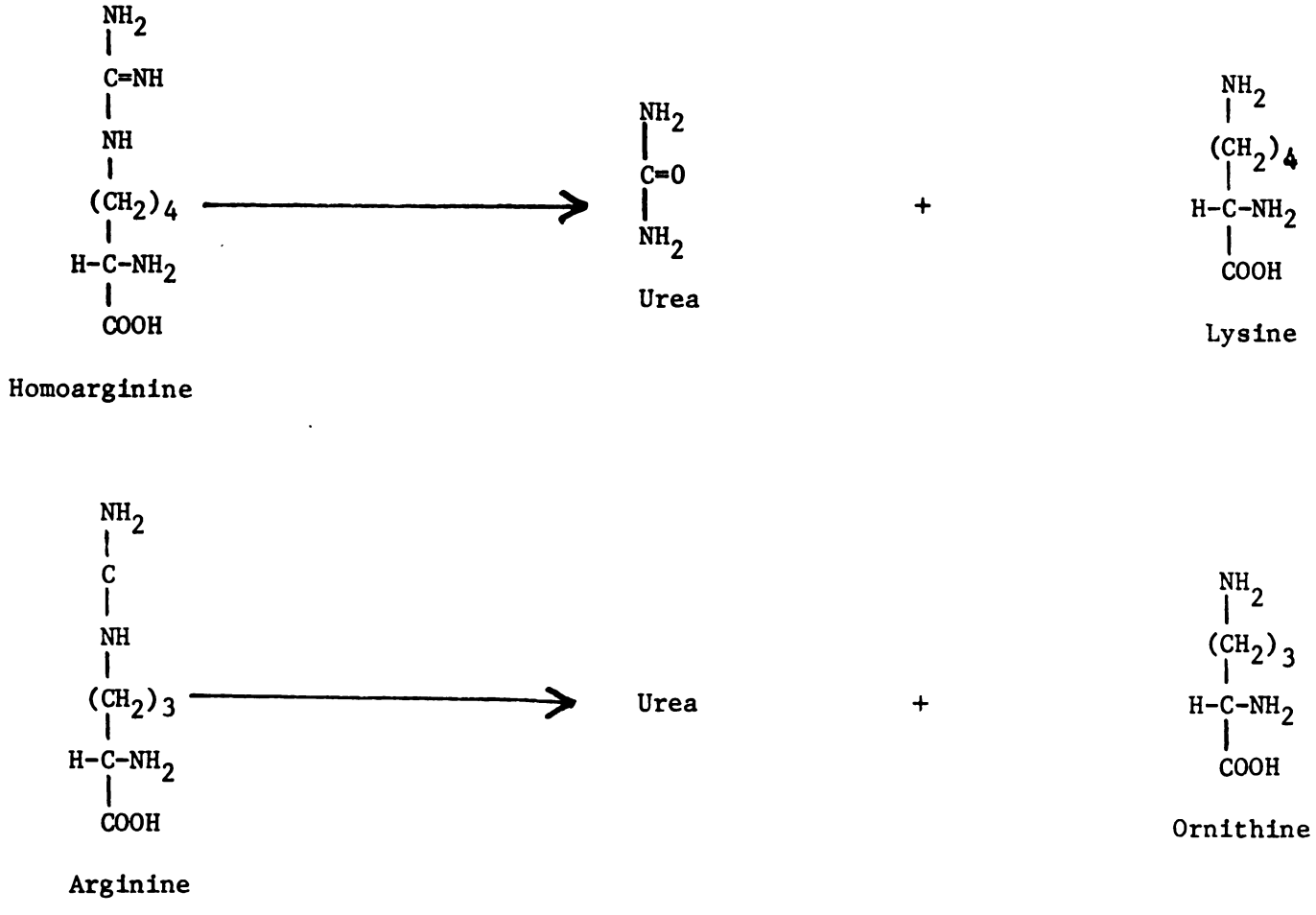


Figure 12

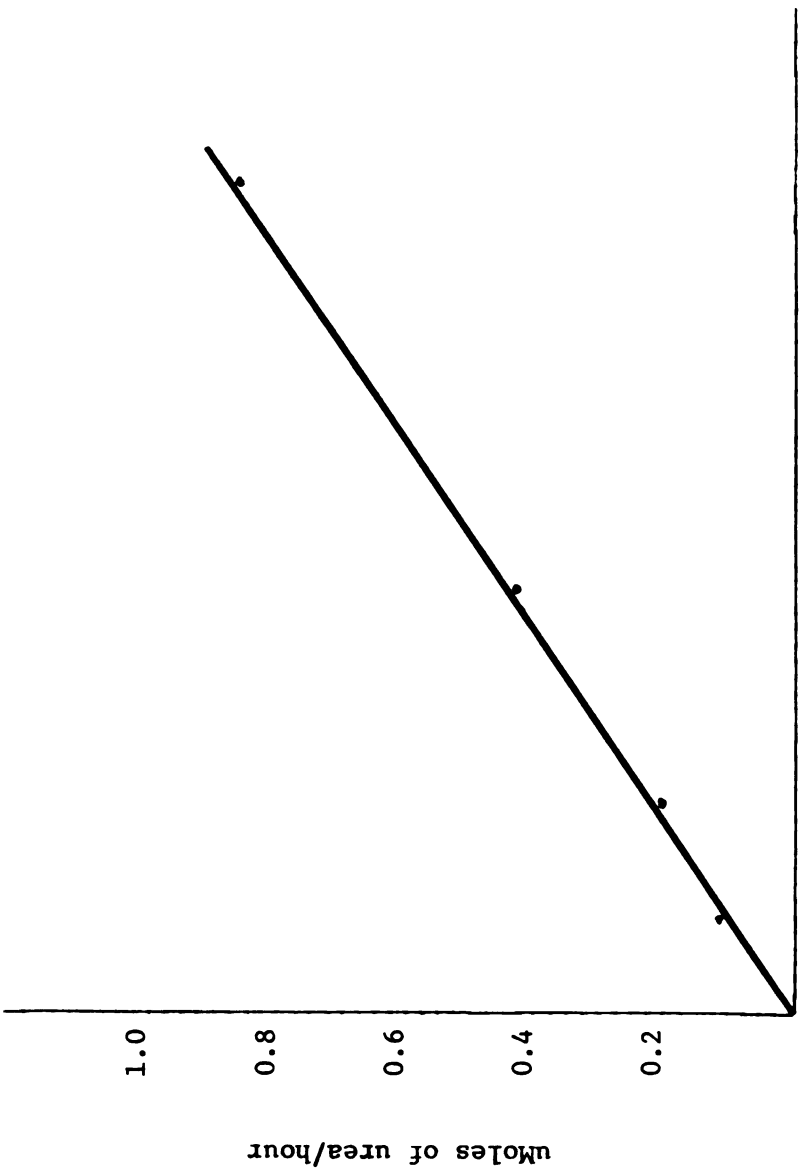


Figure 13: Homoarginine conversion to urea as a function of enzyme concentration

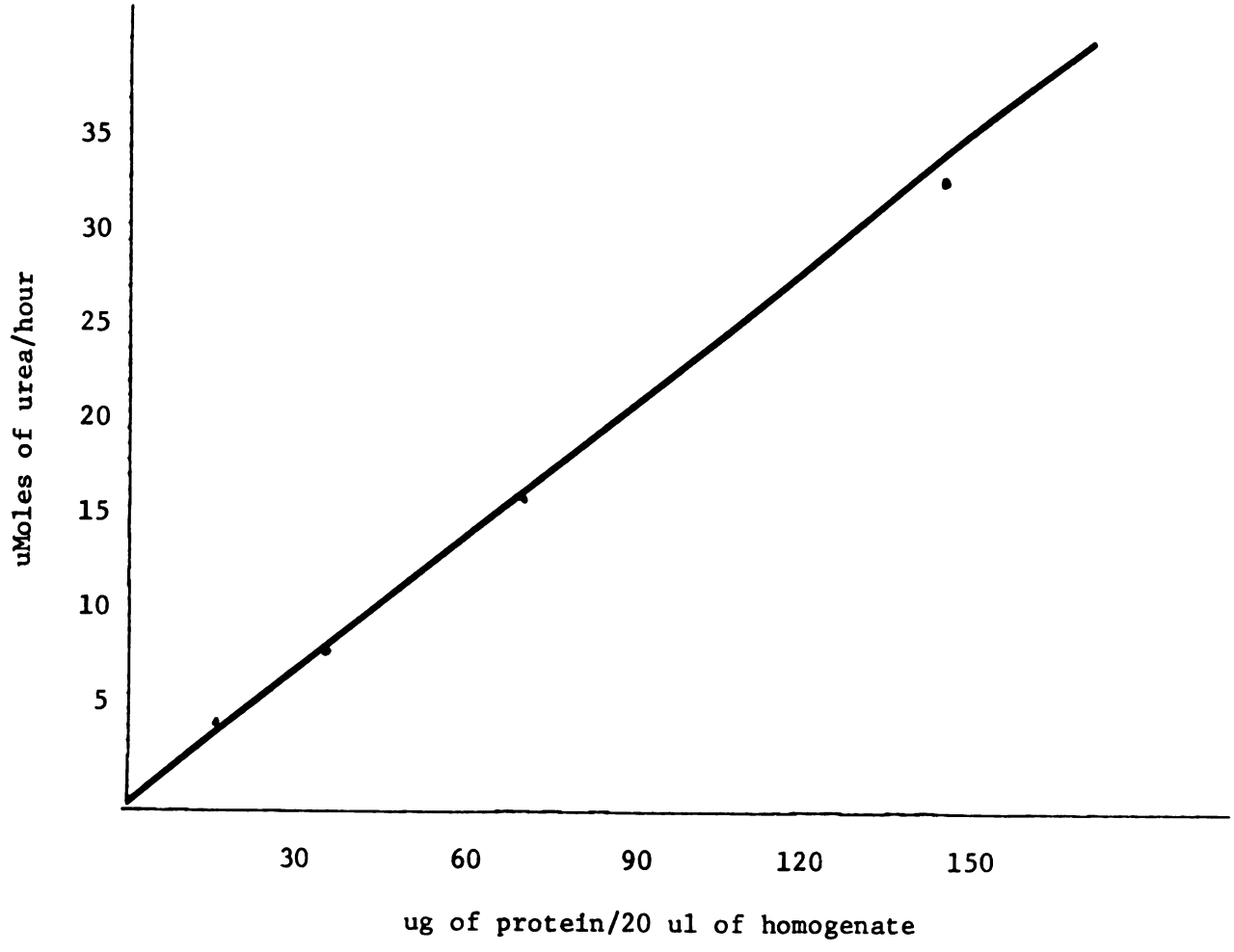


Figure 14: Arginine conversion to urea as a function of enzyme concentration

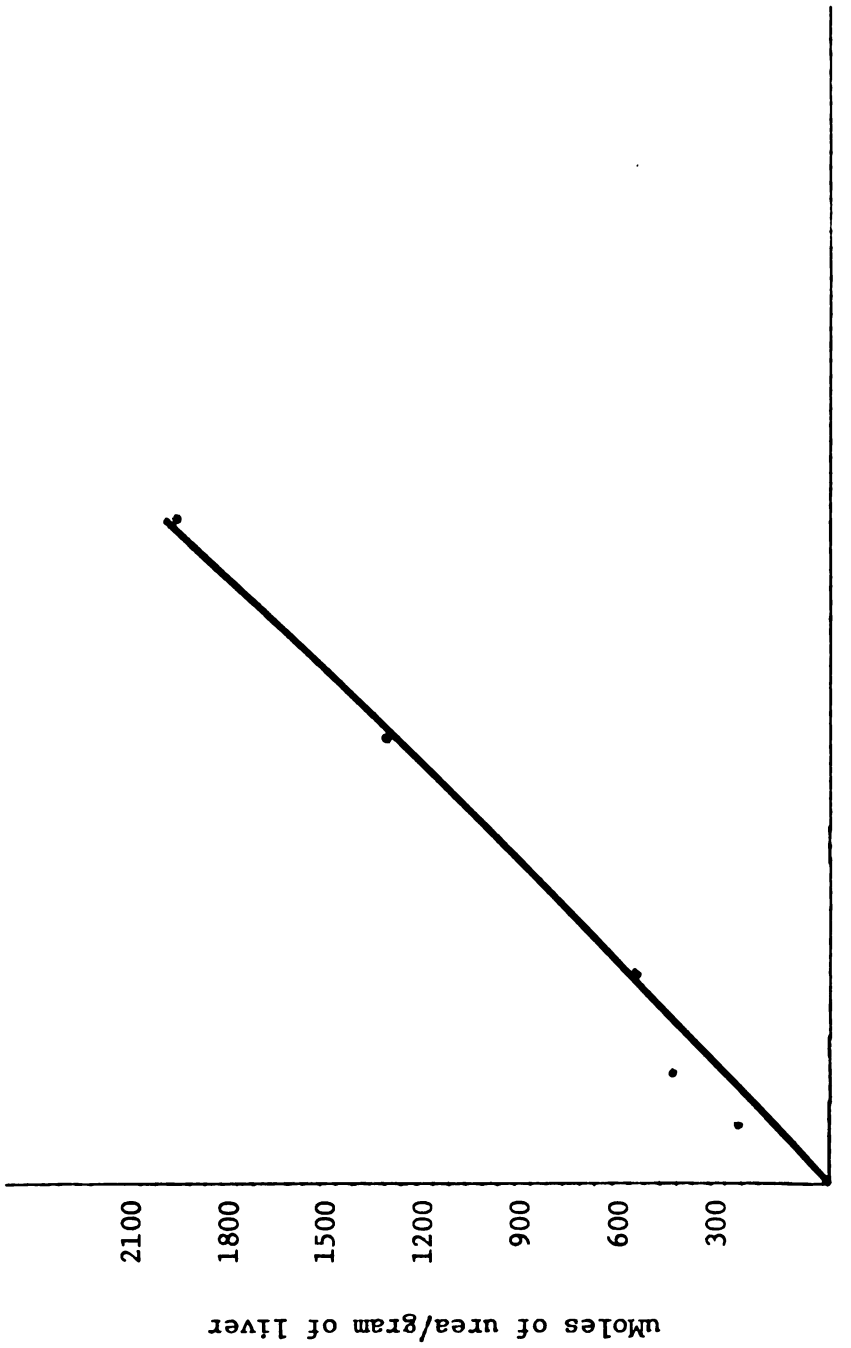


Figure 15: Homoarginine conversion to urea as a function of time

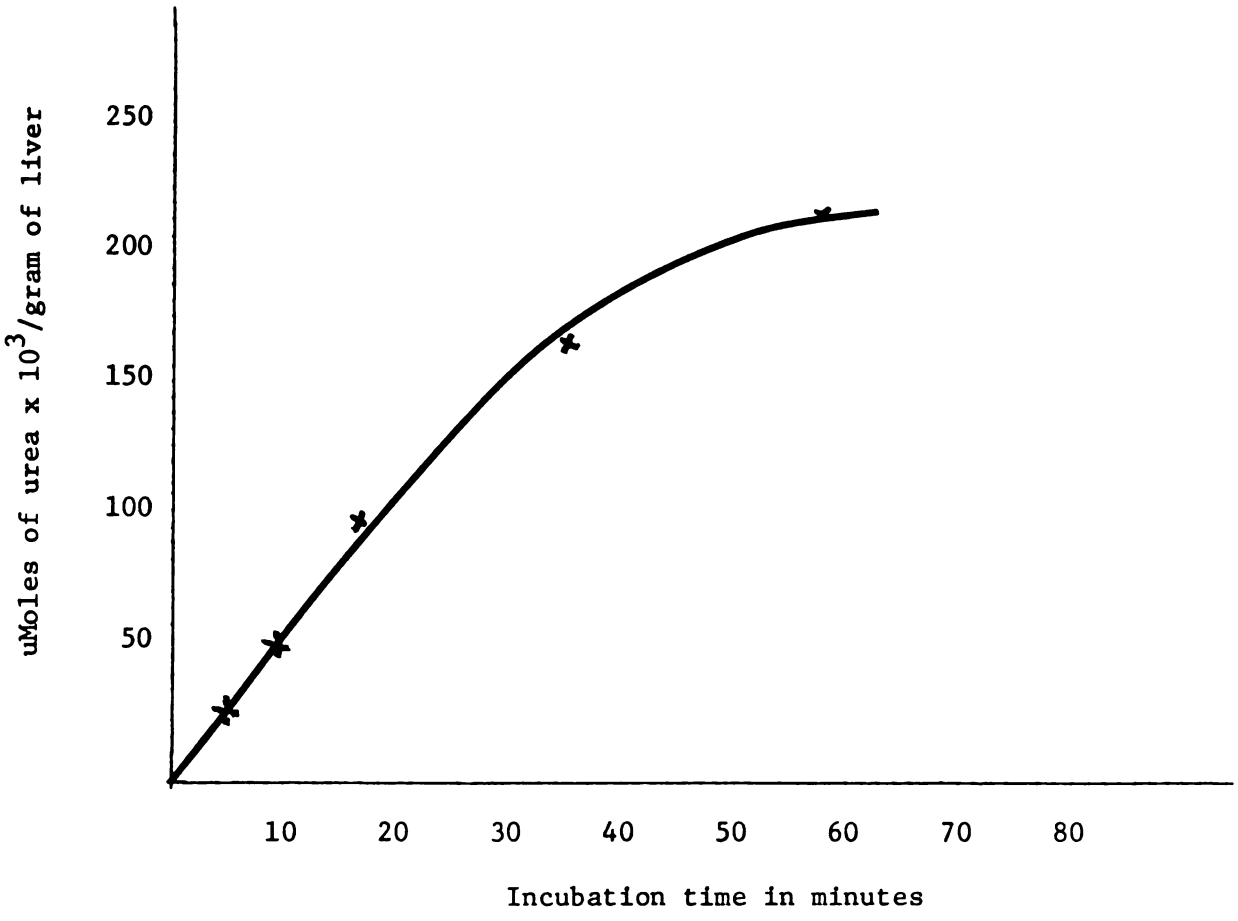


Figure 16: Arginine conversion to urea as a function of time

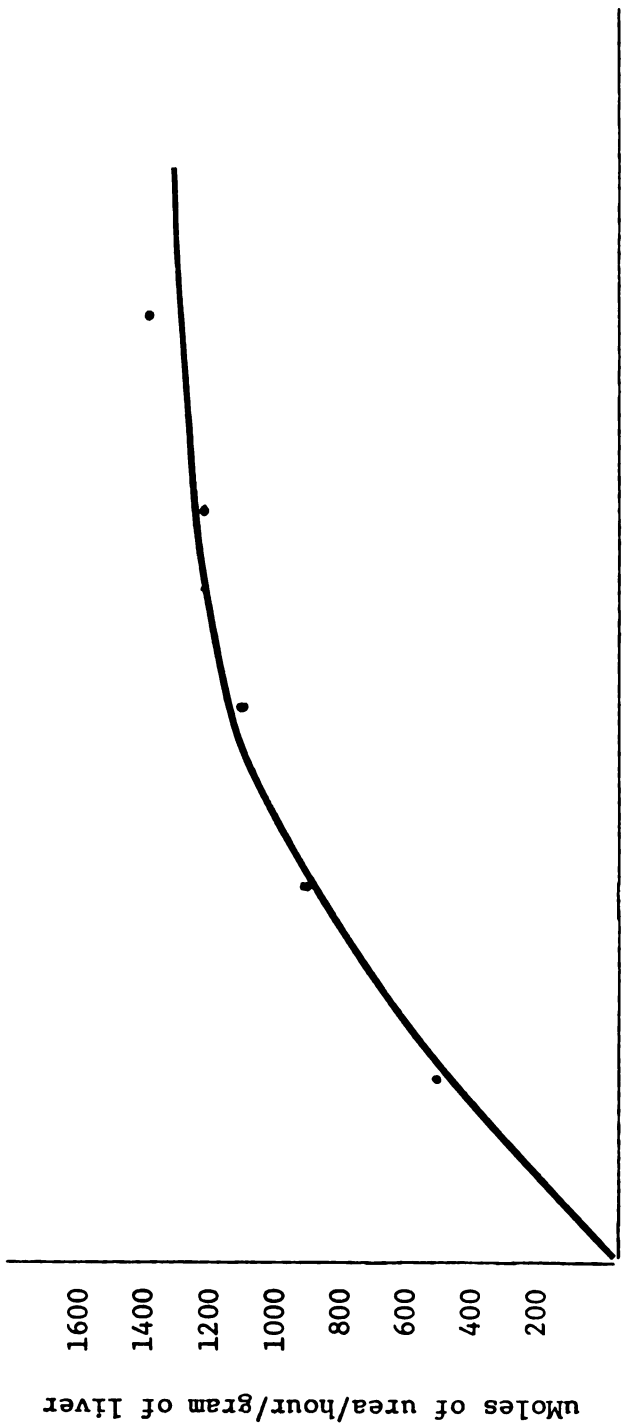


Figure 17: Urea formation as a function of homoarginine concentration

production was determined for increasing concentrations of homoarginine. It is seen that maximum velocity is approximately reached at 0.250 M concentration of homoarginine. Figure 18 shows the results of this same experiment when arginine is substrate. Here it is evident that the maximum velocity is reached at a concentration of 0.200 M. When velocities are compared for the two substrates, it is again shown that the speed of the reaction is approximately 1/30 of that of arginine.

Homocitrulline to Urea

Table 4 demonstrates the conversion of homocitrulline to urea. This conversion was measure by observing homocitrulline depletion and the formation of urea was confirmed by detecting the difference in duplicates with and without urease. The results are shown for two concentrations of homocitrulline. (These results were obtained using the ATP regeneration system.) It is evident that there is a significant depletion of homocitrulline in the presence of protein. There was only slight or no depletion measurable when urease was not present which indicates that urea was interfering with the colorimetric determination of homocitrulline. In the presence of urease, the color production was reduced and an accurate measure of homocitrulline depletion (free from urea interference) was obtained. The

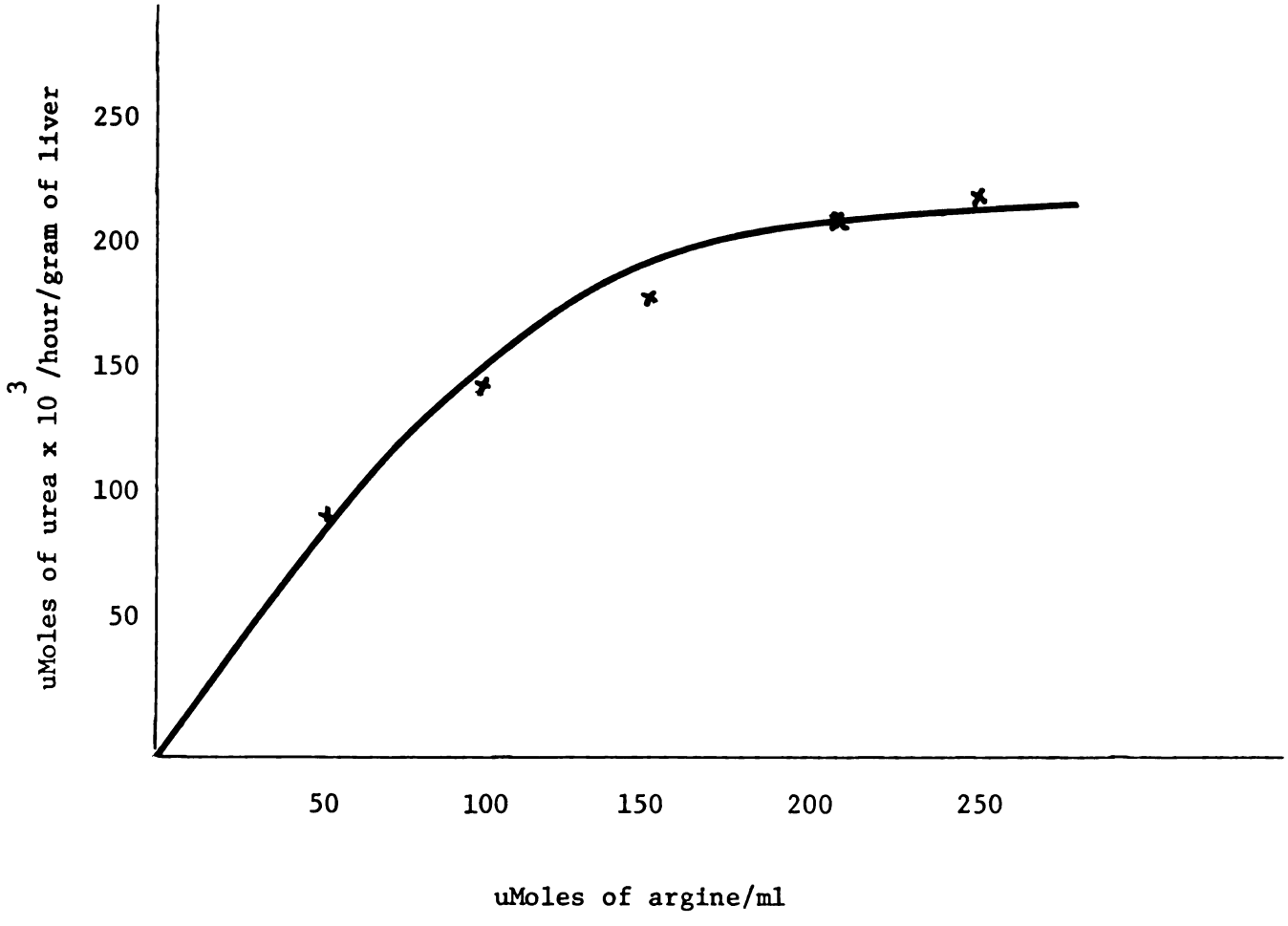


Figure 18: Urea formation as a function of arginine concentration

TABLE 4

Conversion of Homocitrulline to Urea

Substrate	uMoles depleted/hr./gram wet weight of liver			
	With Urease	Without Urease	No ATP	No Aspartate
Homocitrulline				
2.0 uMoles	7.2	2.4	1.30	0.95
3.0 uMoles	8.8	0.0	1.43	1.12

table also shows that when ATP or aspartate is removed from the medium only a slight depletion in homocitrulline was detectable. Unfortunately, due to the lack of sensitivity in the assay, it was not possible to obtain a response to increasing protein. It appears as though depletion is only measurable at maximum activity.

ANIMAL FEEDING EXPERIMENTS

Food Consumption and Growth

The effects of the two treatment diets on growth and food consumption in adult rats are given in Table 5. The food consumed is a mean calculated from nine rats in each group. It is a total measure for the 10 days on the diets. The rats in the 60% protein diet consumed less food than either the control (22% protein) group or the high lysine group. The high protein diet had a much reduced sugar content and its consistency was very dry compared to the other diets. It can be seen that although the high protein group consumed less food, it did have an increased protein intake. The final weight of the animals in the three groups was similar. This weight is tabulated as a mean weight per rat.

THE SPECULATED METABOLISM OF HOMOCITRULLINE

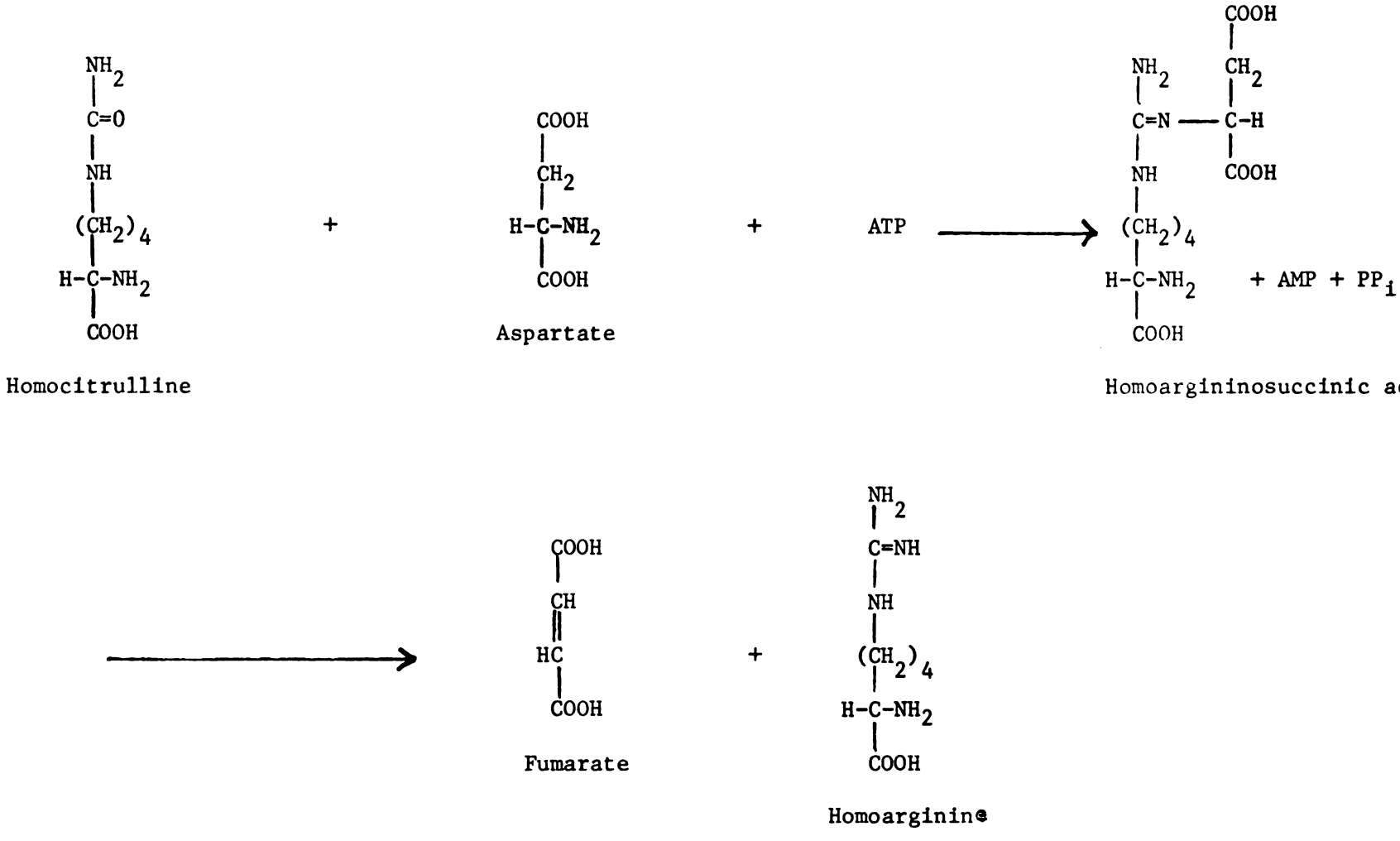


Figure 19

TABLE 5

Food Consumption and Growth of Rats on Three Different Diets

	22% Protein	60% Protein	22% Protein + 5% Lysine
Number of Rats	9	9	9
Food Consumed grams/rat/10 days	368 ± 18.11	255 ± 18.13	322 ± 21.21
Protein Intake grams/rat/day	8.10	15.30	7.10
Final weight per rat (g)	339 ± 20.68	315 ± 21.45	352 ± 22.21

Urea Cycle Enzyme Activities

The effects of the high protein diet on the activity of the reactions of the ornithine urea cycle and the lysine urea cycle are listed in Table 6. The results are tabulated according to the substrate used in the assay. All assays were run using liver homogenates, therefore, the activities listed only represent the ability of a liver homogenate to catalyze the reactions. Lysine, homocitrulline, and homoarginine are not necessarily to be considered as substrates of ornithine transcarbamylase, argininosuccinic acid synthetase, or arginase. The high protein diet significantly increased the activity of ornithine transcarbamylase when ornithine was the substrate. The difference from the 22% protein group was significant at $P < .01$. On the high protein diet when lysine was the substrate, the ability of the liver homogenate to convert lysine to homocitrulline also was significantly increased ($P < .01$) when compared to the 22% protein group. The increases detected are proportionately the same for both substrates. The conversion of citrulline to argininosuccinate also showed a significant ($P < 0.01$) increase in rats on the high protein diet. This increase is proportionately the same as those for ornithine and lysine. There was no significant increase in the ability of the liver homogenate

TABLE 6

Urea Cycle Enzyme Activities in Rats on Three Diets

Reactions	Activity[a] 22% Protein	60% Protein	22% Protein + 5% lysine	$\sqrt{MS_e/9}$[b]
ornithine to citrulline	12,853	15,426**	10,631**	± 508.31
lysine to homocitrulline	10.70	18.59**	13.33	± 0.99
citrulline to ASA	158.75	268.80**	104.35**	± 9.51
homocitrulline to homoASA	14.04	15.77	17.11*	± 0.78
arginine to urea	137,866	212,533**	287,133**	± 11,351
homoarginine to urea	1,890	2,580**	2,342**	± 84.68

a--each value is a mean for that treatment group (9 rats/treatment)
b--an estimate of the standard error
*--$P < 0.05$
**--$P < 0.01$

to convert homocitrulline to urea. The arginine to urea reaction is significantly increased ($P < 0.01$) in rats on the high protein diet. When homoarginine is the substrate, the activity is also significantly increased ($P < 0.01$). These increases are again proportionate to each other and to those detected for the other substrates.

The effects of the high lysine diet on the activity of these same reactions are also listed inTable 6. When considering ornithine urea cycle substrates, the level of activity drops significantly for both the ornithine and citrulline reactions, while the level of activity for the arginine reaction increases. All of these findings are significant at $P < 0.01$. The ability of the liver homogenate to convert the homocompounds of the urea cycle increases significantly or remains at the control level for all three homo-compounds. There is no significant decrease for any of these three assays as there was for two of the ornithine urea cycle substrates. The increase for homocitrulline is significant at $P < 0.05$ and for homoarginine at $P < 0.01$. The increase seen for lysine is not significant at $P < 0.05$ but the change in activity is in line with both homocitrulline and homoarginine. Figure 20 graphically depicts these results as the percentage of the difference from the control.

CHANGES IN ENZYME ACTIVITY IN RATS

ON HIGH LYSINE DIETS

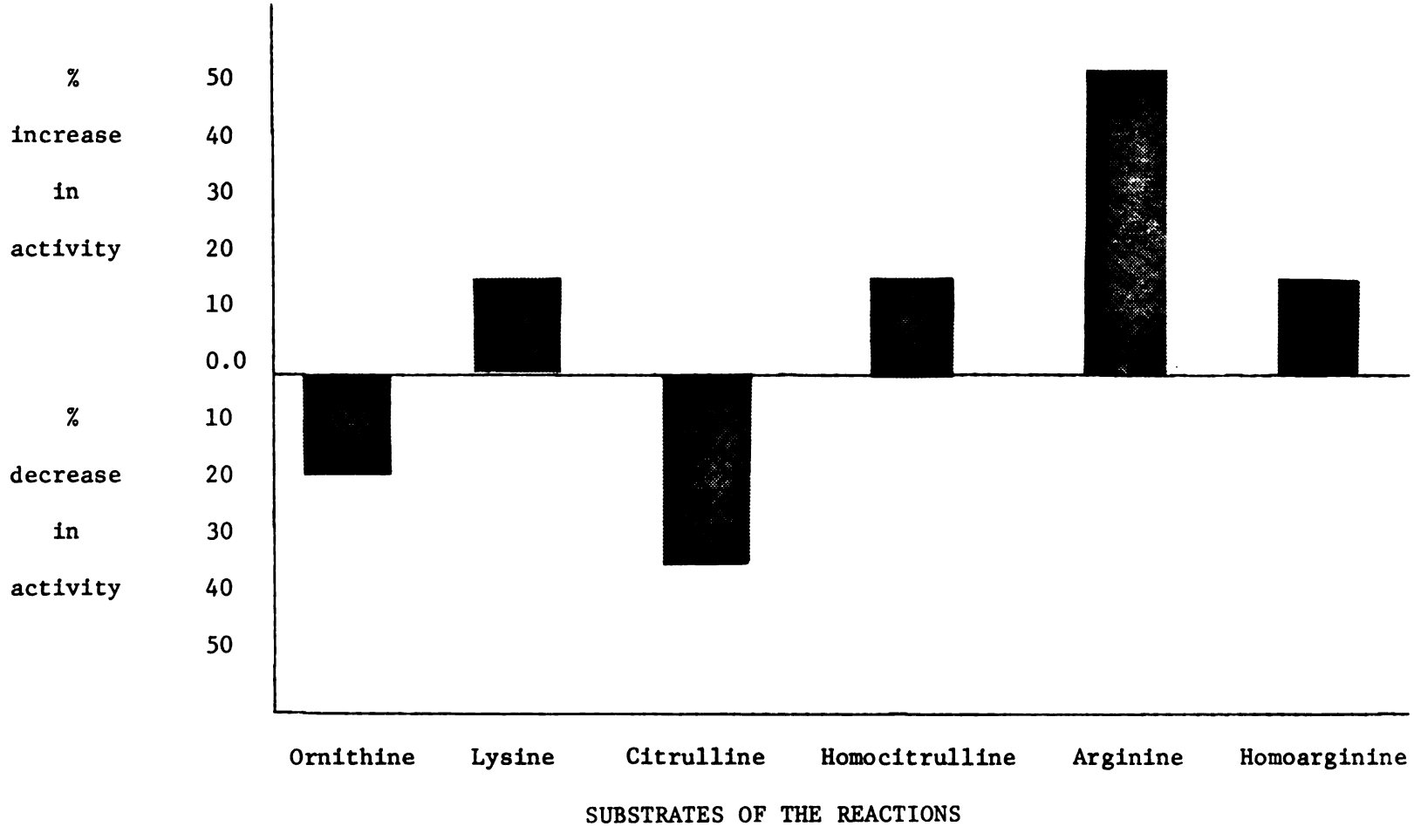


Figure 20

Serum Determinations

Serum urea nitrogen and ammonia nitrogen were determined on all three treatment groups (Table 7). There were no significant changes in blood ammonia nitrogen in either the high protein or high lysine groups. It is interesting to note that the blood urea nitrogen significantly increased in both treatment groups. The increase in the high lysine group is significant at $P < 0.05$.

Protein determinations on the livers of all the animals were found not to show any significant differences between the treatment groups.

TABLE 7

Serum Urea and Ammonia

	22% Protein	60% Protein	22% Protein + 5% Lysine	$\sqrt{MS_e/9}$
Urea--N mg%	20.83	45.66**	26.33*	± 1.29
NH_3--N ug/ml	2.32	2.71	2.97	± 0.22

*--P < .05
**--P < .01

DISCUSSION

In early 1969, as the result of a screening program of all the institutionalized mentally retarded in the State of Michigan, an individual was detected who excreted an excessive amount of citrulline. This patient has citrullinemia, an inborn error of urea biosynthesis (Scott-Emuakpor et al., 1972). This patient has normal urea and ammonia concentrations in the blood, and normal urea excretion, in the presence of an enzymatic error in the Krebs-Henseliet urea cycle. These findings, along with the blood and urine amino acid patterns, were the basis for speculating about an alternate pathway of urea synthesis that utilizes the homocompounds of the substrates of the classic urea cycle in reactions parallel to those of the ornithine urea cycle. The results of the experiments of Scott-Emuakpor (1970) supported the existence of this alternate pathway. The present research was undertaken in an effort to gain further insight on this hypothesized mechanism of urea production.

ENZYME STUDIES

An attempt was made to demonstrate the overall lysine - urea cycle by demonstrating the conversion of C^{14} - lysine to C^{14} - homoarginine. Although the results of that assay were supportive of the cyclic nature of the speculated reactions, the assay system was not sensitive enough to demonstrate the individual reactions. Because of this, each individual reaction was studied using colorimetric assay techniques. In this way, the existence of the overall cycle can be shown by demonstrating each individual reaction.

Lysine to homocitrulline

Gerritsen *et al*. (1963) believed that homocitrulline was not synthesized by the body or formed by intestinal bacteria. The results reported here demonstrate the production of homocitrulline from lysine. The reaction was shown to be linearly dependent on protein, carbamyl phosphate and lysine concentrations. These data are in agreement with the findings of Scott-Emuakpor (1970). The data indicate that the enzymatic conversion of lysine to homocitrulline demonstrated by Ryan *et al*. (1968) probably proceeds by a transcarbamylation. Ryan *et al*. did not demonstrate the carbamyl phosphate dependence of

this reaction in their studies. Marshall and Cohen (1963) found a very minor activity for ornithine transcarbamylase with lysine as substrate but Colombo (1971) could not detect any activity for OTC with lysine as substrate. The studies by Colombo were done on human liver while those reported here were done on rat liver. It is, therefore, possible that the human liver does not have the capability of transcarbamylating lysine to homocitrulline. Colombo did not describe his technique for the assay and therefore it is possible because of the minor conversions noted in these experiments that his technique may not have been sufficiently sensitive to detect the activity.

Homocitrulline to Homoargininosuccinic Acid

This reaction has not previously been demonstrated in any tissue of any organism. Ryal et al. (1968) could not detect any metabolism of homocitrulline in rat liver. The conversion of homocitrulline to homoargininosuccinic acid is indirectly shown by the results of the experiments reported here. The conversion is assayed as the depletion of homocitrulline and the formation of urea is inferred by a depletion of color after the addition of urease. In addition, evidence that this reaction proceeds by way of homoargininosuccinic acid is the finding of Strandholm et al. (1971) that homoargininosuccinic acid is formed in an

enzymatic reaction from fumarate and homoarginine. The reaction demonstrated by Strandholm et al. is the third reaction in the postulated lysine - urea cycle.

Homoarginine to Urea

Arginase has been shown to cleave homoarginine to urea and lysine (Ryan et al., 1968, Scott-Emuakpor, 1971). This reaction occurs in rat liver and also with commercial arginase (bovine). Colombo was unable to demonstrate this conversion in human liver. The results of the experiments described here confirm the ability of rat liver to metabolize homoarginine to urea.

It appears that from the results of these experiments and those published in the literature that all of the hypothesized reactions of the lysine - urea cycle can be shown to occur. Because the evidence gained supports this alternate mechanism of urea synthesis, animal feeding experiments were designed to gain further information on the enzymes and adaptive characteristics of the lysine - urea cycle.

ANIMAL FEEDING EXPERIMENTS

High Protein Diets

The results obtained in the studies presented here show that the reactions of the ornithine - urea cycle increased in activity in rats on the 60% protein diet. The activities of two of the lysine - urea cycle reactions also increased although the homocitrulline reaction showed no significant change. This is the first reported experiment on the effect of high protein diets on the speculated lysine - urea cycle reactions. The effect of high protein diets on the ornithine - urea cycle reactions has previously been reported (Schimke, 1962, Nazum and Snodgrass, 1971) and the results found in the experiments here compare favorably. The noted response of the lysine - urea cycle enzymes may be an indication that the individual reactions are indeed cyclic. Although the activities were determined *in vitro*, the findings of increased blood urea nitrogen and normal serum ammonia are indications that the *in vitro* activity is a measure of the *in vivo* activity. The increased blood urea nitrogen can be assumed to reflect increased urea production because in animals on high protein diets the glomerular filtration rate is increased,

thereby, eliminating the possibility of increased blood urea nitrogen due to decreased filtration rate.

High Lysine Diets

Relationship of lysine and arginase

It is known that lysine inhibits arginase *in vitro* (Hunter and Downs, 1945). However, the results of the experiments presented here indicate that arginase activity is increased in rats fed high lysine diets. That this increased activity occurs *in vivo* is in doubt because the increased blood urea nitrogen may not be due to increased urea production but to impaired renal function. There is no evidence in rats on the effects of high lysine on renal function. However, in patients with hyperlysinemia it has been shown that renal function is normal (Colombo *et al.*, 1967) giving evidence that blood urea nitrogen may be indicative of increased urea production. If this is assumed, then the experiments presented here show an *in vivo* stimulation of arginase by lysine, which would seem to be in conflict with the findings of Colombo (1971). He described a patient with hyperlysinemia, high arginine, and hyperammonemia and explained these findings by showing an *in vivo* inhibition of arginase. His patient, however, was described as having an abnormally functioning arginase.

Although this is the first study in rats on the activity of arginase after lysine feeding, studies on chick livers have shown that arginase activity increases when the diet is high in lysine.

Significantly Increased Blood Urea Nitrogen

The increased concentration of urea noted in the blood of rats fed the high lysine diet occurred in the presence of significantly decreased ornithine transcarbamylase and argininosuccinic acid synthetase activities. This increased urea concentration could have arisen from one or a combination of the following:

1) The lysine diet may have impaired renal function, thereby causing increased blood urea nitrogen. There are no data available in rats to indicate that this is or is not the case. If it is assumed that the lysine diet did not affect the kidney function, then the increased blood urea nitrogen is a measure of urea production and may be explained by the following (2,3, and 4).
2) The enzymes of the ornithine - urea cycle which are significantly reduced in activity in vitro are still capable of producing normal levels of urea because of increased substrate forcing the reactions or due to

the increased arginase activity balancing the reduced activity of the other enzymes. It has been found that ornithine is indeed increased when the diet is high in lysine (Shu-heh Wang et al., 1973).

3) The direct transamidination of lysine to homoarginine by argine - lysine amidinotransferase. This reaction is present in rat kidney (Ryan et al., 1969). The homoarginine is then cleaved to lysine and urea by arginase which was shown to have a significantly increased activity for this substrate, in the experiments reported here.

4) Lysine is converted to homocitrulline which is in turn metabolized to homoarginine. The homoarginine thus produced is cleaved to urea and lysine. The experiments reported here have shown an in vitro increase in activity for homocitrulline and homoarginine metabolism.

Assuming that renal function is normal, an increase in urea production per day per rat of 368 mg must be explained. Making calculations from the activities of the reactions of these mechanisms, at most 100 mg of this increased urea can be explained by the above. Because of this, it appears as though the noted increase in urea is due to a combination of these mechanisms or a mechanism as yet unknown.

Normal blood ammonia in the presence of reduced activities in the Ornithine - Urea Cycle.

This finding could have arisen due to any of the following mechanisms or any combinations thereof:

1) Ammonia could be detoxified by any of the other reactions as discussed in the Literature Review.
2) The ornithine - urea cycle although reduced in activity is still capable of detoxifying ammonia. It is known that there is a greater than usually necessary capability of the urea cycle enzymes to detoxify ammonia (Hutchinson and Labby, 1965), therefore, a partial reduction in urea cycle enzyme activities will not lead to hyperammonemia.
3) Ornithine could be excreted in a higher concentration than normal due to its possible buildup because of reduced ornithine transcarbamylase activity. This in itself could serve as an ammonia detoxication mechanism. As noted previously, ornithine is increased in the blood when lysine is increased in the diet.

4) In the presence of a defective ornithine urea cycle, the lysine - urea cycle could enhance in activity and therefore function as an ammonia detoxication mechanism. The results here did show an increase in the activity of two of the reactions of the lysine urea cycle and at the same time decreased activities in ornithine transcarbamylase and argininosuccinic acid synthetase.

EVIDENCE FOR THE LYSINE - UREA CYCLE

From the previous sections of the discussion it is obvious that there is substantial evidence supporting the existence of the lysine - urea cycle. The following is a summary of that evidence.

1) The demonstration of the ability of a rat liver homogenate to catalyze the individual reactions of the hypothesized cycle.
2) The demonstration of the concerted response of these reactions when lysine is fed to rats in higher than normal concentrations. This coupled with (1) indicates the possible interrelationship of the individual reactions.
3) The demonstration of an increased concentration of blood urea nitrogen when

the activity of the ornithine urea cycle is decreased.

4) The demonstration of normal blood ammonia when the activity of the urea cycle is decreased.

5) The findings associated with patients who have a defect in the ornithine - urea cycle (This is discussed in the Literature Review).

SPECULATION ON THE ENZYMES

Although no conclusive evidence as to the enzymes that catalyze the reactions of the lysine - urea cycle has been found, the results of these studies do lend themselves to some interesting speculation. The data suggest that at least a portion of the lysine - urea cycle is catalyzed by distinct enzymes other than those for the ornithine - urea cycle. This is suggested because of the increased activity for lysine and homocitrulline when the decreased activity for ornithine and citrulline when rats are fed high lysine diets. It is possible that the same enzymes are catalyzing the reactions but that there are different active sites for the two substrates or the two substrates are competing for one site. The data also suggest that

the two cycles join at a common point, namely arginase. When rats are fed high lysine diets increased activity for arginine as well as homoarginine occurs. Whatever the case, it is evident that lysine is capable of stimulating the reactions of the lysine urea cycle.

Although these data only represent evidence for this alternate pathway of urea biosynthesis in the rat liver, it should initiate studies on these reactions in human tissues. It is possible that this pathway, although very minor in the rat, is capable of maintaining normal blood ammonia and of synthesizing normal amounts of urea in patients with urea cycle disorders. It is possible that given the right environmental stimulus, this minor pathway could be enhanced. Indeed, it has been shown in these experiments that lysine stimulates the activity of these reactions in a very short period of time. This, therefore, could be a possible treatment for individuals with inborn errors of the urea cycle. This points to the need for gaining further knowledge of the lysine - urea cycle in humans.

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