THE BIOCHEMICAL CHARACTERIZATION OF A PECULIAR KIND OF CITRULLINEMIA (AN INBORN ERROR OF METABOLISM IN MAN): A POSSIBLE NEW PATHWAY FOR UREOGENESIS

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AJOVI B. SCOTT-EMUAKPOR

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This is to certify that the

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THE BIOCHEMICAL CHARACTERIZATION OF A PECULIAR KIND OF CITRULLINEMIA (AN INBORN ERROR OF METABOLISM IN MAN): A POSSIBLE NEW PATHWAY FOR UREOGENESIS

presented by

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has been accepted towards fulfillment
of the requirements for

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ABSTRACT

THE BIOCHEMICAL CHARACTERIZATION OF A PECULIAR KIND OF CITRULLINEMIA (AN INBORN ERROR OF METABOLISM IN MAN): A POSSIBLE NEW PATHWAY FOR UREOGENESIS

by

Ajovi B. Scott-Emuakpor

A mentally retarded middle aged white male, who has excessive citrulline, homoarginine and lysine in his blood and excretes large amounts of citrulline, homocitrulline, ornithine, homoarginine and (occasionally) arginine in his urine, has been analyzed biochemically for the purpose of characterizing his syndrome. The assumption has been made that all of the aminoacidopathies found in this patient are the result of a single genetic defect in his metabolism: the deficit or complete absence of argininosuccinic acid synthetase, an enzyme that catalyzes the conversion of citrulline to argininosuccinic acid. This syndrome appears to be distinct from all other citrullinemics because of his abnormal pattern of amino acids and because, unlike the previously described citrullinemics, he has normal ammonia levels in his serum. His serum citrulline levels are always more than 60 times normal, and his urinary citrulline levels are always many thousandfold above normal under various dietary conditions. Evidence from urea and creatinine clearances show that his kidney function is normal. He excretes normal amounts of urea. Both of his parents, his only sib (a brother), and his two nieces and a nephew were available for analysis,

which failed to show any similar defect in the family. Analysis of the pedigree showed low likelihood of consanguinity.

An attempt to explain the patient's complex defects led to a series of biochemical assays using rat liver. These assays have revealed what could possibly be a minor secondary pathway for ammonia disposal and for urea synthesis. This pathway involves the enzymatic conversion of lysine to homocitrulline in the presence of carbamyl phosphate. Homocitrulline is then converted to homoarginine in an enzymatic reaction that requires ATP and aspartic acid. The aspartic acid dependence indicates that an unstable intermediary, homoarginino-succinic acid, exists between homocitrulline and homoarginine. The homoarginine is apparently then hydrolyzed to urea and lysine. Thus, a lysine-urea cycle, with substrates that have an extra CH_2 group relative to substrates of the ornithine-urea cycle, is thought to exist.

With this cycle, it was possible to understand the biochemical characteristic of this patient as well as several other previously unexplained phenomena observed by other workers in patients manifesting errors of the urea cycle. In the patient observed in this study, the secondary pathway probably enables him to avoid serious continuing ammonia intoxication.

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by

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To my Family

and

To my Teachers

and Friends who have made my

academic endeavors

intellectually stimulating

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INTRODUCTION

Within the last few decades, our knowledge of the relationship between biochemical errors and mental retardation has increased enormously. There is considerable evidence that most of the biochemical errors are inherited, hence the name "inborn errors of metabolism" is applied to them collectively. Although we have made great advances in this area of genetics, only a tiny fraction of the mentally retarded population has problems that can be associated with known errors of metabolism, and our knowledge of the exact relationship between most of the metabolic errors and mental retardation is still not clear. Most of the enzymatic processes that lead to these errors are still not properly delineated. The relationship of the primary defects to other metabolic intermediaries is still a matter of considerable speculation. The possibility of back-up pathways in place of blocked primary pathways has not been adequately explored. It is for these, and many more reasons, that the study of biochemical genetics has attained its present level of interest.

Diseases of the urea cycle fall into this category of poorly understood inborn errors of metabolism, perhaps most of which are associated with mental retardation. We do not know a lot of things that we should know if we are to help these individuals in adapting to their own unusual internal environment. The urea cycle is believed to be the only pathway for the biosynthesis of urea in man, and hence for the disposal of ammonia, which would otherwise reach toxic levels.

A mentally retarded patient has been found who has an apparent urea cycle disease known as citrullinemia (a block, partial or total, in one of the steps in the urea pathway), and yet he has normal ammonia levels in his serum and is able to synthesize normal amounts of urea. Besides having enormously elevated amounts of citrulline in his blood and urine, he has high levels of closely related amino acids.

The purpose of this study is to characterize this syndrome, to try to explain the reason for normal blood ammonia levels and normal urea production despite an apparent block in the pathway for ureogenesis, and to try to answer some of the questions posed by the abnormally high levels of some amino acids.

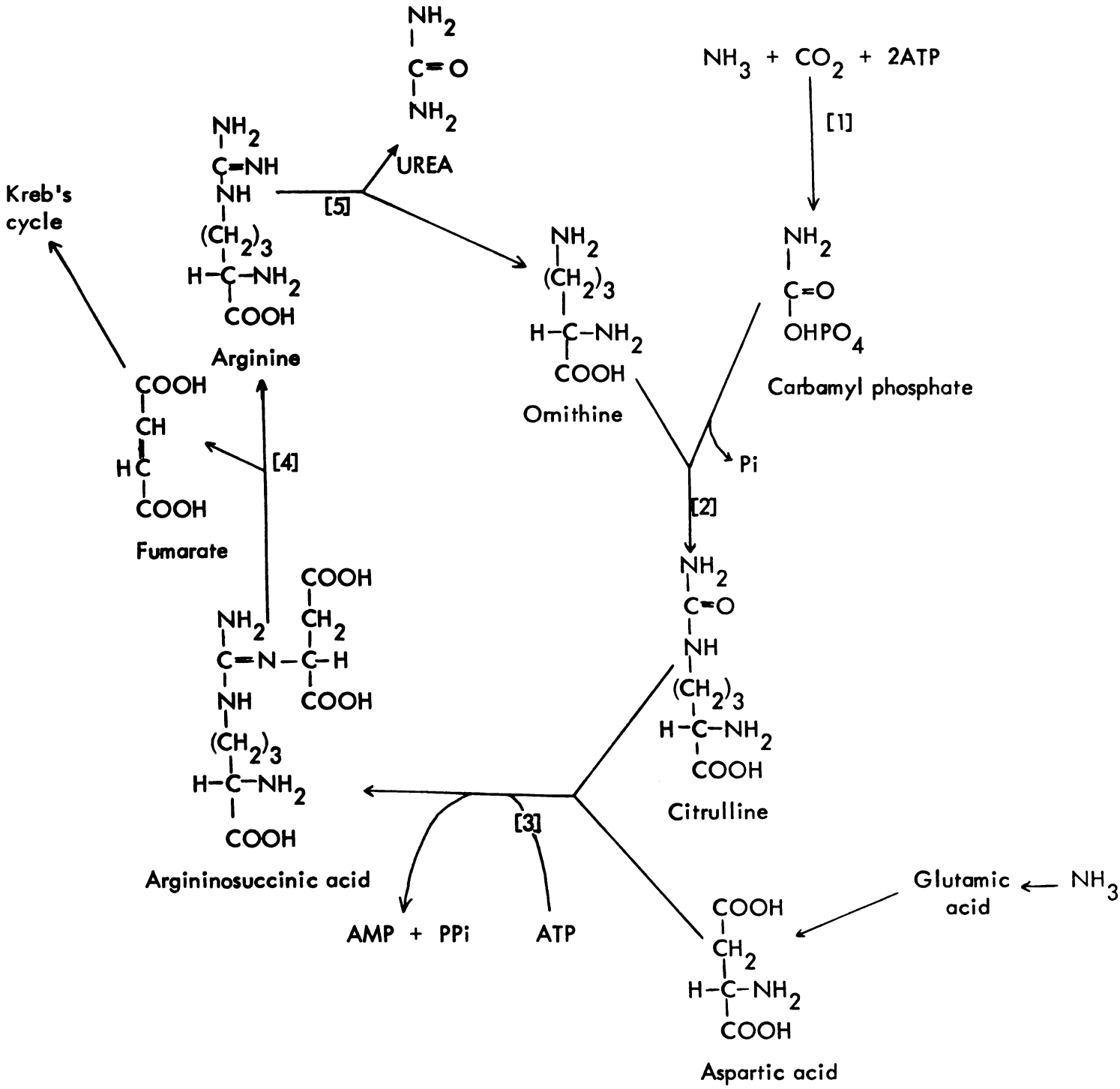
REVIEW

AMMONIA DISPOSAL

Unlike fats and carbohydrates, which are oxidized to water and carbon dioxide that are easily excreted, man hydrolyzes proteins to produce various nitrogenous compounds - amino acids. One of the metabolic fates of the amino acids is deamination, with the consequent production of ammonia.

Ammonia must be kept very low in the blood of man because of its toxicity when sustained at levels higher than 100μg/100ml (Conway and Cooke, 1939). Thus, ammonia has to be excreted as fast as it is produced or else converted to some less toxic compound. In most mammals, very little ammonia is excreted directly, but it is converted to urea, which is less toxic and more readily soluble in water.

The liver of man contain an enzyme, arginase, which hydrolyzes the amino acid arginine to urea with the formation of another amino acid, ornithine. Ornithine can be converted through three steps back to arginine, thus forming a complete cycle (Fig. 1). Ammonia enters this cycle at two points. One is in the formation of a fairly high energy phosphate compound, carbamyl phosphate, which combines with ornithine to form citrulline. The second is in the formation of glutamic acid which, through aspartic acid, combines with citrulline to form argininosuccinic acid in the presence of ATP (adenosine triphosphate). Thus, this cycle constitutes an effective way of ammonia disposal in man.



[1] Carbamyl phosphate Synthetase

[2] Ornithine Transcarbamylase

[3] Argininosuccinate Synthetase

[4] Argininosuccinase

[5] Arginase

Fig. 1 : The Urea Cycle (Simplified)

(Ratner et al., 1953a, 1953b, 1953c)

INBORN ERRORS OF THE UREA CYCLE

Defects of the five enzymes known to take part in the process of ammonia conversion to urea (see Fig. 1) have been reported in man, except for arginase (enzyme 5). Phenotypically, all four errors are associated with ammonia intoxication; the blood ammonia being markedly elevated, usually after a meal of protein, but sometimes even in the fasting state (Efron, 1967). Affected patients are usually ataxic, often with a history of seizures, and most have severe mental retardation. A biochemical analysis is necessary for the purpose of distinguishing the four types.

Deficiency of the enzyme carbamyl phosphate synthetase (enzyme 1) has been suggested for patients with hyperglycinemia (Efron, 1967). As would be expected, this deficiency leads to the accumulation of ammonia in the blood and consequent ammonia intoxication. Of particular interest is the excessive accumulation of glycine, which plays no known role in the synthesis of carbamyl phosphate. As of now, only one confirmed case of a hyperglycinemic patient with carbamyl phosphate synthetase deficiency has been reported. However, because of the hyperammonemia found in all hyperglycinemic patients, it has been suggested that this is the enzyme defect present in all of the patients.

A family having three members (an identical twin set and their cousin) with hyperammonemia and no amino acid abnormality was described by Russell et al. (1962). They were found to be deficient in the enzyme ornithine transcarbamylase (OCT) (enzyme 2). One member of the twin set and the cousin were severely affected, and both died of this condition.

Enzyme assays done on liver biopsies from both of them gave very perplexing results (Levin, 1967). One of the patients showed only ten per cent OCT activity when compared with normal subjects. The other showed two defects - five per cent of normal activity of OCT and twenty per cent of normal activity of carbamyl phosphate synthetase. Of particular interest is the lack of elevation of ornithine in the plasma or urine of the patients. This is understandable because of the possibility of ornithine conversion to glutamic acid and proline (Roloff et al., 1940). An increase in blood glutamine was found in these patients, which could be due to the stimulation of glutamine production because of ammonia accumulation in the blood (Tigerman and MacVicar, 1951).

Citrullinemia, which results from the deficiency in the activity of argininosuccinic acid synthetase (ASA synthetase) (enzyme 3), has been confirmed in two patients (McMurray et al., 1962; Morrow et al., 1967). This is perhaps the best documented of all the defects of the urea cycle. McMurray et al. found the defect in a mentally retarded child whose parents were first cousins. Citrulline levels were markedly elevated in the blood, cerebrospinal fluid (CSF), and urine. There was an insignificant elevation of some neutral amino acids which could be the result of renal competition with citrulline for clearance (Webber, 1962). Urinary citrulline for this patient was over 1000 times that of normal subjects. The patient's plasma citrulline was about 40 times normal and his CSF citrulline level was about 50 times normal (Efron, 1966). Enzyme assays from a liver biopsy showed this patient to be

lacking in the enzyme ASA synthetase (McMurray et al., 1963; Mohyuddin et al., 1967). The case described by Morrow et al. (1967) was that of a 21-month-old female who showed all the clinical symptoms of a urea cycle disease. Upon the analysis of some urinary and plasma constituents, it was found that the patient was a citrullinemic with the high ammonia levels which are often found to be associated with all urea cycle defects. Morrow's patient was interesting from the point of view that she produces very little urea (the significance of this comment will become obvious later). Her ASA synthetase was found to be defective in cultured skin fibroblast cells (Tedesco and Mellman, 1967). They found that at very high citrulline concentrations, the synthesis of arginine was normal, but at low citrulline concentrations, arginine synthesis was only ten per cent of normal. The Michaelis constant (Km) for citrulline in her cell lines was between 25 and 250 times that in normal cell lines. These results suggest very strongly that the defect is only a partial block of the enzyme activity. There are no data available on the activities of the urea cycle enzymes of a third possible case from Northern Ireland (Carson and Neill, 1962). The parents of this patient were thought to be first cousins (Efron, 1966).

A defect of the splitting enzyme, argininosuccinase (enzyme 4) leads to the accumulation of argininosuccinic acid (ASA), resulting in the disease argininosuccinicaciduria. This defect has been reported in about thirteen patients, some of whom have shown very severe mental retardation and hyperammonemia, while others show mild retardation and one case approaches normal intelligence (Efron, 1966). The first cases

reported were by Allan et al. (1958). They described two siblings in a family of six children who excreted very high levels of a ninhydrin-positive compound in their urine. This compound was later identified as argininosuccinic acid (Westall, 1958, 1960). Whereas normal subjects do not excrete identifiable amounts of ASA, these patients excreted about 3000 mg per day in their urine. The CSF concentrations of ASA in these patients were more than two times the plasma level - about 4 mg per 100 cc in plasma and 9.5 mg per 100 cc in CSF (Levin et al., 1961). Other patients were subsequently described with the same condition (Dent, 1959; Carson and Neill, 1962; Coryell et al., 1964; Armstrong et al., 1964; Moser et al., 1967). One phenotypic feature which has regularly been found in argininosuccinicaciduria but not in the other urea cycle defects is that the patient's hair was short, grew irregularly, gave a tufted appearance, and seemed to break off easily (Efron, 1966). Scriver (1962) reported that Dent has identified the hair lesion histologically as "trichorexis nodosa."

The enzyme argininosuccinase, which has been found to be active in the red blood cells of normal subjects, was found to be completely absent in three argininosuccinicaciduric patients, and to be at about half the normal level in both parents of two of these patients (Tomlinson and Westall, 1964). When a citrulline or ornithine load was administered to these patients, there was a corresponding increase in ASA excreted. These results, therefore, justify the conclusion that this disease is due to blockage of argininosuccinase.

EFFECT OF THESE DISEASES ON UREA PRODUCTION

All the cases of carbamyl phosphate synthetase deficiency, OCT deficiency, and argininosuccinase deficiency that have been described excrete normal amounts of urea. Of the two cases with ASA synthetase deficiency that have been studied in detail, one excretes normal amounts of urea and the other has defective urea production (Morrow et al., 1967). In an attempt to explain the reason for normal blood urea in the presence of an obvious deficiency of the enzyme that catalyzes one of the steps of the known urea cycle, many investigators have proposed interesting speculations.

One of these speculations is that the enzyme defect is a partial one; and that urea synthesis is carried out along the normal pathway, but that the rate of synthesis is insufficient to get rid of the ammonia rapidly. This point seemed to have been supported by Tedesco and Mellman when they found that at very high citrulline concentrations, the skin fibroblasts from a citrullinemic patient are able to synthesize normal amounts of urea; but at low citrulline concentrations, these same skin fibroblasts only had ten percent efficiency. Their data on enzyme kinetics is also in agreement with this point. They found that the Km value for citrulline in citrullinemic patients was between 25 and 250 times as much as the value in normal subjects. This means that fibroblasts from citrullinemic patients need between 25 and 250 times more citrulline than those from normal subjects in order to have normal urea synthesis. However, the hypothesis that normal urea production in many urea cycle defects is due to partial enzyme activity has major

weaknesses. One weakness is that the patients with argininosuccinase deficiency whose red cell enzyme activity was investigated, have been found to have zero activity (Tomlinson and Westall, 1964; Moser et al., 1967). This means that the enzyme block is complete and yet they synthesize normal amounts of urea. Another major weakness of this hypothesis is the fact that it was formed to explain the results for the only patient who has defective urea production, although he has more than 50 times the normal level of plasma citrulline.

Another speculation is that the enzymes of the urea cycle exist in different organs as isoenzymes and that each isoenzyme is genetically independent. By this hypothesis, each one of the biochemical defects reviewed above exists in at least one organ, but not in all organs. Indeed, it has been suggested that in argininosuccinicaciduria, two genetically independent argininosuccinase isoenzymes exist: that one exists in the brain and the other in the liver and that while the brain enzyme is affected, the liver enzyme continues to function (Dent, 1959; Westall, 1960). No experimental evidence was found which lends support to this hypothesis.

A third speculation has to do with the presence of a completely new pathway for ureogenesis. This speculation was made by Russell et al. (1962) when they described a patient with OCT deficiency who has normal levels of urea in his plasma. Since that time, it has been found that homoarginine can be hydrolyzed to urea by arginase, the same enzyme that hydrolyzes arginine to urea (Ryan et al., 1968, 1969).

The same workers have found that the source of homoarginine as well as homocitrulline is lysine. They found that kidney enzymes can convert lysine to homoarginine by transamidation, but the mode of lysine conversion to homocitrulline was not known. Thus, when rats, as well as human subjects, are given a lysine load, there are corresponding increases in the urinary homoarginine and homocitrulline levels (Ryan and Wells, 1964). However, they were not able to demonstrate the conversion of homocitrulline to homoarginine, and hence were not able to prove the existence of a true cycle.

Support for the existence of an alternate cycle may be found in lysinemia, a disease that sometimes has symptoms of the urea cycle defects (Colombo et al., 1967). They described a patient with ammonia intoxication who had elevated lysine in both his blood and urine. Following an oral load of lysine, there was an enormous elevation of the patient's plasma ammonia. This elevation of the plasma ammonia level following lysine ingestion was not observed in the patient's parents, a sibling, and an unrelated child. During periods of high protein intake, this patient's plasma lysine rose from 2.7 to 6.8 mg per cent. Associated with lysine elevation was a rise in plasma arginine from 2.2 to 6.2 mg per cent. Because of this apparent relationship between lysine and ammonia disposal, all of the urea cycle enzymes were assayed from a liver biopsy taken from the patient. All the enzymes showed normal activity. The first step in the degradation of lysine in man is not very well known, but it is thought to involve an NAD-dependent oxidation reaction catalyzed by lysine dehydrogenase (or lysine: NAD oxido-reductase) (Rothstein and Miller,

1954a, 1954b). Buergi et al. (1966) have found this enzyme to be defective in their lysinemic patient, with one-fourth of the activity found in normal subjects. The relationship between lysine and arginine in their patient has led to the belief that lysine is a potent inhibitor of arginase. The exact mode of this inhibition has not been worked out. However, in the hyperlysinemic patient described by Ghadimi et al. (1964, 1967) there was no ammonia intoxication. Only 58 per cent of the lysine administered is metabolized by the patient compared with more than 99 per cent for controls. Homoarginine and homocitrulline were found in some of this patient's plasma samples, implying that the pathway(s) for converting lysine to these compounds is (are) intact. Unfortunately, they did not report the arginine levels of their patient.

INHERITANCE

The family described by Russell et al. (1962) with three apparent cases with OCT deficiency leads to the presumption that the condition, being familial, is an inherited disease. The mode of inheritance is still obscure since enzyme assays were not done on any unaffected relatives.

The first case of citrullinemia (ASA synthetase deficiency) described was of a child of a consanguineous marriage (Mohyuddin et al. 1967). One could postulate an autosomal recessive inheritance because of this consanguinity. However, preliminary enzyme studies of a patient in Oregon have shown the enzyme to be deficient in skin fibroblasts of one parent and near normal activity in the other parent (Buist, 1970). This could be an indication of dominant inheritance.

Argininosuccinicaciduria (argininosuccinase deficiency) has been reported among siblings in four families (Allen et al., 1958; Carson and Neil, 1962; Armstrong et al., 1964; Moser et al., 1967). Each one of these cases had zero activity of the enzyme and in each case investigated both parents had below normal function of the same enzyme. The familial occurrence of this error is consistent with an inherited disease, and the subnormal level of enzyme activity in the parents suggests that they are heterozygous for the allele responsible for this error. It seems, therefore, that argininosuccinicaciduria is inherited as an autosomal recessive trait.

The inheritance of lysinemia seems to be the best documented of all. Ghadimi et al. (1964) reported a familial case with no parental consanguinity. Woody (1964a) described two siblings with the syndrome who are offspring of an incestuous mating (father-daughter). Another family with two affected siblings and an affected cousin was also described, and each one of the affected patients in this case had consanguineous parents (Woody, 1964b; Woody et al., 1966; Ghadimi et al., 1967). We therefore have strong evidence that this is a genetic error that is inherited as an autosomal recessive trait.

Careful studies of enzyme abnormalities of the type that lead to urea cycle defects have almost always indicated that they are recessively inherited in man. Carriers have reduced enzyme activity, when this can be measured accurately.

MATERIALS AND METHODS

IDENTIFICATION OF ERROR

Starting in 1967, patients in the state institutions for the mentally retarded in Michigan have been screened for metabolic errors. A random sample of urine was collected from each patient and blood flowing from a finger prick was collected in two capillary tubes. In this section, methods that are fully described in the cited reference will not be given in detail; but those that have been modified from any printed description will be more fully described. Each urine sample was analyzed for the following:

(1) Mucopolysaccharides:

Two methods for the detection of mucopolysaccharides were used. The first is a modification of Renuart's method (1966). One ml of clear urine (centrifuged if cloudy) was placed in a test tube at room temperature and 1 ml of cetyl-trimethylammonium bromide (CTAB) reagent was added. The urine was allowed to stand at room temperature for 5 minutes. A cloudy precipitate constituted a positive test. The CTAB reagent was prepared by dissolving 5 grams of the salt in 100 ml of 1 M citrate buffer, pH 6. The second method used is the Toluidine Blue Spot Test as described by Berry and Spinanger (1960). Various mucopolysaccharidoses are detectable by these tests.

(2) Keto acids and most of their derivatives:

The ferric chloride test described by Renuart (1966) was used. This test gives positive reactions (usually a greenish

color) with urines from patients who have phenylketonuria (PKU), maple syrup disease, homogentisicaciduria, and tyrosinosis The 2,4-dinitrophenylhydrazine test was also used for identifying keto compounds. The technique employed was described by Penrose and Quastel (1937).

(3) Compounds with disulfide bonds:

The test used was the sodium nitroprusside (sodium nitroferricyanide) test as described by Carson et al. (1963) and Knox (1966). Cystinuria and homocystinuria are the most common diseases detected by this test.

(4) General amino acids:

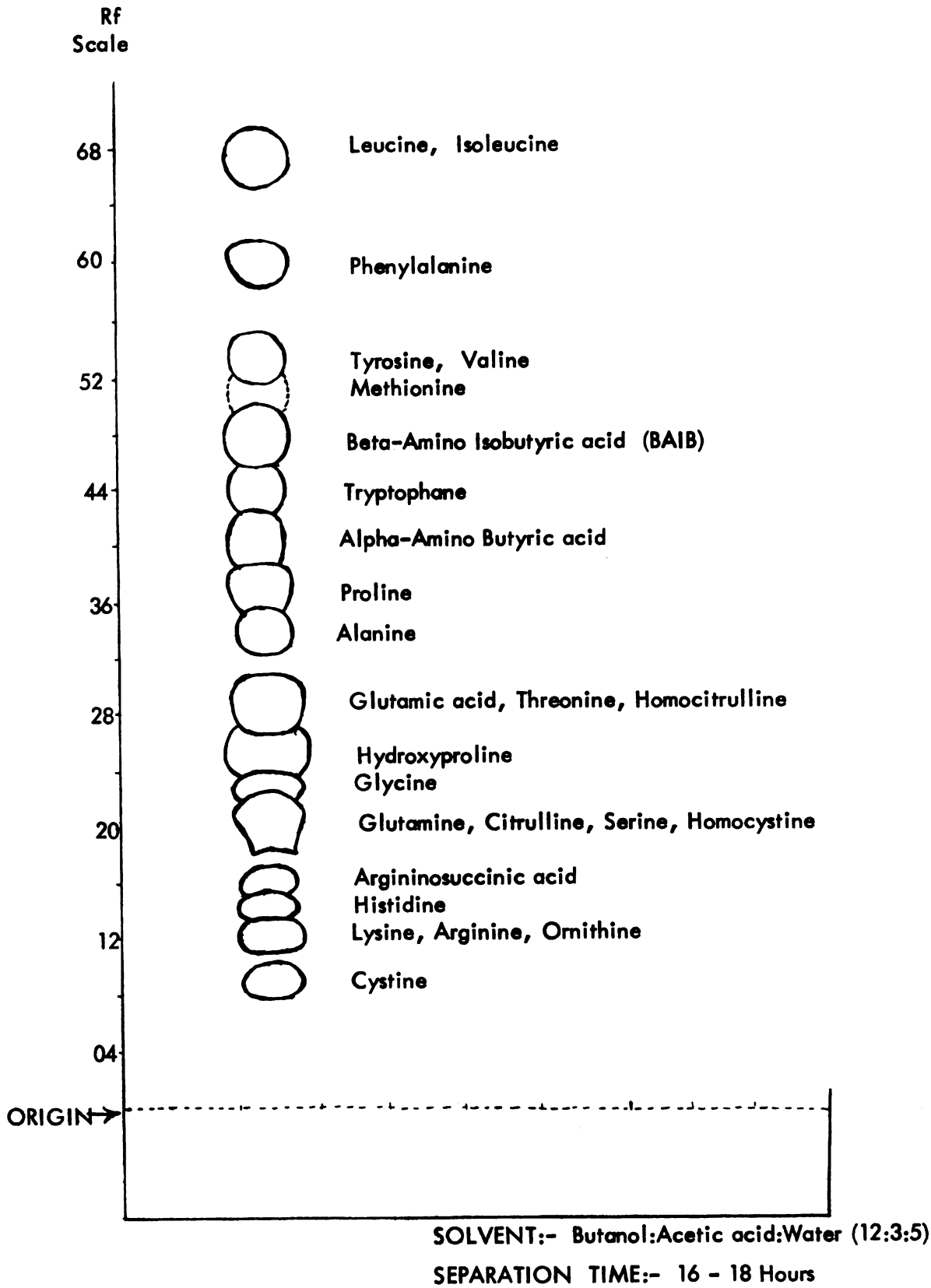
A simple one dimension chromatographic procedure was used for the detection of various amino acid abnormalities. The method employed was a slight modification of that described by Smith (1960a) and Efron et al. (1964). Between 5 and 70 microliters of each urine (depending on creatinine content) was spotted along one edge of a 23 x 57 cm Whatman 3 mm paper. This size paper takes eight samples at a time. The chromatograms were developed in a descending fashion using butanol:acetic acid: water (12:3:5) solvent at room temperature for approximately 16 hours. The chromatograms were then dried in a fume hood for one hour, after which they were dipped through a ninhydrin/isatin stain (2.5 grams ninhydrin, 0.1 gram isatin, 1000 cc acetone), and heated in an oven at 80-90^{o}C for 10 minutes. The pattern of

separation of the amino acids is represented in Fig. 2. Many deviations from the normal pattern are readily identified.

After eye-scanning the ninhydrin positive spots for abnormalities, each chromatogram was cut into two just above the histidine spot. The bottom part was dipped into Pauly's reagent (described by Scriver, 1964) for evidence of a histidine abnormality while the top half was dipped into Ehrlich's reagent (Scriver, 1964) for evidence of proline, hydroxyproline, citrulline and homocitrulline disorders. The urea content of each sample can also be crudely analyzed from the Ehrlich's reaction.

The blood sample was analyzed only for general amino acid disorders. The capillaries were spun in a centrifuge to separate red cells from serum and about 10 microliters of serum were spotted on Whatman 3 mm paper and chromatographed as described for urine amino acids.

Fig. 2: The pattern of separation of amino acids by descending paper chromatography



THE SUBJECT

With the above screening method, a patient was discovered with a rare aminoacidopathy - citrullinemia.

The patient, R.D., was born in Warren, Michigan, on March 15, 1937. His mother reports that he was a full-term product of an undisturbed pregnancy and normal delivery. His growth and development were apparently normal until, at the age of one, he suffered from what a doctor diagnosed as encephalitis and spinal meningitis. The symptoms that led to the diagnosis were attacks of vomiting and profound lethargy. He was hospitalized in a semi-comatose state. Upon "recovery" he was no longer able to talk and did not resume talking until the age of 2 1/2, according to his mother's report. A few other attacks followed this recovery, but only lasted a few hours. Two of these subsequent attacks followed a breakfast of eggs, toast and milk. The pattern of attack in each of these two cases was similar, with the patient standing motionless, growing increasingly lethargic and reaching for support, his eyes rolling backwards, with mild convulsions following.

A complete physical examination has been given to this patient in many instances, and each time no physical anomaly was detected.

The patient's IQ was tested in 1967 with the following results:

verbal IQ	53
performance IQ	54
full scale IQ	50

Wais, Bender-Gestalt test

The patient is thus classified as operating within moderate range of retardation.

The patient's maternal grandfather is of Swiss extraction and his maternal grandmother is of German extraction. On the paternal side of the patient, his grandfather is of German and Austrian extraction, and his paternal grandmother is of Polish extraction. Although both the patient's maternal grandmother and his paternal grandfather have a similar ancestry, there is no knowledge of any blood relationship between them. Close consanguinity has been ruled out.

The patient has one brother who was available for biochemical analysis and was found to be normal. (See complete pedigree in appendix.)

ANALYSIS OF THE SUBJECT'S METABOLISM

To gain information that would lead to an understanding of the patient's metabolism, he was hospitalized for three weeks and maintained under each of the following conditions for at least four days:

(1) Normal institution feeding conditions

(2) Very low protein diet conditions

(3) Very high protein diet conditions, with milk

(4) Very high protein diet conditions, without milk

Serum and 24-hour urine samples were collected daily during each test, and each sample was subjected to the following analysis:

(a) Amino acid determination

The qualitative and quantitative determination of amino acids was done with the aid of a two-channel automatic amino acid analyzer

(Technicon Model). The elution of amino acids was carried out with 0.05M sodium citrate buffer, pH 2.875, pH 3.8, and pH 5.0 (Fig. 3 shows the pattern of amino acid separation). The urine was not pre-treated before use. 0.2 ml of the urine was applied on the column for analysis. It was necessary to dilute the urine (1:10) in order to quantitate citrulline and to apply 1 ml of undiluted urine to quantitate homoarginine. The serum, however, had to be deproteinized. About 10 cc of venous blood was drawn in heparin tubes and centrifuged for about 15 minutes at 300 rpm. The serum was decanted into a centrifuge tube and 65 mg of sulfosalicylic acid was added for every ml of serum. After thorough mixing, it was centrifuged for 15 minutes at 3000 rpm. The supernatant is a protein-free filtrate and 0.5 ml of this was applied to the column for chromatography. It was necessary to apply 2.5 ml of serum to quantitate homoarginine.

When these tests were applied to control subjects, the blood was drawn without anticoagulants and allowed to coagulate before centrifugation.

(b) Ammonia determination

Only serum ammonia was determined. This was done daily two hours following lunch. On the last day of each feeding condition, ammonia was determined six times - immediately before lunch, then at 30, 60, 90, 120, and 180 minutes later.

Two methods for ammonia determination were employed. The micro-diffusion method of Conway (1950) involves the liberation of ammonia with alkali (potassium carbonate), the diffusion of ammonia into an acidic indicator (mixed indicator containing 0.066% methyl red and 0.033% bromocresol green in alcohol), and the subsequent titration of

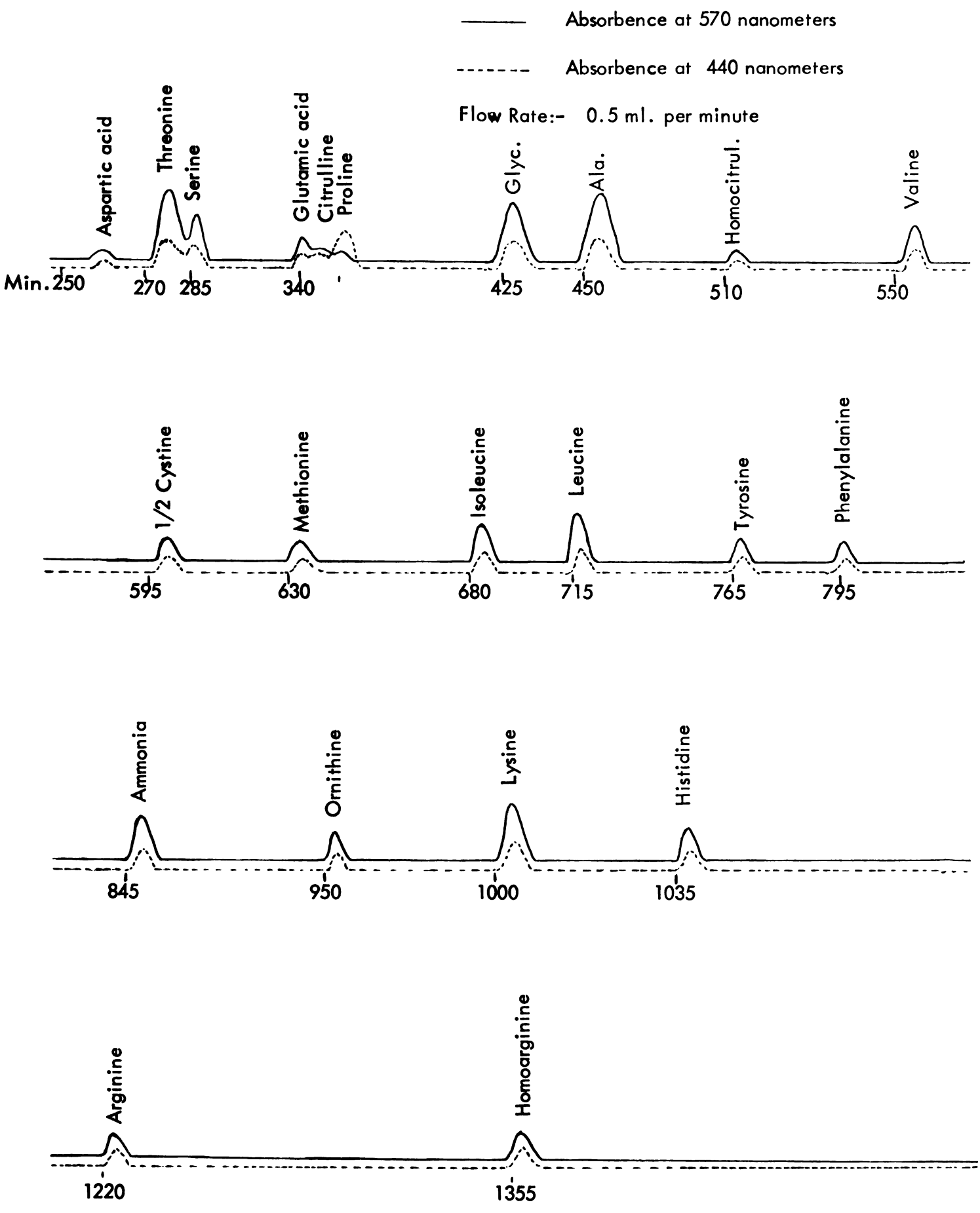


Fig. 3: The pattern of separation of amino acids by automatic amino acid analyzer

the now basic indicator with a very weak acid (0.001 N HCl).

The other method of ammonia determination used in this work is the Betherlot color reaction (Chaney and Marback, 1962) as modified by Caraway (1966). By this method, proteins were precipitated with sulfate and sodium tungstate. The ammonium ions in the protein-free filtrate were coupled with phenol in the presence of hypochlorite to form an indophenol which gave a blue color in an alkaline solution after 10 minutes of incubation at 37°C. This color can be read in a spectrophotometer at 630 nanometers. Because of the reaction of EDTA with copper to form a blue supernatant, EDTA was never used as an anticoagulant. Blood was therefore drawn in heparin tubes.

(c) Creatinine determination

Creatinine determination was by a method which is essentially that of Folin and Wu as described by Caraway (1966). This method is based on the fact that in the presence of creatinine, alkaline picric acid will form a reddish-brown color which has maximum absorbence in a spectrophotometer at 520 nanometers. Serum and urine samples were analyzed in essentially the same way, but the urine sample was diluted 1:100 before use.

(d) Urea nitrogen determination

Blood urea nitrogen (BUN) was determined by a modification of the method of Gentzkow (1942). The principle involves the hydrolysis of urea by urease to ammonium carbonate, precipitation of proteins with sodium tungstate, and the colorometric determination of ammonia using a simple stabilizing reagent - Nessler's reagent (Connerty et al., 1955).

The Nessler's reagent was prepared in the following way:

Reagent 1: 15 grams of potassium iodide were dissolved in 10 ml of water. 20 grams of mercuric iodide were added, stirred to dissolve, and made up to 100 ml with water.

Reagent 2: 2.50 N carbonate-free sodium hydroxide

Reagent 3: To 80 ml of Reagent 1, 390 ml of Reagent 2 were added slowly with mixing. It was allowed to stand for a day, and the clear supernatant was decanted and stored as Nessler's reagent.

To 1 ml of blood, 4 drops of urease solution (prepared according to Gentzkow's method) were added. 0.5 ml of disodium phosphate buffer (3.55 gm anhydrous Na_2HPO_4 in 500 ml water) and 1.5 ml of water were added and allowed to stand at room temperature for 20 minutes. Then 5 ml of water, 1 ml of 0.66 N sulfuric acid and 1 ml of 10% aqueous solution of sodium tungstate were added, thoroughly mixed and centrifuged. 0.5 ml of the supernatant was taken, 9 ml of water and 0.5 ml of Nessler's reagent were added, allowed to stand for 5 minutes, and read in a spectrophotometer at 500 nanometers.

Urine urea was determined in the same way as serum urea except that the 24-hour urine sample had to be diluted 1:10 or 1:100 depending on its volume.

ENZYME ASSAYS

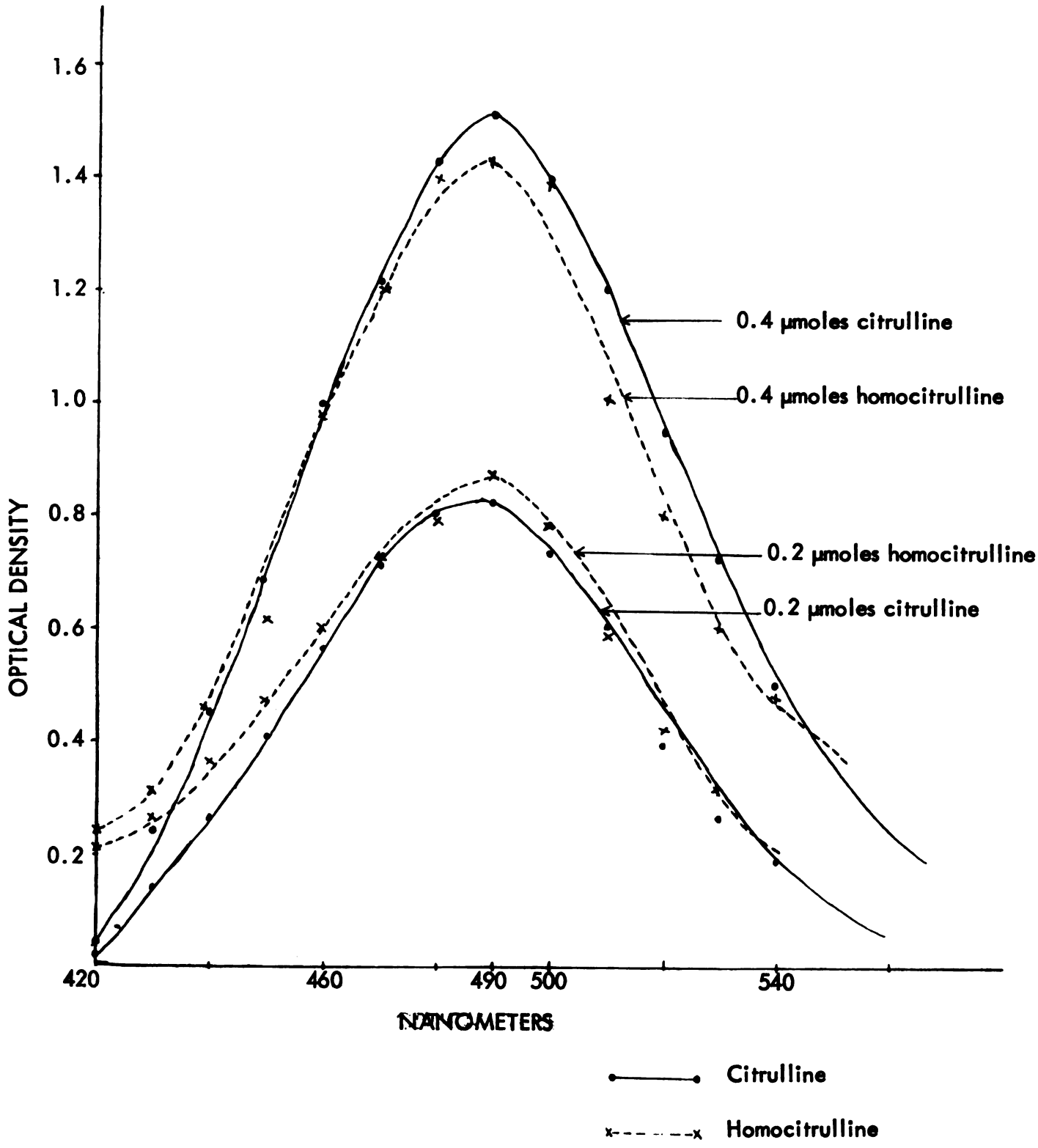
Liver biopsy specimens were unavailable from this patient. Appropriate enzyme assays were performed using liver specimens from

inbred colonies of Sprague-Dawley and Long-Evans strains of rats. They were routinely maintained on Wayne laboratory block diet until used. For an experiment, one rat was sacrificed by decapitation, the liver was quickly removed and placed in a beaker surrounded by ice. One gram of the liver was weighed out and homogenized in 19 cc of ice cold distilled water for about 1 minute. Enzyme analyses were made within the following few minutes. This homogenate gave a reasonable protein concentration for the assays (Schimke, 1962).

With the exception of argininosuccinase, commercial reagents were employed. Argininosuccinase was partially purified from cow liver according to the method of Ratner (1955). All assays were performed in duplicate, and each assay had its own set of standards.

The assays were based on the colorimetric determination of urea and citrulline by the method of Archibald (1944) as modified by Ratner (1955). Homocitrulline was determined colorimetrically the same way as citrulline with the same reagents. Figure 4 shows that the maximum absorption of homocitrulline, like citrulline, is at 490 nanometers. The color reagents were 1-phenyl-1, 2-propanedione-2-oxime for urea determination and 2,3-butanedione-2-oxime for citrulline and homocitrulline determinations. The methods used for the assays were patterned after those of Brown and Cohen (1959) as modified by Schimke (1962). The unit of activity of the enzymes was expressed as uMoles of products formed per hour per gram wet weight of liver.

Fig. 4: Absorption curve for citrulline and homocitrulline



ORNITHINE TRANSCARBAMYLASE

This enzyme activity was determined as the rate of citrulline formation. Whenever lysine was used as a substrate instead of ornithine, the enzyme activity was measured as the rate of homocitrulline formation. The assay medium contained 20 µMoles of L-ornithine or L-lysine, pH 8.0 (Sigma Chemical Company), 90 µMoles of glycyl-glycine buffer, pH 8.3, and the liver homogenate. The reaction was started with 20 µMoles of dilithium carbamyl phosphate (Sigma Chemical Company). The final volume was 2.0 ml containing 1.0 ml of the homogenate. Incubations were at 37.5°C for 15 minutes, at the end of which the reactions were stopped with 2 ml of 30% perchloric acid. A medium treated with perchloric acid before incubation served as zero time control. For the ornithine reactions, 150 µLiters of the incubated medium were taken for citrulline color development; and for the lysine reactions, 1.5 ml of the incubated medium were taken for homocitrulline color development.

ARGININOSUCCINIC ACID SYNTHETASE (CONDENSING ENZYME) AND ARGININOSUCCINASE (SPLITTING ENZYME)

The activities of both of these enzymes were assayed together using two different assay methods. One method contained ATP regenerating system and the other did not. The assay medium with ATP regenerating system contained 1.25 µMoles of citrulline or homocitrulline, pH 7.8; 1.25 µMoles of aspartate, pH 7.8; 12.5 µMoles of potassium phosphate buffer, pH 7.8; 0.5 µMoles of ATP, pH 7.8; 2.5 µGrams of pyruvate kinase; 0.75 µMoles of magnesium sulfate; 2.5 µMoles of phosphoenol pyruvic acid (PEP); 20 units of arginase; and 50 µLiters of liver homogenate. The

final volume was 250 µLiters, and the incubation time was one hour at 37.5°C. The reaction was stopped at the end of incubation by the addition of 1 ml of 15% perchloric acid. A medium treated with perchloric acid before incubation served as a zero blank. 1 ml of reaction medium was taken for urea color development. This assay condition was modified from Schimke (1962).

The assay medium without ATP regenerating system contained 50 µMoles of potassium phosphate buffer, pH 7.0; 5 µMoles of L-citrulline or L-homocitrulline, pH 7.0; 5 µMoles of L-aspartate, pH 7.0; 5 µMoles of ATP, pH 7.0; 5 µMoles of magnesium sulfate; and 20 units of arginase. The volume of the above medium was 0.5 ml and 0.5 ml of liver homogenate was added to start the reaction. Incubation was for 1 hour at 37.5°C. At the end of incubation, 1 ml of 30% perchloric acid was added to stop the reaction. The zero blank was a medium treated with perchloric acid before incubation. 1 ml of the reaction medium was taken for urea color development. This assay technique was modified from that of Brown and Cohen (1959).

ARGINASE

The assay medium contained 250 µMoles of L-arginine or L-homoarginine, pH 9.8 and 1 µMoles of manganese sulfate ($MnSO_4$) in a volume of 1.0 ml. In order to increase the activity of the enzyme, 50 µMoles of $MnSO_4$ were added for every ml of homogenate, and this was preincubated for 5 minutes at 55°C. This gave about an 80 to 125 per cent increase in enzyme activity (it was not necessary to treat commercially prepared enzyme the same way). 20 µLiters of preincubated homogenate were added to 1 ml of

assay medium and incubated for 15 minutes at 37.5°C. At the end of the incubation, 2.5 ml of 15% perchloric acid were used in stopping the reaction. A medium treated with perchloric acid before incubation served as zero blank. For the reaction with arginine as substrate, 0.1 ml of reaction medium was used for urea color development, while for that with homoarginine as substrate, 1.0 ml of reaction medium was used for urea color development.

THE OVER-ALL REACTION

The activities of three enzymes, ornithine transcarbamylase, argininosuccinate synthetase (condensing enzyme), and argininosuccinase (splitting enzyme), were measured as the amount of urea produced using a coupled system that contained excess arginase. The assay system contained 90 uMoles of glycyl glycine buffer, pH 8.0; 20 μMoles of L-ornithine or L-lysine, pH 8.0; 5 μMoles of ATP, pH 7.8; 2.5 μMoles of $MgSO_4$; 10 μMoles of L-aspartate, pH 7.8; and 1 mg of arginase per ml of assay medium. The reaction was started with 20 μMoles of dilithium carbamyl phosphate. Incubation was carried out for one hour in 2 ml of the medium that contained 1 ml of liver homogenate. At the end of incubation, the reaction was stopped with 2 ml of 15% perchloric acid. Zero blanks were gotten the same way as in the other assay systems.

ATP regenerating system was not used because between 5 and 10 uMoles of ATP proved to be equally as satisfactory.

RADIO-ISOTOPE ASSAY

Because the conversion of L-lysine to urea is inefficient, the colorimetric quantification of urea was not possible. Therefore, for the overall reaction, L-lysine-C^{14} was used as substrate.

Activities of the enzymes were measured as amount of homoarginine produced. Homoarginine was separated by low-voltage paper electrophoresis as described by Smith (1960b) using 0.53 per cent sodium carbonate buffer, pH 11.5. By this method, lysine and homocitrulline moved toward the anode, and homoarginine moved a short distance from the origin toward the cathode.

The preparation of reagents and the procedure for the radio-isotope overall reaction were as follows:

Reagents

(1) 100 mM potassium phosphate (KH_2PO_4) buffer, pH 7.8;

(2) 450 mM glycyl glycine buffer, pH 8.0;

(3) 150 mM aspartate, pH 7.8, prepared in Reagent 1 (10 µLiters of this solution contain 1.5 µMoles of aspartate);

(4) 100 mM ATP, pH 7.8, 400 mM KCl, and 24 mM $MgSO_4$, all prepared in Reagent 1. (10 µLiters of this solution contain 1 µMole of ATP, 4 µMoles of KCl, and 0.2 µMoles of $MgSO_4$);

(5) 200 mM phosphoenol pyruvic acid (PEP) and 50 µGrams/ml pyruvate kinase, prepared in water. 1 mg of arginase was added for each ml of assay medium where necessary. (10 µLiters of this solution contain 2 µMoles of PEP and 0.5 µg of pyruvate kinase. Where arginase is used, it contains 10 µg);

(6) 40 mM carbamyl phosphate prepared in water. (50 µLiters of this solution contains 2 µMoles of carbamyl phosphate);

(7) 300 mM L-lysine, pH 8.0. 548.1 mg of L-lysine were dissolved in 10 ml of Reagent 2 and adjusted to pH 8.0;

(8) Before use, 100 μLiters of Reagent 7 were added to 100 μLiters of L-lysine-C^{14} (New England Nuclear). (20 μLiters of this mixture contain 3 μMoles of L-lysine and 1 μCurie of L-lysine-C^{14}).

Procedure

0.5 gm of liver was homogenized in 10 ml of Reagent 1. 100 μLiters of the homogenate were added to the reaction tube (5 ml test tube), followed by 10 μLiters of Reagent 3, 10 μLiters of Reagent 4, 20 μLiters of Reagent 8, and 10 μLiters of Reagent 5. 50 μLiters of Reagent 6 were added to start the reaction.

The entire reaction medium was incubated for an hour at 37.5°C after which the reaction was stopped by boiling. Preboiled medium served as zero blank.

After the reaction was stopped, the tubes were centrifuged for 10 minutes and 100 μLiters of medium were spotted on Whatman no. 4 paper for an electrophoretic run. The dimensions of the paper were 30 x 18 cm and the electrical current was 3 volts per cm of paper.

Column chromatography was also used for clearly isolating homoarginine. Technicon resin (chromobeads) were used in a 150 cm column and 0.05 M sodium citrate buffer of pH 6.8 was used as eluent. One hundred microliters of incubated medium were chromatographed and at a flow rate of 0.5 ml per minute, all amino acids were eluted by the eighth hour. The last amino acid, homoarginine, was eluted between 7 1/2 and 8 hours. Authentic homoarginine was added as a carrier, and a

ninhydrin color reaction was allowed to take place before collection, so that the smallest possible volume that contained homoarginine was collected. Between 30 and 50 cc volume was required to collect all of the homoarginine, and 5 cc of the total eluent was added to 15 cc of the counting fluid (aqueous and room temperature counting fluid) to be counted.

RESULTS

GENERALIZED METABOLIC SCREENS

The results of the generalized metabolic screens are given in Table 1. There was no evidence that the patient excretes mucopolysaccharides as the CTAB and toluidine blue spot tests were negative. The ketone tests, ferric chloride and 2,4-dinitrophenyl hydrazine, were negative, implying that the patient is free of errors like PKU, branched chain ketonuria, homogentisicaciduria and tyrosinuria, to name the more common ketone diseases. No common sulfur disease, such as cystinuria and homocystinuria, was present in the patient, as indicated by the negative cyanide-nitroprusside test.

AMINO ACID SCREEN

Figure 5a shows the amino acid pattern of the urine and plasma compared with a normal pattern. The position occupied by citrulline is indicated. Migrating to the same position as citrulline are glutamine and serine, but it is easy to identify citrulline (Fig. 5b) because with Ehrlich's reagent only citrulline will stain from yellow to pink, depending on its concentration. Urea will stain bright yellow with Ehrlich's reagent, hence a crude estimate of how much urea is excreted could be made from paper chromatography.

DIETARY MANIPULATION OF THE PATIENT'S METABOLIC PROCESSES

The patient was hospitalized for three weeks to permit a series of studies of his responses to specialized diets. For the first seven days (Monday through Sunday) he was maintained on a normal diet. On the

	Test	Result	Comments
1.	CTAB test	No precipitate	Patient normal for mucopolysaccharides
2.	Toluidine Blue test	Negative	Patient normal for mucopolysaccharides
3.	Ferric chloride test	Negative	Patient normal for ketonurias
4.	2,4-dinitrophenyl hydrazine test	No precipitate	Patient normal for ketonurias
5.	Sodium cyanide - Sodium nitroprusside test	Negative	Patient is normal for most of the known sulfur diseases

Table 1: General metabolic screens

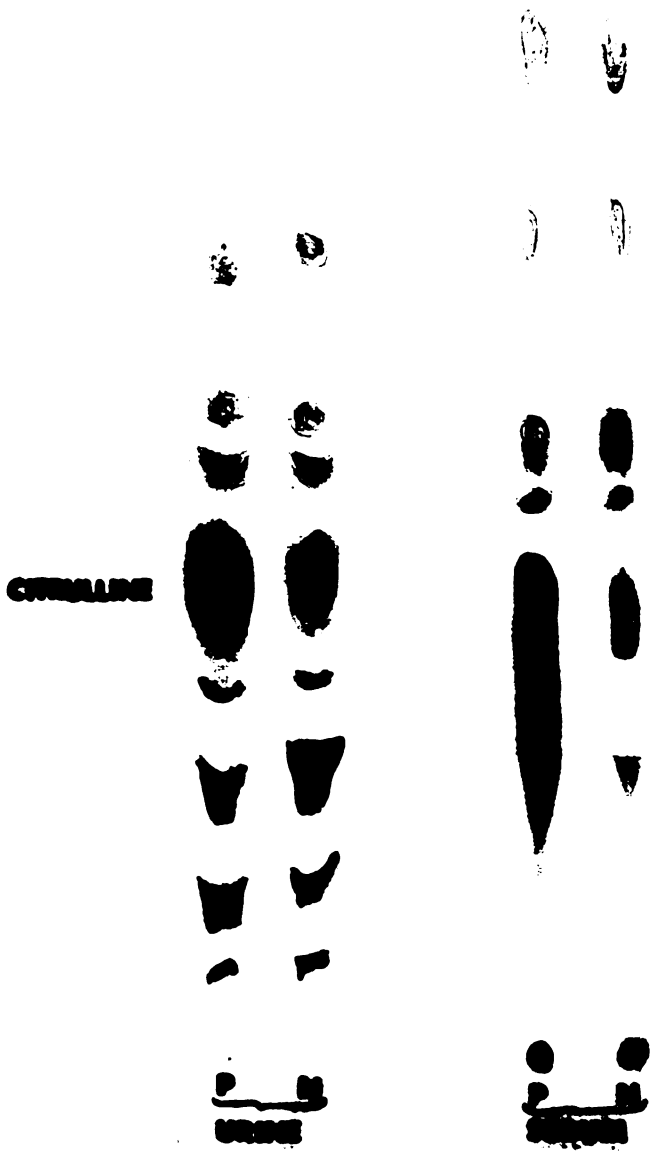


Fig. 5a: Paper chromatographic separation of the patient's urine and serum amino acids compared with a normal subject's (NINHYDRIN STAIN). P is the patient; N is normal

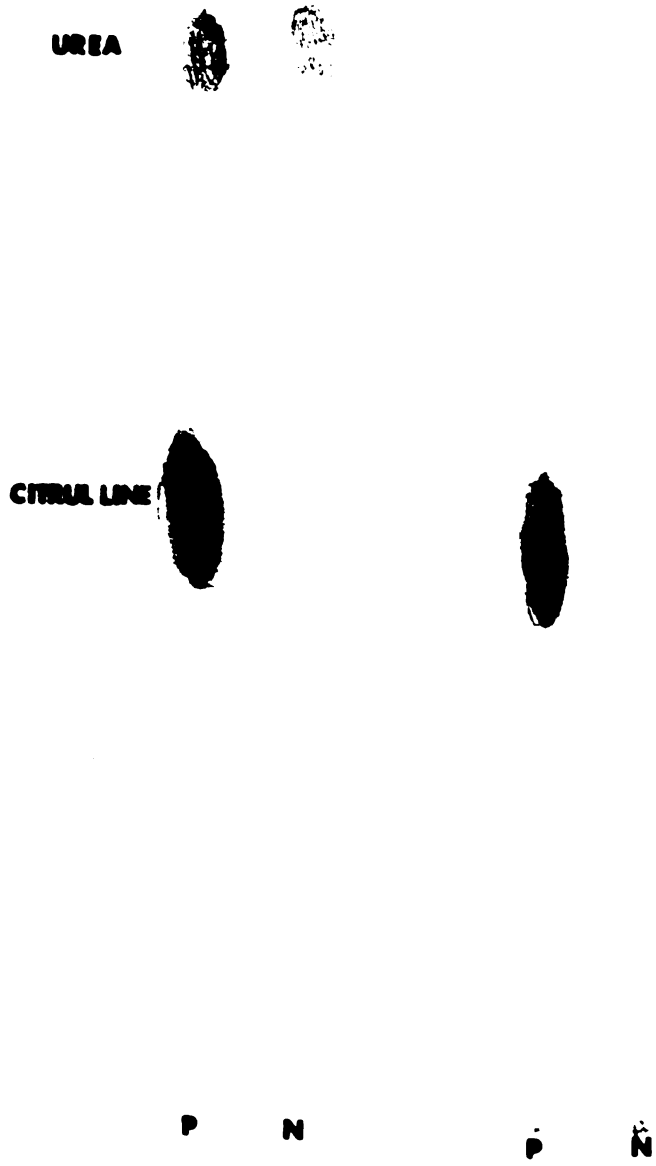


Fig. 5b: Over-dip of chromatogram in Fig. 5a with Ehrlich's reagent. (Citrulline is stained pink and urea is stained bright yellow)

eighth day (Monday) he was placed on a low protein diet, which was maintained for a total of five days (through Friday). One day was then spent on a normal diet (Saturday), and the next eight days (Sunday through Sunday) were on a high protein diet, with the first four days without milk and the last four days with milk. Milk was omitted from the first four days on the high protein diet because of the large amounts of citrulline and homocitrulline that it contains.

Urea and creatinine clearances

Each of these two tests was performed on three successive days while the patient was hospitalized, but on a normal diet. The tests checked the patient's kidney function to see if any of his biochemical anomalies could be attributed to poor kidney function. Tables 2 and 3 give the clearance values for urea and creatinine, respectively, for each of the three successive days. The urea clearance values ranged from 91 to 148 ml per minute (normal range is described by Caraway, 1966, as 75 to 125 ml per minute) with an average clearance of 125.6 ml per minute. This mean value is acceptable as approximately normal. The creatinine clearance levels ranged from 121.4 to 161.6 ml per minute (normal range is described by Caraway, 1966, as 100 to 125 ml per minute) with an average clearance of 142.8 ml per minute. This mean value is by no means unusual. Based on the urea and creatinine clearances, the patient's kidney can be assumed to be functioning normally.

Normal diet

The urea levels in the patient's urine are given in Table 4 for each day of his hospitalization. The patient's average urea excretion,

Blood Urea Nitrogen in mg per cent	Urine Urea-N at 10 am (mg per cent)	Urine Urea-N at 11 am (mg per cent)	Urine Urea-N per day (mg per cent)	Clearance ml per minute 10 am	11 am	24-hr.
15.5	1250 (58 ml.)	1300 (56 ml.)	- (695 ml.)	138	141	-
9.5	350 (177 ml.)	510 (96 ml.)	1050 (160 ml.)	135	119	91
9.0	420 (152 ml.)	540 (60 ml.)	540 (1705 ml.)	148	104	113

Patient's Average Urea Clearance = 125.6 ml per minute

NOTE: Normal urea clearance value = 75 - 125 ml per minute

Values in parenthesis indicate the volume of urine voided

Table 2: The patient's urea clearance

Blood Creatinine mg per cent	Urine Creatinine mg per 24-hr.	Creatinine Clearance ml. per minute
1.00	2080.1 (1705 ml.)	145.6
1.05	1822.4 (1340 ml.)	121.4
0.90	2076.4 (1160 ml.)	161.6

Patient's Average Creatinine Clearance = 142.8 ml per min.

NOTE: Normal creatinine value in blood = 0.5 - 1.5 mg per cent
Normal creatinine value in urine = 100 - 1800 mg per 24 hours
Normal creatinine clearance = 100 - 125 ml. per minute

Table 3: The patient's creatinine clearance

	Vol. of 24-hour Sample	Grams per day*
Normal Diet	1160	12.18
	1705	9.20
	1340	8.04
	870	9.57
	785	9.03
	1600	10.48
	740	9.25
Low Protein Diet	850	8.67
	1620	8.42
	2225	6.68
	1620	5.35
	1365	4.64
	720 normal diet	2.11
High Protein Diet (without milk)	1390	9.48
	1745	8.55
	1240	6.08
	1305	7.70
High Protein Diet (with milk)	1080	6.90
	1285	8.60
	1060	8.05
	1430	11.20

*Normal values for urine urea - 10 - 15 gm per day

Table 4: Urea levels in the urine of patient

9.67 grams per day, is close to the generally accepted normal values of 10 to 15 grams per day.

The ammonia levels in the patient's serum are given in Table 5. There are no data for the last two days on the normal diet and for the day on normal diet following the low protein diet. It appears that on a normal diet, his ammonia level is generally within an acceptable range (30 to 80 µg per cent, according to Conway, 1950), although one value below and several values above this range are also observed.

Table 6 shows the values for the patient's serum amino acids. His serum citrulline level was 1.474 µMoles per ml, compared with a normal level of 0.015 µMoles per ml (thus, his level is about 100 times that of normal). His homocitrulline levels varied by a factor of two, and were generally above those observed among the controls (one control individual is a high excretor of homocitrulline, valine, leucine, and isoleucine, and he has disproportionately contributed to high values for the means and standard deviations for these four amino acids). There is a wide variation in the patient's ornithine levels, but the mean value of 0.096 µMoles per ml of serum appears to be within the normal range. The patient's serum lysine level of 0.379 µMoles per ml and serum arginine level of 0.133 µMoles per ml are generally above the values observed among the controls. Homoarginine was not detected in any control subjects, but the patient had values ranging from 0.024 to 0.041 µMoles per ml. This indicates that the patient's homoarginine levels are extremely elevated.

After Lunch

	0 min*	30 min	60 min	90 min	120 min	180 min
Normal Diet	105.0	-	-	-	-	-
	40.0	-	-	-	-	-
	20.7	-	-	-	-	-
	36.5	85.0	94.0	125.0	78.0	70.1
	-	-	-	-	31.5	-
Low Protein Diet	-	-	-	-	65.8	-
	-	-	-	-	23.1	-
	-	-	-	-	20.0	-
	51.6	17.5	22.5	13.3	9.1	4.1
	N.V**	1.7	13.3	N.V	N.V	N.V
High Protein Diet (without milk)	-	-	-	-	24.0	-
	-	-	-	-	33.0	-
	-	-	-	-	45.6	-
	32.0(21.4)	40.0(35.5)	85.0(64.5)	82.0(57.5)	92.0(83.0)	136.0(142.0)
High Protein Diet (with milk)	-	-	-	-	155.0	-
	-	-	-	-	123.0	-
	-	-	-	-	90.0	-
	66.0(83.0)	72.0(92.5)	81.5(83.0)	69.0(72.5)	89.0(62.5)	153.0(187.5)

*0 minutes means just before lunch. The other times are measured from this point.

**N.V (no values) means that ammonia levels were too low to be measured.

Values in parenthesis were got using Caraway's colorimetric method. All other values were got with Conway's microdiffusion method.

Normal values: a) With Conway's microdiffusion method - 35 - 80 µg per cent
b) With Caraway's colorimetric method - less than 100 µg per cent

Table 5: The patient's ammonia levels (micrograms per cent)

	Serum Amino Acids µMoles per ml Normal values*		The Patient's Serum Amino Acids µMoles per ml (Normal Institution Diet Condition)						
	Mean	S.D						Mean	S.D
Aspartic Acid	0.026	0.021	0.025	trace	trace	trace	trace	tr.-0.025	-
Threonine	0.502	0.047	0.470	0.404	0.340	0.280	0.240	0.346	0.092
Serine	0.202	0.041	0.958	0.159	0.148	0.130	0.127	0.144	0.015
Glutamic Acid	0.133	0.091	0.100	0.095	0.103	0.170	0.114	0.116	0.030
Citrulline	0.015	0.013	1.400	1.400	1.570	1.570	1.430	1.474	0.088
Proline**	0.271	0.052	-	-	-	-	0.290	0.290	-
Glycine	0.331	0.085	0.207	0.200	0.201	0.240	0.264	0.222	0.028
Alanine	0.498	0.111	0.420	0.530	0.560	0.480	0.558	0.509	0.059
Homocitrulline	0.022	0.036	0.020	0.036	0.043	0.035	0.040	0.034	0.008
Valine	0.312	0.131	0.220	0.260	0.237	0.238	0.280	0.247	0.023
1/2 Cystine	0.099	0.024	0.130	0.130	0.148	0.134	0.160	0.140	0.013
Methionine	0.027	0.010	0.050	0.040	0.034	0.041	0.060	0.045	0.010
Isoleucine	0.103	0.062	0.080	0.063	0.059	0.092	0.084	0.080	0.014
Leucine	0.207	0.110	0.120	0.113	0.119	0.143	0.150	0.129	0.016
Tyrosine	0.069	0.017	0.100	0.100	0.110	0.112	0.093	0.103	0.007
Phenylalanine	0.073	0.022	0.080	0.070	0.090	0.075	0.074	0.077	0.007
Ornithine	0.124	0.036	0.106	0.103	0.093	0.055	0.127	0.096	0.026
Lysine	0.275	0.069	0.330	0.360	0.392	0.440	0.375	0.379	0.040
Histidine	0.130	0.027	0.120	0.116	0.140	0.130	0.160	0.133	0.017
Arginine	0.104	0.018	0.145	0.109	0.137	0.130	0.146	0.133	0.015
Homoarginine	0.000	-	0.031	0.024	0.041	0.030	0.037	0.032	0.006

*Normal values were determined from data on 6 men and 4 women who work in the Human Genetics Laboratory

**The patient's proline peak was obscured by his excessive amounts of citrulline.

Table 6: Patient's serum amino acids under conditions of normal diet

The patient's urinary amino acids under the normal diet conditions are reported in Table 7. His urinary citrulline is many thousandfold higher than normal. Homocitrulline, ornithine, arginine and homoarginine are strikingly higher than normal. His lysine level is in the high range of normal excretion. Except for ornithine, the amino acids elevated in the urine appear to be elevated in the serum.

Low protein diet

The patient's urine urea level became progressively lower each day on the low protein diet, and even continued to drop on the day when he was returned to a normal diet (Table 4). This result is not unexpected because of the dependence of urea production on the level of protein metabolism. Like the urine urea, the serum ammonia level dropped on this diet, until, on the fifth day, levels were too low to be measured except shortly after eating (Table 5).

Urinary citrulline, homocitrulline, and homoarginine levels dropped sharply on the low protein diet, but they remained much higher than the values for normal individuals (Table 8). The urinary lysine, ornithine, and arginine levels are well within the range set by the normals. In the patient's serum, only citrulline appears to be unusually high (Table 9).

High protein diets

On a high protein diet, the normal urine urea levels for this patient were rapidly restored, but they did not increase to compensate for the unusually high levels of ingested nitrogen (Table 4). Serum

Urine Amino Acids µMoles per day Normal Values*	The Patient's Urine Amino Acids µMoles per day (Normal Institution Diet Condition)								
	Range							Mean	S.D
Aspartic Acid	tr.-174.7	trace	trace	trace	trace	trace	trace	trace	tr.	-
Threonine	129.6-1558.4	764.5	681.4	702.5	231.0	645.2	721.3	742.4	641.1	185.0
Serine	129.6-1387.1	403.1	473.5	788.6	254.6	321.1	549.5	452.8	463.3	173.6
Glutamic acid	tr.-230.4	trace	trace	trace	trace	trace	trace	trace	tr.	-
Citrulline	0.000-trace	14178	13629	17819	14293	12110	14052	14448	14361	1717.6
Proline	0.000	-	-	-	-	-	-	-	-	-
Glycine	576.0-3493.6	1911	1679	2285	803.3	2644	2041	2128	1927	580.3
Alanine	129.6-894.0	299.5	323.4	399.8	301.4	483.7	293.6	496.0	371.0	88.8
Homocitrulline	tr.-57.6	340.5	246.0	401.8	377.3	499.7	370.9	420.0	379.4	77.4
Valine	tr.-72.0	67.4	56.6	56.1	45.2	62.6	31.4	42.9	51.7	12.5
1/2 Cystine	28.8-334.6	143.8	139.7	185.8	197.4	370.9	113.8	97.9	178.4	92.0
Methionine	16.6-61.7	38.2	30.4	26.6	37.0	30.2	28.1	31.0	31.6	4.3
Isoleucine	tr.-57.6	134.1	70.5	66.7	41.7	58.9	49.9	51.0	67.5	31.0
Leucine	28.8-85.6	71.6	38.6	47.7	51.8	43.7	75.3	40.8	52.7	14.8
Tyrosine	67.1-232.2	166.8	100.5	226.8	117.0	280.1	153.0	219.2	180.4	64.4
Phenylalanine	tr.-100.8	124.4	84.6	92.1	85.7	98.1	121.7	308.8	130.7	80.1
Ornithine	tr.-52.7	159.8	83.2	156.4	34.8	182.0	192.3	115.2	131.9	57.1
Lysine	28.8-537.7	410.3	280.7	494.5	252.1	678.6	265.3	364.8	392.3	153.6
Histidine	216.0-2613.3	2641	2056	3395	1628	3793	2433	2672	2660	742.2
Arginine	tr.-288.0	686.6	418.1	886.6	220.4	873.5	537.7	816.0	634.1	252.9
Homoarginine	0.000	32.0	51.4	34.1	190.3	103.8	71.8	59.9	77.6	55.3

*The "normal values" were estimated from data on two control subjects. From one of the subjects, samples were collected under conditions of very low protein diet and very high protein diet.

Table 7: Patient's urine amino acids under conditions of normal diet

	Urine Amino Acids µMoles per day Normal Values*	The Patient's Urine Amino Acids µMoles per day (Condition of Low Protein Diet)							
	Range							Mean	S.D
Aspartic acid	tr.-174.7	trace	trace	trace	trace	trace	trace	trace	-
Threonine	129.6-1558.4	332.6	216.8	210.6	182.4	353.2	135.1	238.4	86.1
Serine	129.6-1387.1	189.8	200.8	226.8	298.1	152.3	226.6	215.7	48.8
Glutamic acid	tr.-230.4	trace	trace	trace	trace	trace	196.6	tr.-196.6	-
Citrulline	0.000-trace	6781	5525	4779	4984	4666	4054	5164.7	1009.1
Proline	0.000	-	-	-	-	-	-	-	-
Glycine	576.0-3493.6	747.0	714.0	844.0	1024	1047	1066	906.9	158.3
Alanine	129.6-894.0	145.0	164.9	132.1	111.3	184.7	214.3	158.7	37.2
Homocitrulline	tr.-57.6	252.2	136.0	128.0	100.9	165.2	82.5	144.1	60.1
Valine	tr.-72.0	trace	trace	trace	trace	trace	trace	trace	-
1/2 Cystine	28.8-334.6	88.1	51.3	95.2	59.7	66.4	49.8	68.4	19.1
Methionine	16.6-61.7	trace	trace	trace	trace	trace	trace	trace	-
Isoleucine	tr.-57.6	trace	trace	trace	trace	trace	trace	trace	-
Leucine	28.8-85.6	trace	trace	trace	trace	trace	trace	trace	-
Tyrosine	67.1-232.2	trace	trace	trace	trace	trace	trace	trace	-
Phenylalanine	tr.-100.8	trace	trace	trace	trace	trace	trace	trace	-
Ornithine	tr.-52.7	trace	83.9	32.7	trace	trace	46.1	tr.-83.9	-
Lysine	28.8-537.7	164.5	102.0	156.3	148.9	110.2	116.0	132.9	26.6
Histidine	216.0-2613.3	1280	994.5	1170	1023	1597	1102	1194.4	222.7
Arginine	tr.-288.0	218.1	162.4	184.7	100.2	220.3	213.6	183.2	46.5
Homoarginine	0.000	31.9	25.6	trace	trace	trace	trace	tr.-31.9	-

*The "normal values" were estimated from data on two control subjects. From one of the subjects, samples were collected under conditions of very low protein diet and very high protein diet.

Table 8: Patient's urine amino acids under condition of low protein diet

Serum Amino Acids µMoles per ml Normal Values*			The Patient's Serum Amino Acids µMoles per ml (Condition of Low Protein Diet)						
	Mean	S.D						Mean	S.D
Aspartic acid	0.026	0.021	trace	trace	trace	0.019	trace	tr.-0.019	-
Threonine	0.502	0.047	0.368	0.170	0.146	0.203	0.366	0.250	0.108
Serine	0.202	0.041	0.033	0.086	0.052	0.138	0.108	0.083	0.042
Glutamic acid	0.133	0.091	0.175	0.064	0.058	0.132	0.220	0.129	0.061
Citrulline	0.015	0.013	1.722	0.972	0.500	1.050	1.300	1.108	0.448
Proline**	0.271	0.058	-	-	-	0.240	-	0.240	-
Glycine	0.331	0.085	0.232	0.159	0.108	0.240	0.245	0.196	0.060
Alanine	0.498	0.111	0.630	0.408	0.300	0.680	0.686	0.540	0.176
Homocitrulline	0.022	0.036	0.035	0.013	trace	0.011	trace	tr.-0.035	-
Valine	0.312	0.131	0.210	0.123	0.078	0.180	0.153	0.148	0.051
1/2 Cystine	0.099	0.024	0.144	0.096	0.065	0.140	0.132	0.115	0.033
Methionine	0.027	0.010	0.031	0.014	0.020	0.026	0.015	0.021	0.007
Isoleucine	0.103	0.062	0.059	0.036	0.031	0.047	0.046	0.043	0.010
Leucine	0.207	0.110	0.121	0.070	0.048	0.099	0.076	0.082	0.028
Tyrosine	0.069	0.017	0.194	0.055	0.039	0.065	0.043	0.079	0.064
Phenylalanine	0.073	0.022	0.062	0.038	0.035	0.048	0.061	0.048	0.012
Ornithine	0.124	0.036	0.090	0.062	0.122	0.041	0.053	0.073	0.032
Lysine	0.275	0.069	0.372	0.190	0.075	0.255	0.233	0.225	0.107
Histidine	0.130	0.027	0.170	0.117	0.066	0.105	0.107	0.113	0.037
Arginine	0.104	0.018	0.110	0.070	0.058	0.102	0.128	0.093	0.028
Homoarginine	0.000	-	0.023	trace	0.000	0.000	0.000	0.000-0.023	-

*Normal values were determined from data on 6 men and 4 women who work in the Human Genetics Laboratory.

**The patient's proline peak was obscured by the excessive amounts of citrulline.

Table 9: Patient's serum amino acids under condition of low protein diet

ammonia levels were restored more gradually (Table 5). While on a low protein or normal diet, the peak serum ammonia level appears to be within 90 minutes of eating, on the high protein diets the ammonia levels rose continually for 180 minutes, the last observation in each series.

The serum citrulline and homoarginine remained high on both high protein diets (Tables 10 and 11). The serum lysine is above average on the high protein diet without milk and increases when milk is added. The other amino acids appear to be within an expected range, except that arginine, inexplicably, became quite low on the high protein diet with milk.

The urinary citrulline, homocitrulline and homoarginine levels are very high on the high protein diets (Tables 12 and 13). Ornithine is above the normal range and lysine is in the high normal range. Lysine, citrulline, homocitrulline, and ornithine may have increased somewhat during the course of the high protein diet with milk, as if in response to a progressive change in the patient's metabolism, and, at the same time, the patient's arginine level appeared to drop. As noted in the previous paragraph, there is no apparent explanation of the drop in arginine on this diet.

ENZYME STUDIES

Lysine to homocitrulline

Figure 6 indicates that lysine conversion to homocitrulline is an enzyme catalyzed reaction. Homocitrulline formation increased approximately linearly with the protein (enzyme) concentration up to about 80 μGrams of protein per ml of homogenate. The response to enzyme concentration

	Serum Amino Acids µMoles per ml Normal Values*		The Patient's Serum Amino Acids µMoles per ml (Condition of High Protein Diet Without Milk)					
	Mean	S.D					Mean	S.D
Aspartic acid	0.026	0.021	trace	trace	trace	0.029	tr.-0.029	-
Threonine	0.502	0.047	0.444	0.232	0.250	0.280	0.301	0.097
Serine	0.202	0.041	0.136	0.086	0.114	0.153	0.122	0.028
Glutamic acid	0.133	0.091	0.178	0.128	0.214	0.240	0.190	0.048
Citrulline	0.015	0.013	1.280	0.860	1.140	1.510	1.197	0.271
Proline**	0.271	0.052	-	-	-	-	-	-
Glycine	0.331	0.085	0.266	0.156	0.202	0.252	0.219	0.050
Alanine	0.498	0.111	0.580	0.420	0.606	0.580	0.546	0.085
Homocitrulline	0.022	0.036	0.022	0.038	0.022	0.034	0.029	0.008
Valine	0.312	0.131	0.260	0.214	0.286	0.302	0.265	0.038
1/2 Cystine	0.099	0.024	0.132	0.084	0.118	0.144	0.119	0.025
Methionine	0.027	0.010	0.060	0.042	0.048	0.024	0.043	0.015
Isoleucine	0.103	0.062	0.110	0.084	0.106	0.084	0.096	0.013
Leucine	0.207	0.110	0.166	0.137	0.162	0.156	0.155	0.012
Tyrosine	0.069	0.017	0.102	0.076	0.095	0.101	0.093	0.012
Phenylalanine	0.073	0.022	0.086	0.058	0.075	0.066	0.071	0.012
Ornithine	0.124	0.036	0.060	0.043	0.058	0.088	0.062	0.018
Lysine	0.275	0.069	0.366	0.260	0.340	0.384	0.337	0.054
Histidine	0.130	0.027	0.144	0.104	0.140	0.147	0.133	0.020
Arginine	0.104	0.018	0.130	0.090	0.110	0.128	0.114	0.018
Homoarginine	0.000	-	0.024	0.048	0.050	0.064	0.046	0.016

*Normal values were determined from data on 6 men and 4 women who work in the Human Genetics Laboratory.

**The patient's proline peak was obscured by the excessive amounts of citrulline.

Table 10: Patient's serum amino acids under condition of high protein diet without milk

	Serum Amino Acids µMoles per ml Normal Values*		The Patient's Serum Amino Acids µMoles per ml (Condition of High Protein Diet With Milk)					
	Mean	S.D					Mean	S.D
Aspartic acid	0.026	0.021	trace	trace	0.012	0.016	tr.-0.016	-
Threonine	0.502	0.047	0.320	0.356	0.325	0.358	0.339	0.020
Serine	0.202	0.041	0.081	0.102	0.078	0.122	0.095	0.020
Glutamic acid	0.133	0.091	0.188	0.230	0.178	0.191	0.196	0.022
Citrulline	0.015	0.013	1.120	1.167	1.150	1.268	1.176	0.064
Proline**	0.271	0.052	-	-	0.271	0.297	0.274	-
Glycine	0.331	0.085	0.153	0.162	0.179	0.163	0.164	0.010
Alanine	0.498	0.111	0.400	0.451	0.378	0.498	0.431	0.053
Homocitrulline	0.022	0.036	0.020	0.022	0.022	0.026	0.022	0.002
Valine	0.312	0.131	0.220	0.213	0.203	0.219	0.213	0.007
1/2 Cystine	0.099	0.024	0.096	0.082	0.088	0.092	0.089	0.005
Methionine	0.027	0.010	0.042	0.047	0.038	0.040	0.041	0.003
Isoleucine	0.103	0.062	0.094	0.055	0.065	0.065	0.069	0.016
Leucine	0.207	0.110	0.138	0.130	0.125	0.119	0.128	0.064
Tyrosine	0.069	0.017	0.071	0.085	0.079	0.067	0.075	0.008
Phenylalanine	0.073	0.022	0.050	0.053	0.023	0.039	0.041	0.013
Ornithine	0.124	0.036	0.066	0.037	0.028	0.041	0.043	0.016
Lysine	0.275	0.069	0.420	0.402	0.381	0.393	0.400	0.016
Histidine	0.130	0.027	0.119	0.109	0.126	0.122	0.119	0.007
Arginine	0.104	0.018	0.092	0.060	0.079	0.041	0.068	0.022
Homoarginine	0.000	-	0.055	0.050	0.048	0.052	0.051	0.002

*Normal values were determined from data on 6 men and 4 women who work in the Human Genetics Laboratory.

**The patient's proline peak was obscured by the excessive amounts of citrulline.

Table 11: Patient's serum amino acids under condition of high protein diet with milk

Urine Amino Acids µMoles per day Normal Values*	The Patient's Urine Amino Acids µMoles per day (Condition of High Protein Diet Without Milk)						
	Range					Mean	S.D
Aspartic acid	tr.-174.7	250.2	trace	trace	trace	tr.-250.2	-
Threonine	129.6-1558.4	478.2	145.4	492.1	465.0	783.0	472.7	225.7
Serine	129.6-1387.1	490.3	219.2	326.3	427.8	690.0	430.7	177.7
Glutamic acid	tr.-230.4	431.6	304.8	trace	trace	trace	tr.-431.6	-
Citrulline	0.000-trace	13010	5814	10051	11160	18812	11769.4	4741.3
Proline	0.000	-	-	-	-	-	-	-
Glycine	576.0-3493.6	4436	1788	2530	2396	3895	3009.1	1108.6
Alanine	129.6-894.0	442.4	217.8	289.7	483.6	549.4	396.5	138.2
Homocitrulline	tr.-57.6	297.8	187.2	266.7	261.4	407.8	284.1	80.1
Valine	tr.-72.0	trace	trace	36.4	49.9	91.2	tr.-91.2	-
1/2 Cystine	28.8-334.6	203.0	117.6	97.7	90.5	184.7	138.7	51.7
Methionine	16.6-61.7	trace	trace	trace	48.9	46.7	tr.-48.9	-
Isoleucine	tr.-57.6	63.2	67.8	56.7	142.8	137.3	93.5	42.6
Leucine	28.8-85.6	trace	trace	55.1	136.4	139.0	tr.-139.0	-
Tyrosine	67.1-232.2	156.4	43.4	141.4	91.8	182.7	123.1	55.5
Phenylalanine	tr.-100.8	52.1	38.6	84.3	88.0	94.4	71.4	24.5
Ornithine	tr.-52.7	347.7	95.1	63.8	47.2	223.8	155.5	127.8
Lysine	28.8-537.7	235.3	122.4	309.7	240.0	632.9	308.0	193.5
Histidine	216.0-2613.3	3059	1527	2134	1942	2858	2304.3	640.0
Arginine	tr.-288.0	172.7	194.4	385.6	294.6	573.7	324.2	163.3
Homoarginine	0.000	58.9	67.8	54.5	31.0	90.2	60.4	21.4

*The "normal values" were estimated from data on two control subjects. From one of the subjects, samples were collected under conditions of very low protein diet and very high protein diet.

Table 12: Patient's urine amino acids under condition of high protein diet without milk

	Urine Amino Acids µMoles per day Normal Values*	The Patient's Urine Amino Acids µMoles per day (Condition of High Protein Diet With Milk)					
	Range					Mean	S.D
Aspartic acid	tr.-174.7	trace	trace	trace	80.8	tr.-80.0	-
Threonine	129.6-1558.4	266.8	388.7	428.0	870.5	488.5	263.7
Serine	129.6-1387.1	179.8	244.1	156.2	332.8	228.2	78.9
Glutamic acid	tr.-230.4	trace	trace	trace	trace	trace	-
Citrulline	0.000-trace	5189.4	9091.4	12114	18229	11156.0	5501.6
Proline	0.000	-	-	-	-	-	-
Glycine	576.0-3493.6	950.4	1333	2385	3368	2008.9	1090.0
Alanine	129.6-894.0	140.4	194.7	189.1	329.4	213.4	81.0
Homocitrulline	tr.-57.6	99.1	273.1	466.4	712.5	387.7	263.3
Valine	tr.-72.0	41.0	38.6	63.7	73.8	54.2	17.2
1/2 Cystine	28.8-334.6	37.0	38.2	65.2	146.6	71.4	51.8
Methionine	16.6-61.7	36.4	31.4	37.8	39.0	36.1	3.3
Isoleucine	tr.-57.6	30.3	30.1	37.4	57.9	38.9	13.0
Leucine	28.8-85.6	24.0	39.2	28.4	32.9	31.1	6.4
Tyrosine	67.1-232.2	89.6	98.3	90.0	168.0	111.3	37.9
Phenylalanine	tr.-100.8	89.0	73.1	69.4	49.3	70.2	16.3
Ornithine	tr.-52.7	109.1	142.0	378.8	664.9	323.7	257.2
Lysine	28.8-537.7	215.5	478.7	475.4	736.5	476.5	212.7
Histidine	216.0-2613.3	853.2	1279	1838	2587	1639.0	749.5
Arginine	tr.-288.0	214.4	108.5	90.2	64.1	119.3	65.9
Homoarginine	0.000	86.4	82.3	94.1	98.7	90.3	7.3

*"Normal values" were estimated from data on two control subjects. From one of the subjects, samples were collected under conditions of very low protein diet and very high protein diet.

Table 13: Patient's urine amino acids under condition of high protein diet with milk

in the ornithine to citrulline reaction (Fig. 7) is very similar.

The formation of homocitrulline from lysine is not only dependent on lysine concentration (Fig. 8a), but it is also dependent on the concentration of carbamyl phosphate (Fig. 9a). In the experiments summarized in Fig. 8, the carbamyl phosphate concentration was held constant at 10 µMoles per ml and lysine or ornithine concentration was varied from 2.5 to 15 µMoles per ml. For the carbamyl phosphate dependent reaction, the lysine or ornithine concentration was held constant at 10 µMoles per ml and carbamyl phosphate concentration was varied from 1.25 to 10 µMoles per ml (Figs. 9a and 9b).

The ratio of citrulline to homocitrulline formed in the ornithine or lysine dependent reactions was consistently about 20 at each concentration of substrate. But in the carbamyl phosphate dependent reactions, this same ratio ranged from 50 at the lowest concentration to about 130 at the highest concentrations. The reason for this obvious discrepancy is not clear.

The course of the lysine reaction as a function of time is presented in Fig. 10. Fig. 11a shows a Lineweaver-Burk plot for lysine. From this plot, the Km for lysine in this reaction was estimated as 2.23×10^{-3} M and the Km for ornithine was estimated from Fig. 11b as 1.86×10^{-3} M.

Two Km values were calculated for carbamyl phosphate. For the lysine reaction (Fig. 12a), the Km was 1.50×10^{-2} M and for the ornithine reaction (Fig. 12b), the Km was 2.30×10^{-2} M. Thus, the following

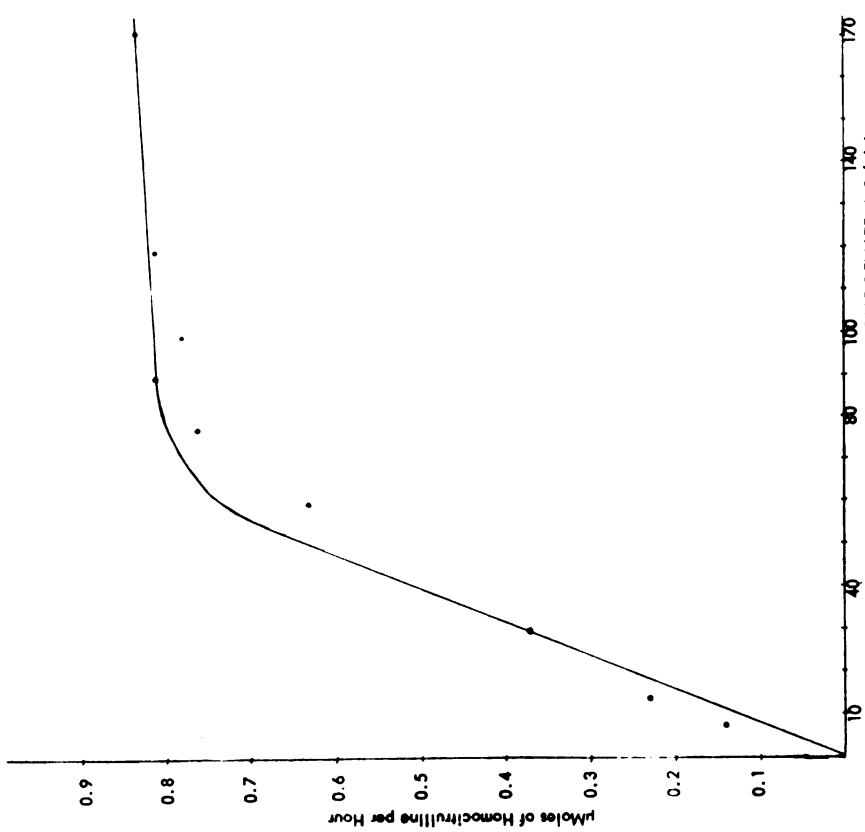


Fig. 6: Graph showing lysine conversion to homocitrulline as a function of enzyme concentration

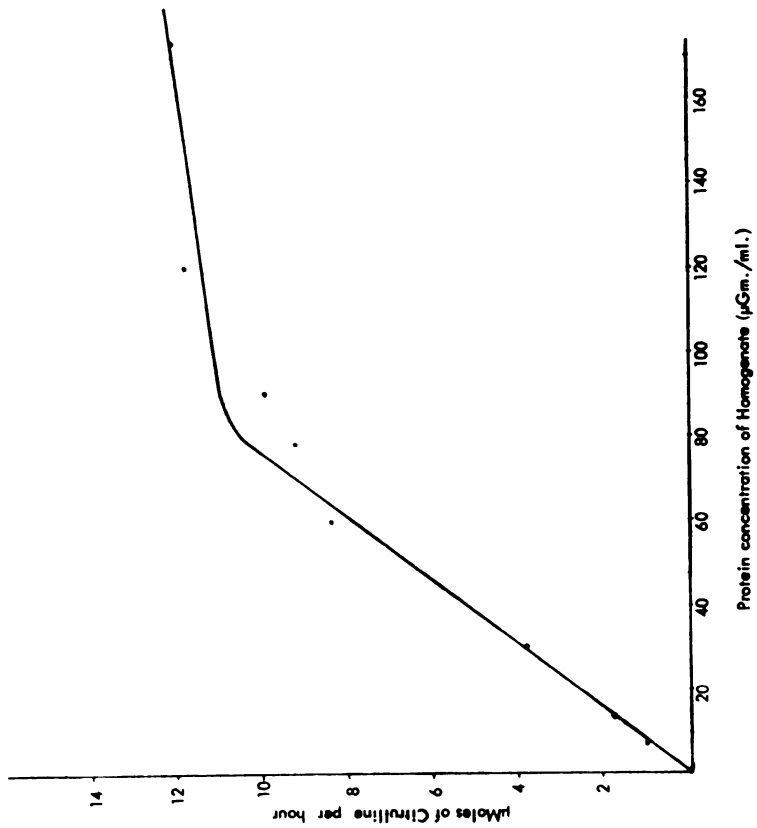


Fig. 7: Graph showing Ornithine conversion to Citrulline as a function of Enzyme concentration

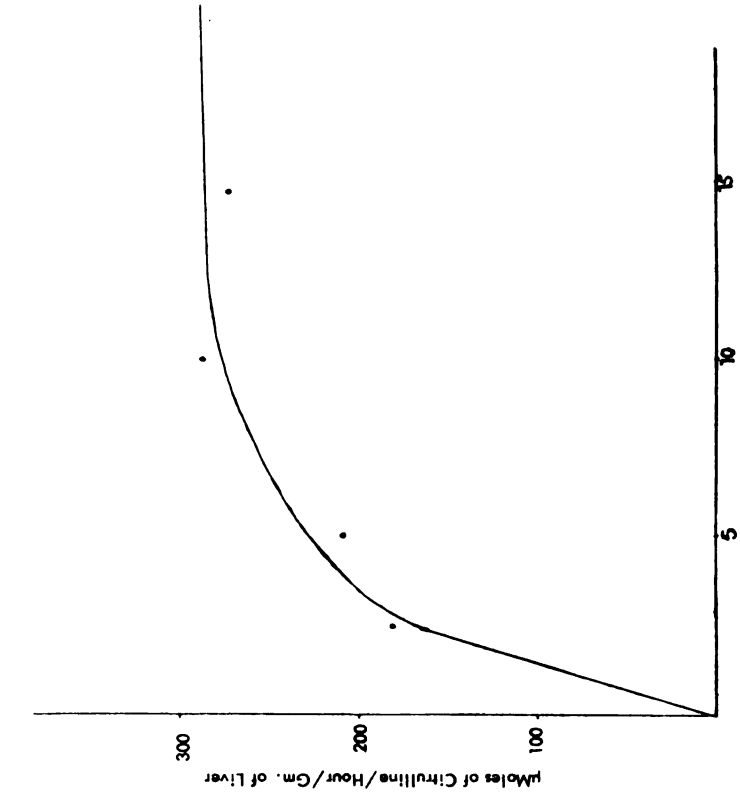


Fig. 8b: Citrulline formation as a function of Ornithine concentration

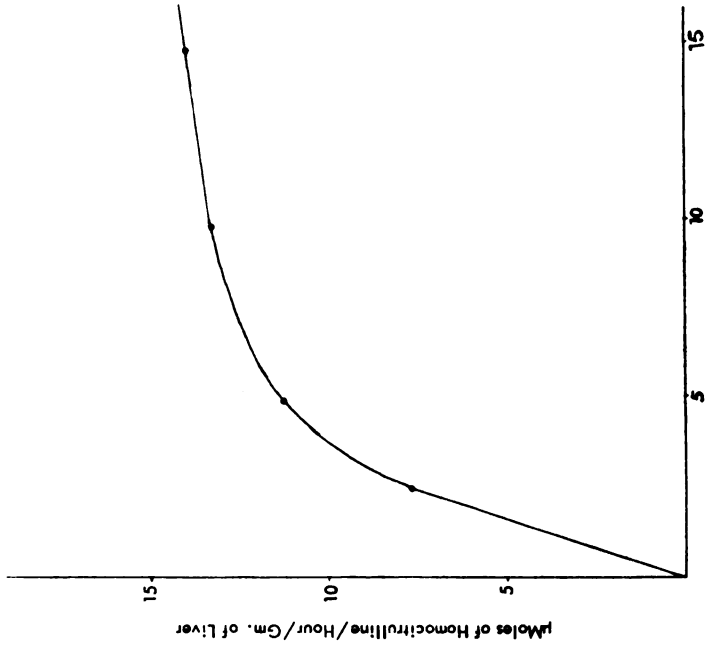


Fig. 8a: Homocitrulline formation as a function of Lysine concentration

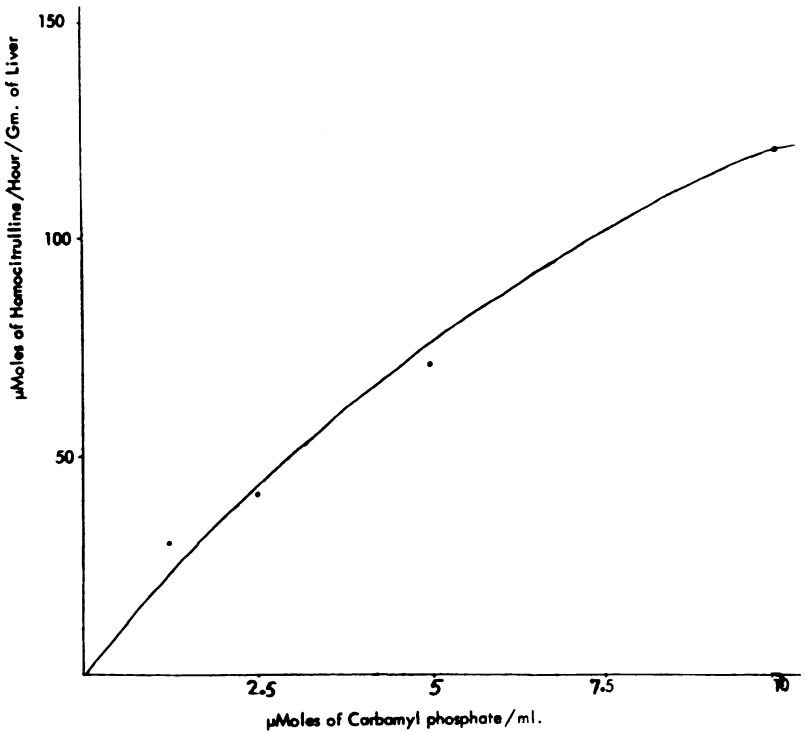


Fig. 9a: Homocitrulline formation as a function of Carbamyl phosphate concentration

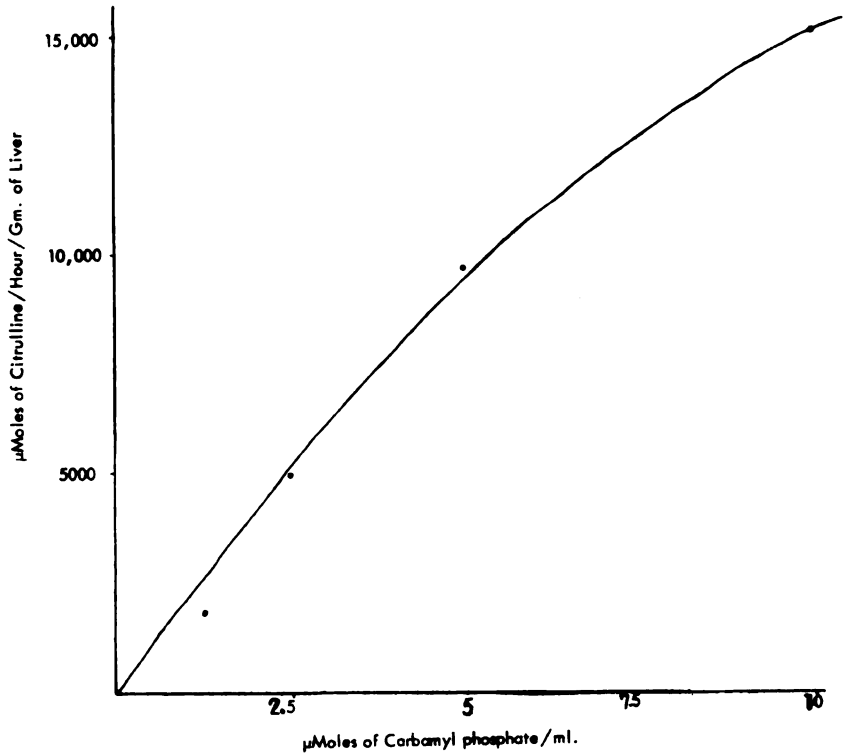
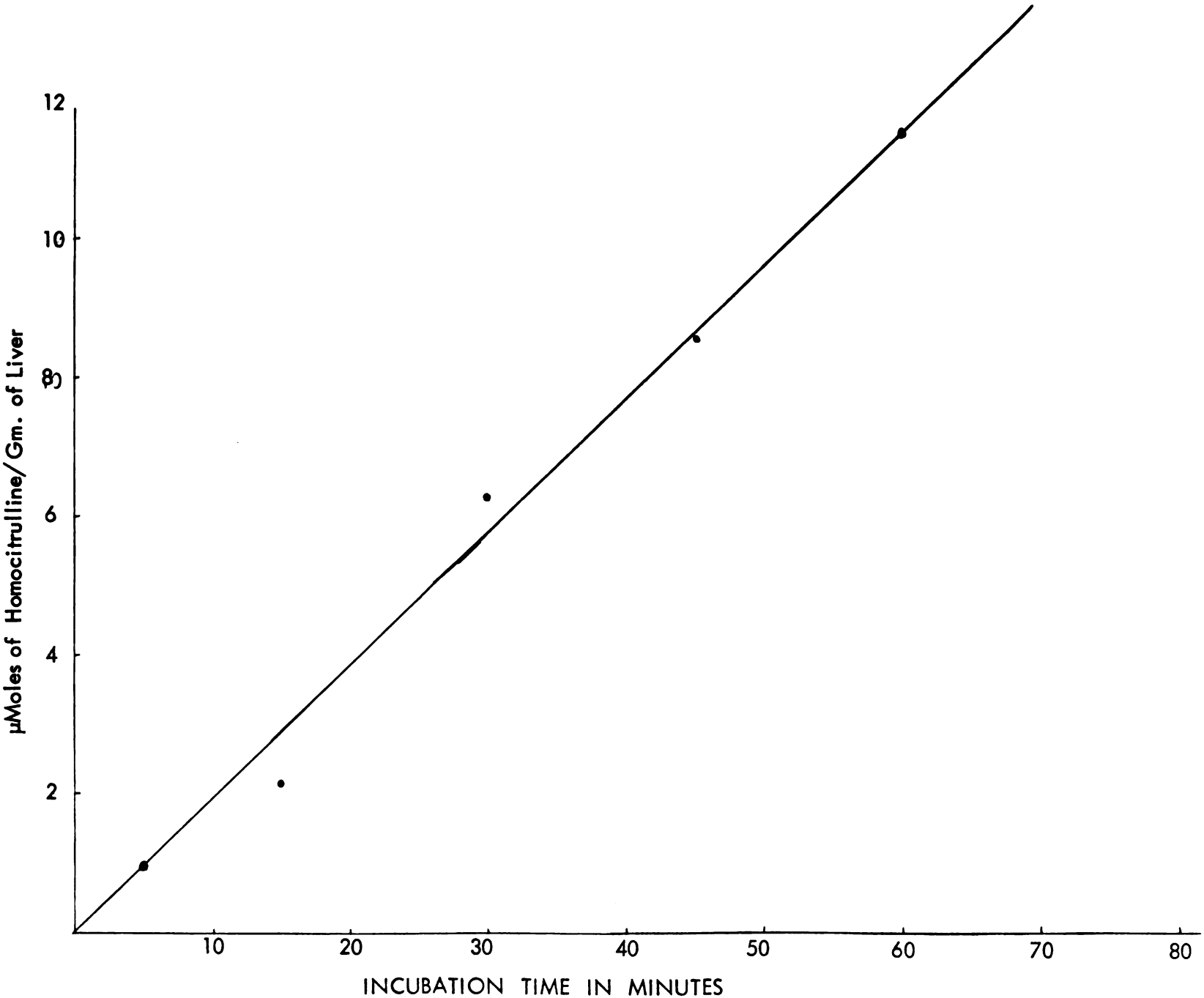


Fig. 9b: Citrulline formation as a function of Carbamyl phosphate concentration

Fig. 10: Graph showing the course of the lysine → homocitrulline reaction as a function of time



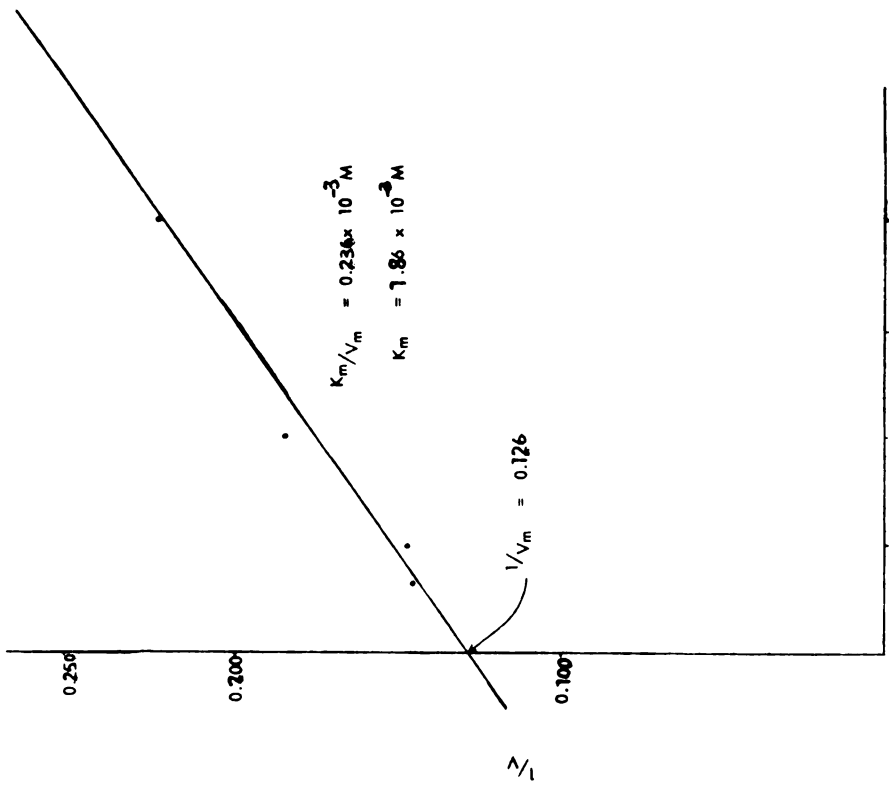


Fig. 11b: Lineweaver-Burk plot for Ornithine

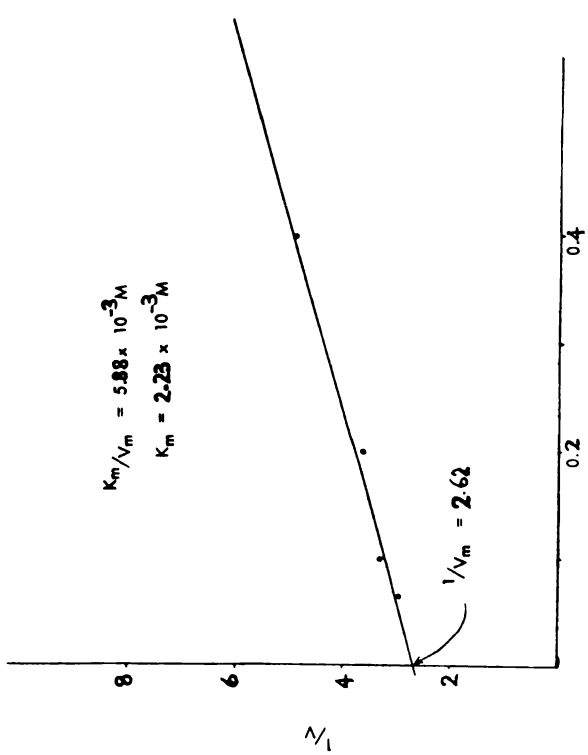


Fig. 11a: Lineweaver-Burk plot for Lysine

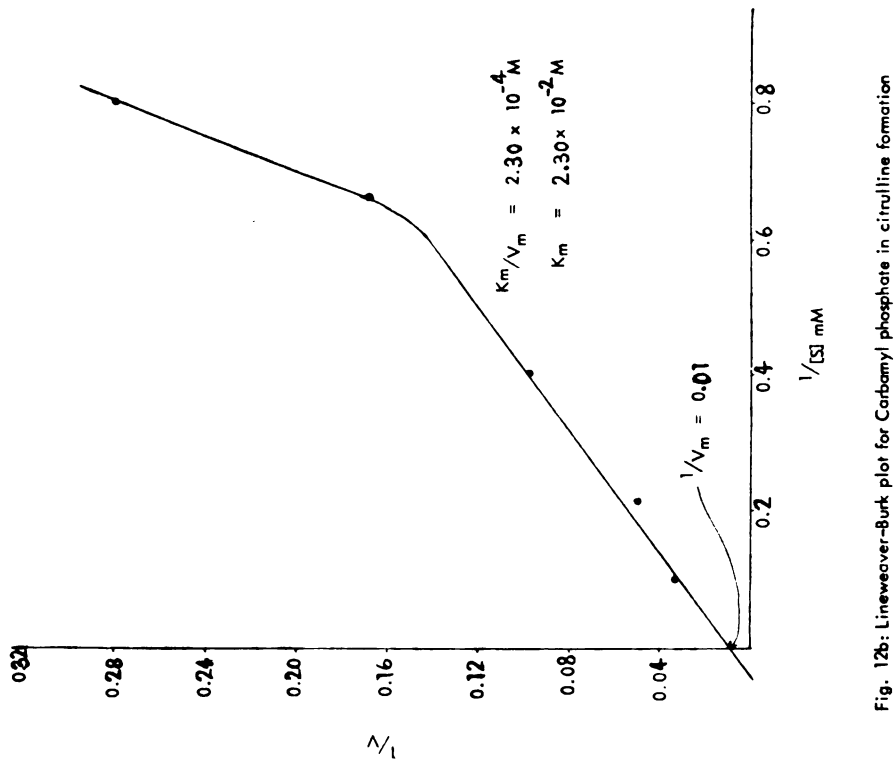


Fig. 12b: Lineweaver-Burk plot for Carbamyl phosphate in citrulline formation

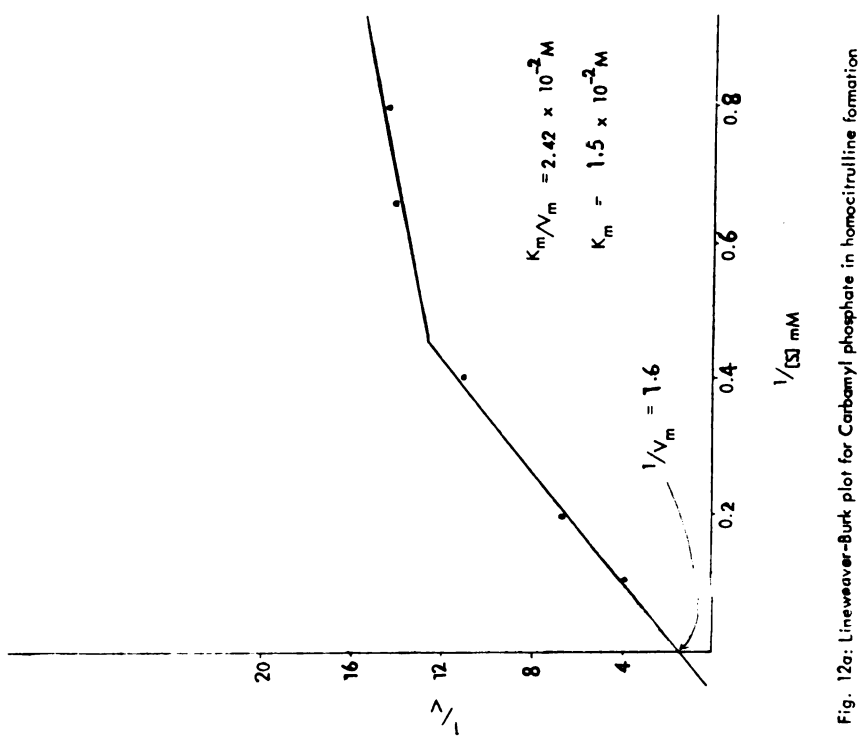
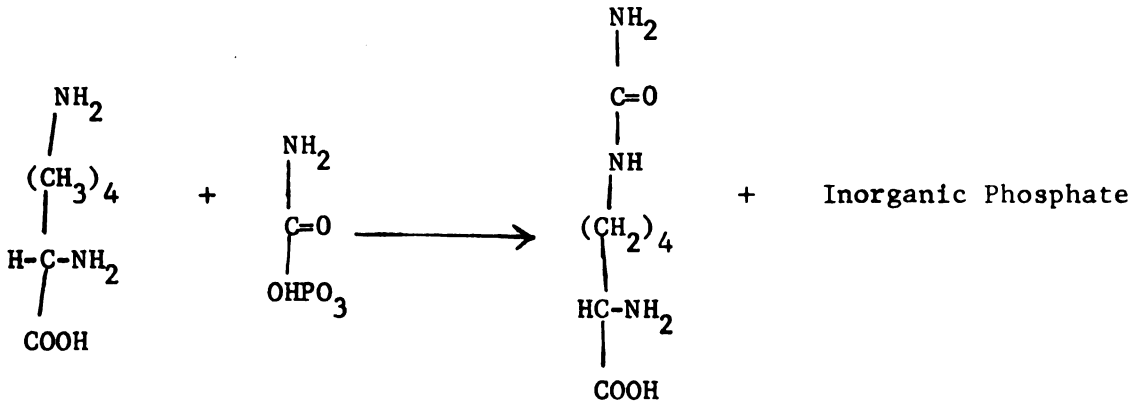


Fig. 12a: Lineweaver-Burk plot for Carbamyl phosphate in homocitrulline formation

reaction may exist:

L-lysine + Carbamyl Phosphate Homocitrulline + Pi



This reaction, in all essential respects, is similar to the ornithine to citrulline reaction.

Experiments have been carried out to determine if there is any competition between lysine and ornithine for an enzyme. In the experiments, ornithine concentration was held constant at Km and various concentrations of lysine were added. The colorimetric method used in these experiments do not distinguish between citrulline and homocitrulline. The experimental results indicate that the total product produced (citrulline plus homocitrulline) decreases as the amount of lysine increases (Table 14). Considering the difference in V_{max} with ornithine and lysine, these results are consistent with one enzyme (presumably OCT) acting on both substrates.

Lysine Concentration	Ornithine Concentration	uMoles of Product/Hour/Gm. of Liver with both substrates	uMoles of Product/Hour/Gm. of Liver with lysine alone
0 mM	Km (1.86 mM)	88.9	-
2.5 mM	Km (1.86 mM)	86.5	3.20
5 mM	Km (1.86 mM)	75.2	7.20
10 mM	Km (1.86 mM)	66.6	10.20

Table 14: Lysine-ornithine competition experiment

In Fig. 12, it appears that the rate of lysine conversion to homocitrulline is substantially increased at small carbamyl phosphate concentrations. For ornithine conversion to citrulline, it appears that the reverse is the case (that is, the rate of citrulline production drops sharply at low carbamyl phosphate concentrations). There is no apparent explanation for these observations.

Homocitrulline Metabolism

In order to find out if homocitrulline can be metabolized, an attempt was made to convert it to urea. Using liver homogenate as the enzyme source, preliminary results indicated that at the substrate concentrations used, homocitrulline could be converted to urea with the same efficiency as citrulline conversion to urea. The unfortunate aspect of these experiments (which were done fifteen times) is that the optical density readings of the urea color reactions were less than 0.1 for both citrulline and homocitrulline. Thus the optical density values were in an unreliable range and little confidence can be placed in these results.

Radio-isotope studies were carried out in order to test for homocitrulline conversion to homoarginine. Because of the commercial unavailability of labelled homocitrulline, labelled lysine was used in these experiments. The test was for the conversion of lysine-C^{14} to homoarginine-C^{14} as a function of carbamyl phosphate concentration. Table 15 shows the results obtained. The tests were run in duplicate (E1 and E2), and the counts were made after paper electrophoresis, which would adequately separate lysine from homoarginine.

Homoarginine Counts per Minute

Carbamyl Phosphate Concentration	E 1	E 2	Z Zero Time Control	No Asp.	No ATP	(Z-E) Average Net Count
0.00M	3303	4966	3492	3504	3470	-
0.00M	2185	2237	2097	2117	2104	106
0.04M	3451	3174	2791	2703	2811	547
0.04M	3278	3129	2636	2712	2680	
0.06M	3471	3584	2638	2692	2612	888
0.06M	3640	3757	2813	2892	2791	
0.08M	8393	7775	6979	6909	6953	1183
0.08M	8521	7756	6964	6903	7004	
0.08M	7922	7336	6356	6369	6391	
0.12M	11353	10235	7953	7903	8013	2888
0.12M	10398	11442	8125	8092	8139	
0.12M	10244	11610	7899	7904	7913	

NOTE: Separation of homoarginine was by low voltage electrophoresis.

Table 15: Table showing the conversion of lysine-C^{14} to homoarginine-C^{14} as a function of carbamyl phosphate concentration

As checks, a zero time control using pre-boiled enzyme, a control omitting aspartate, and a control omitting ATP were also run. All three of these checks gave similar results, and all agreed in that the counts increased substantially with increasing carbamyl phosphate concentrations. These data also indicate that aspartate and ATP are both required for this reaction.

The increase in control counts with increasing carbamyl phosphate level suggests that some non-enzymatic conversion is taking place. If this conversion is in the first step of the sequence, lysine + carbamyl phosphate homocitrulline, the homocitrulline might have interfered with the counts for homoarginine because these two amino acids migrate only a short distance from the origin, although in opposite directions.

Because the electrophoresis employed in the preceding experiment might not have given satisfactory separation, column chromatography was used in clearly isolating homoarginine from every other amino acid (Table 16). Counts for the zero time control are still very large, but they remain relatively constant for all three concentrations of carbamyl phosphate. This supports the postulate that some homocitrulline was counted with the homoarginine in the earlier experiments. However, the remaining large counts in the zero time control were still unexplained, as these counts should have been close to zero.

Experiments were, therefore, carried out to determine the purity of the lysine-C^{14} (Table 17). It can be seen that although lysine

Carbamyl phosphate Concentration	Homoarginine* Counts per Minute E 1	E 2	Z Zero Time Control	Average Net Count
0.00M	1042	1121	1003	78.5
0.04M	1790	1795	1101	691.5
0.06M	2101	2113	1132	980.0

*Homoarginine was separated by column chromatography

Table 16: Further studies showing lysine conversion to homoarginine as a function of carbamyl phosphate concentration

COMPOUND	TREATMENT	MEAN CPM
1μC Lysine-C^{14}	None	965700
1μC Lysine-C^{14}	Chromatographed and Lysine Peak Eluted	378720
1μC Lysine-C^{14}	Chromatographed and Homoarginine Peak Eluted	1416

Per cent Recovery = $\frac{378720}{965700}$ = 0.392 or 39.2%

Table 17: Table of experiment showing the purity of the lysine-C^{14} used in the previous radio-isotope experiments

elutes about four hours before homoarginine, the homoarginine eluted contained radio-activity of about the same magnitude as the zero blanks in Table 16. This suggests that the lysine-C^{14} used in the preceding experiments contained some homoarginine or some other compound that elutes with homoarginine.

For confirmatory purposes, fresh lysine-C^{14} was purchased and the assays were repeated. The radioactivity counts in this set of assays were extremely low, indicating excessive quenching. It was possible that the quenching could be attributed to the salt content of the buffer, which was 0.05 M sodium citrate and also 0.6 M sodium chloride. Therefore, the eluents had to be desalted.

For desalting, 10 ml of the eluent were passed through a 2.5 ml resin bed in a short glass column. 30 ml of water (pH adjusted to about 6) were used in washing the resin, and 15 ml of 28 per cent ammonium hydroxide solution were used in eluting the amino acids. The elution was done directly into counting vials, following which the vials were evaporated to dryness in an oven. 15 ml of dioxane counting fluid (room temperature) were added before counting.

Table 18 shows the number of counts as a function of carbamyl phosphate concentration. In order not to exceed the maximum capacity of the resin, 10 ml of eluent were taken for desalting, and the counts were corrected for the total amount of eluent observed (31 to 40 ml) by multiplying the count by total ml of eluent divided by 10.

The zero time blanks should regularly have given values of zero homoarginine (since they were done with preboiled enzyme), but gave variable counts, all of which happened to be greater than those

Carbamyl Phosphate Concentration	Homoarginine Counts for Preboiled Enzyme Zero Time Blank (10 ml eluent)	(Total)*	Homoarginine Counts for Assays with Arginase (10 ml eluent)	(Total)*	(Net)**	Homoarginine Counts for Assays without Arginase (10 ml eluent)	(Total)*	(Net)**
0.00M	209(36ml)	754.2	152(36ml)	547.2	-	165(34ml)	561.0	-
0.02M	236(46ml)	991.2	193(36ml)	694.8	-	485(35ml)	1697.5	706.3
0.04M	332(33ml)	1095.6	262(33ml)	864.6	-	581(31ml)	1801.1	705.5
0.08M	280(35ml)	980.0	470(33ml)	1551.0	571.0	953(32ml)	3049.6	2069.6
0.12M	270(40ml)	1080.0	433(36ml)	1558.8	478.8	1780(34ml)	6052.0	4972.0

Values in parenthesis represent the total volume of eluent for complete extraction of homoarginine.

*Total counts were gotten by multiplying counts by volume correction factor (the values in parenthesis divided by 10).

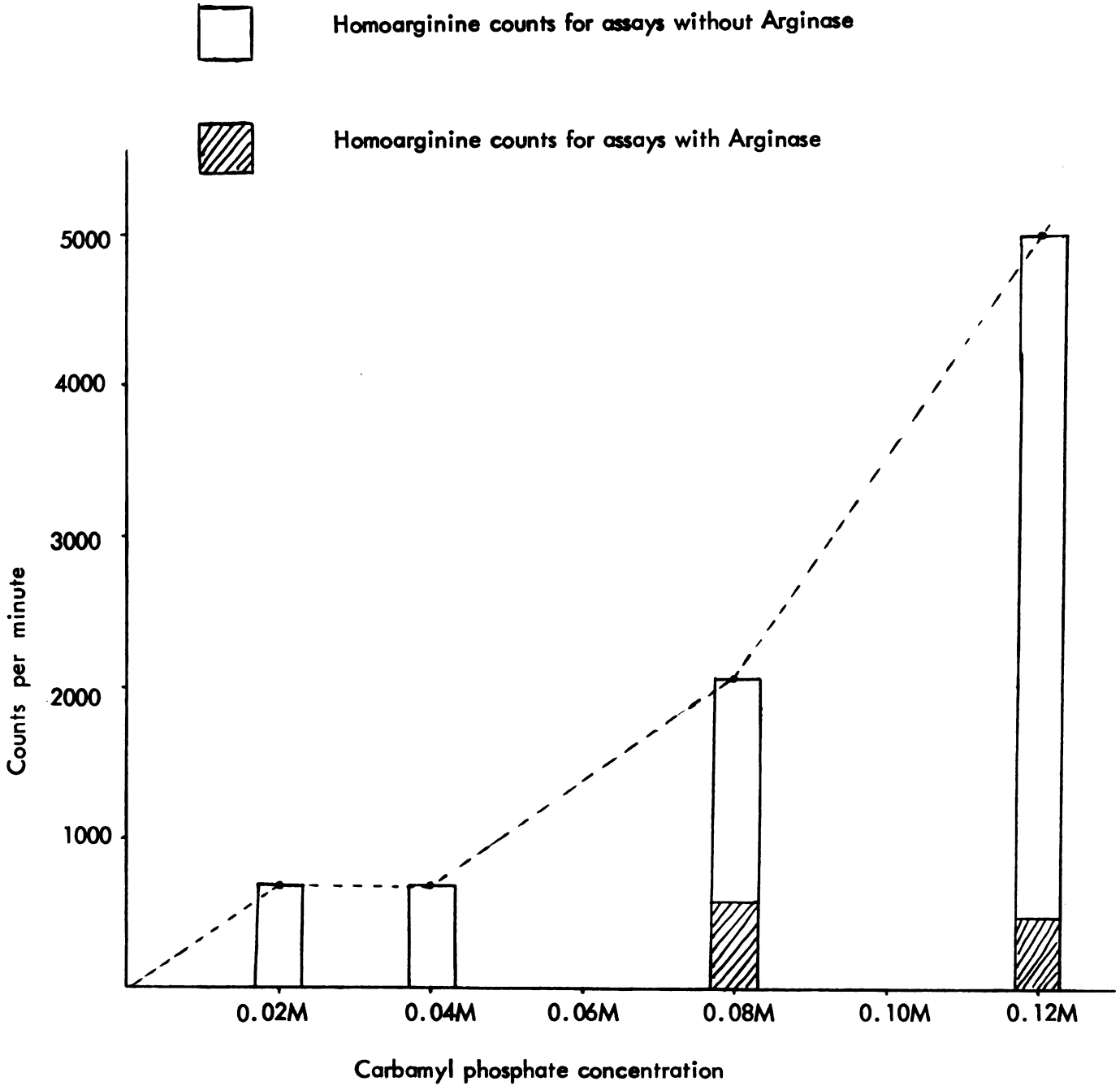
**Net counts were gotten by subtracting the other total counts from the total counts for preboiled enzyme zero time blank.

Table 18: Homoarginine formation as a function of carbamyl phosphate concentration

observed for the lowest runs made with arginase present. The unusually long chromatographic runs needed to extract homoarginine suggest that most of the count in the zero time blank is due to this amino acid, and the drop in the count in the presence of arginase confirms this. The homoarginine might be present due to non-enzymatic conversion and/or to contamination of the lysine-C^{14}. There is no evidence for a dependence of these processes on carbamyl phosphate, as the homoarginine counts do not increase with increasing carbamyl phosphate.

The assays for homoarginine show a general increase with increasing carbamyl phosphate concentration, suggesting that there is an enzymatic conversion of lysine to homoarginine in the presence of carbamyl phosphate. The relationship is roughly linear (Fig. 13). The data of Fig. 13 are corrected by subtraction of the count for the zero time blank from the total count. Because of the variability of the zero time blanks, and perhaps other variation within the system that cannot be identified, the data do not fall into as neat a pattern as would be desired. Nevertheless, the general relationship between carbamyl phosphate concentration and homoarginine level is readily apparent. That this is due largely or completely to homoarginine is evidenced by the fact that the same assays with arginase added give much lower counts at all concentrations of carbamyl phosphate.

Fig. 13: Histogram showing homoarginine formation as a function of carbamyl phosphate concentration



Homoarginine Metabolism

It has been found by Ryan, Barak and Johnson (1968) that the enzyme arginase is capable of hydrolyzing homoarginine to urea. It was earlier found that arginase is a non-specific enzyme because of its ability to hydrolyze arginic acid, canavanine, clupein, alpha-N-methyl-arginine, and octopin (Sumner and Somers as cited by Ryan et al., 1968).

Figure 14a shows the relationship between protein (enzyme) concentration and homoarginine hydrolysis using rat liver homogenate. This relationship appears to be linear up to a concentration of about 180 uGrams of protein per 20 uLiters of homogenate. Figure 14b shows a similar response of arginine to enzyme concentration.

The amount of urea produced from homoarginine and arginine as a function of incubation time is represented in Figs. 15a and 15b, respectively.

Figure 16a shows urea formation as a function of homoarginine concentration. The optimum substrate concentration appears to be about 250 uMoles per ml. It appears that there may be a substrate inhibition above the optimum concentration, as shown in the figure. This apparent substrate inhibition was not observed for urea formation as a function of arginine concentration (Fig. 16b).

Both authentic arginase and liver homogenates were used for the experiments summarized in Table 19. This table gives the ratio of the amount of urea formed from arginine to the amount of urea formed from homoarginine. This ratio varied from 40 (when authentic arginase was used and the substrate was at optimum concentration) to 70 (when the

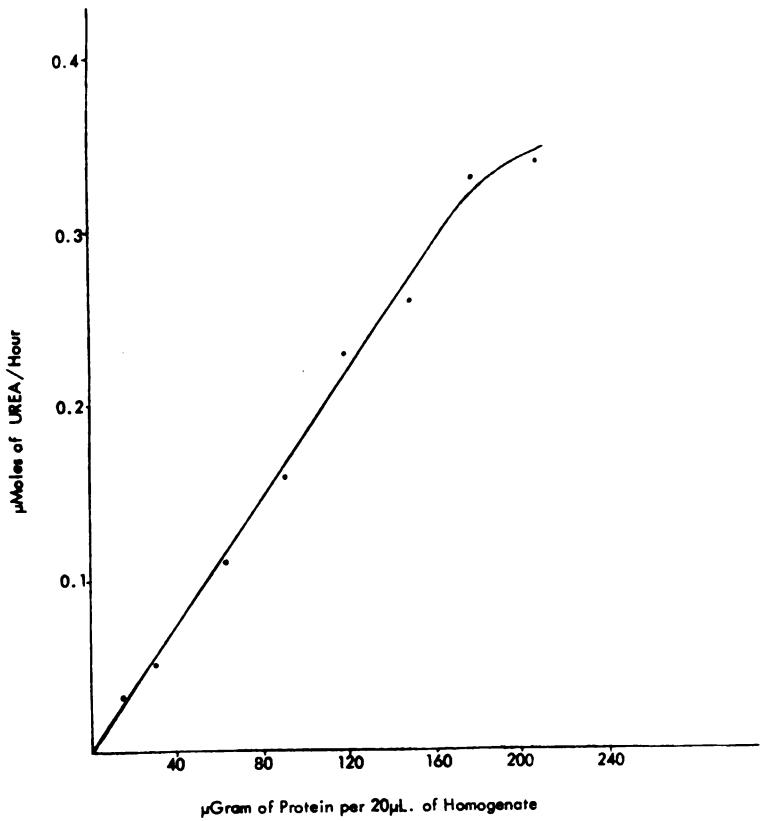


Fig. 14a: Graph showing the course of Homoarginine hydrolysis as a function of Enzyme conc.

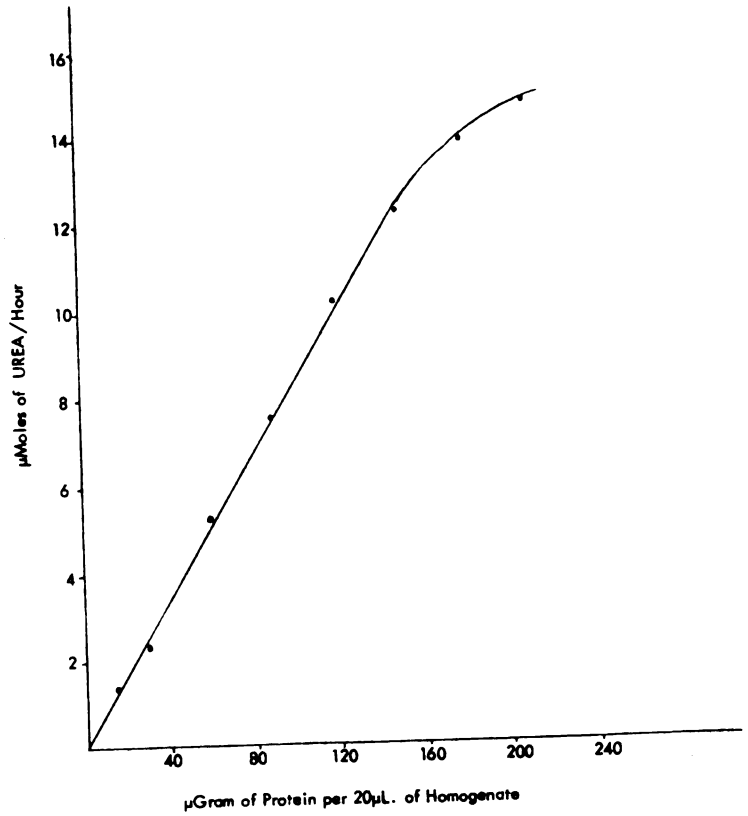


Fig. 14b: Graph showing the course of Arginine hydrolysis as a function of Enzyme conc.

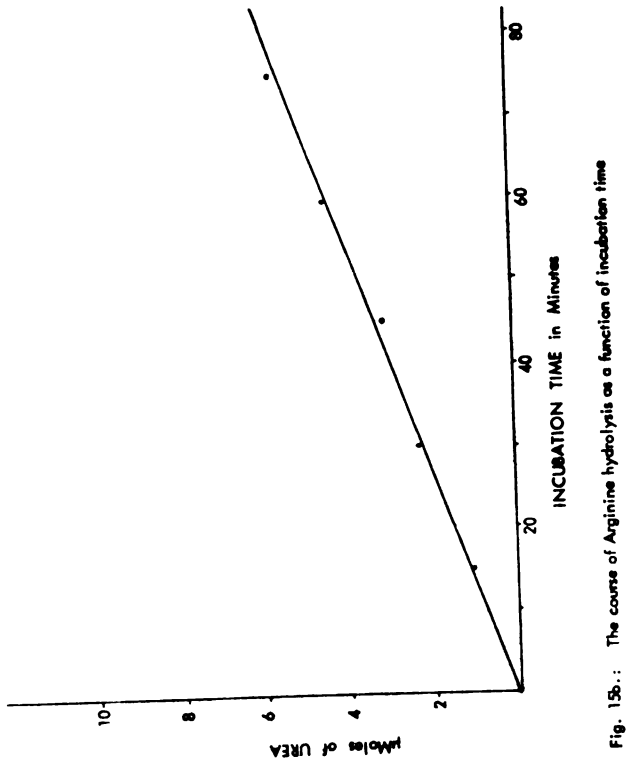


Fig. 15b.: The course of Arginine hydrolysis as a function of incubation time

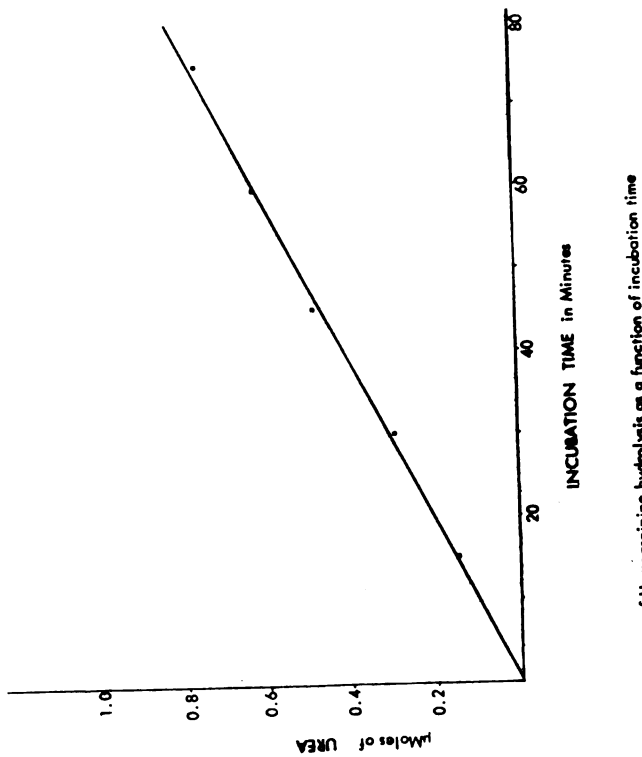


Fig. 15a:The course of Homoarginine hydrolysis as a function of incubation time

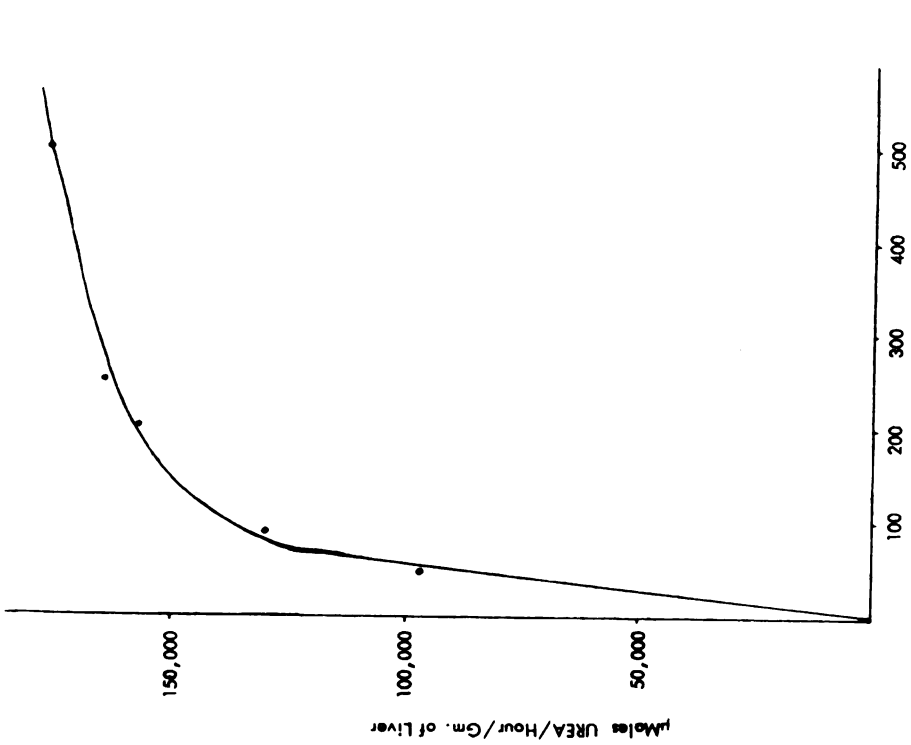


Fig. 16b: The course of Arginine hydrolysis as a function of arginine concentration

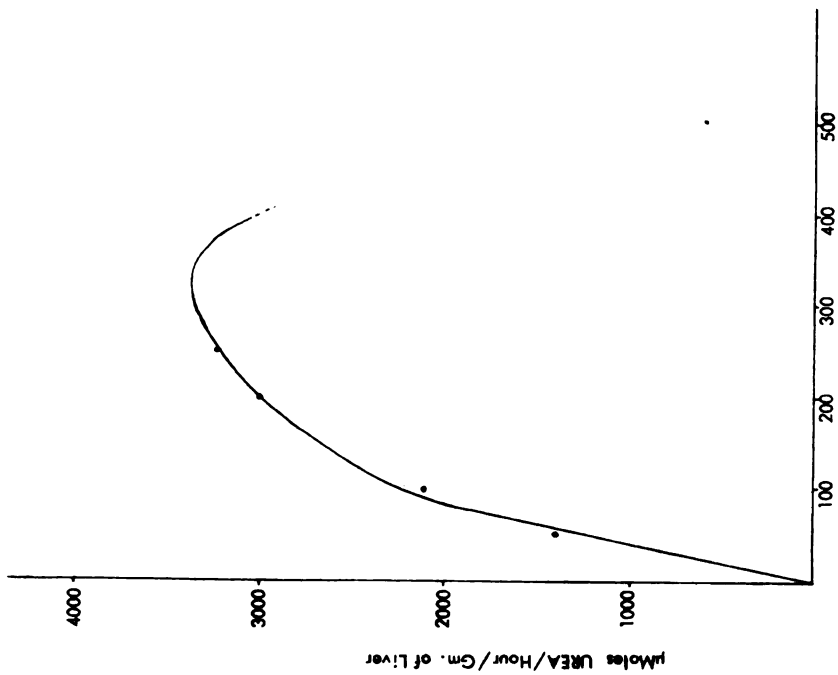


Fig. 16a: The course of homoarginine hydrolysis as a function of homoarginine concentration

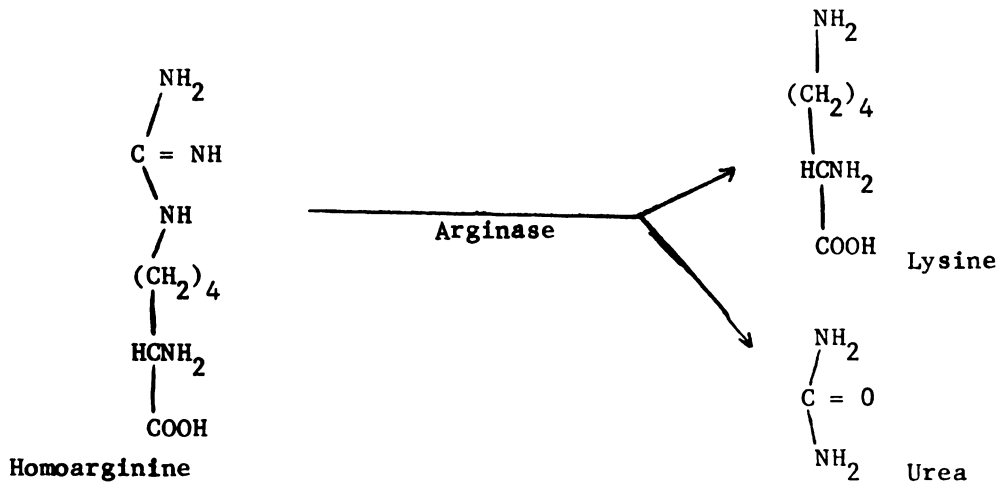
(S) = Substrate Arginine or Homoarginine Concentration in uMoles		V = velocity uMoles Urea/Hour		Specific Activity uMoles Urea/Hour/Gm. of Liver		Ratio Arginine:Homoarginine
		from Arg.	from Homoarg.	from Arg.	from Homoarg.	urea urea
Liver Homogenate	50	4.2	0.602	97860	1400	69.9
	100	5.6	0.923	130480	2146.2	60.7
	200	6.8	1.284	158440	2986.2	53.0
	250	7.2	1.404	167760	3266.2	51.0
	500	7.6	0.152	177080	355.0	?
				uMoles Urea/Hour/mg. of Enzyme		
Authentic Arginase	250	444	11.36	1110	28.4	40.1

Table 19: Table comparing the rate of homoarginine and or arginine hydrolysis

concentration of substrate was 50 uMoles per ml and liver homogenate was used). The value of 40 is probably the most accurate one because of the possibility of interference from several several endogenous factors present in liver homogenate.

From Table 19, Lineweaver-Burk plots were constructed as shown in Fig. 17. The Km value for homoarginine was estimated as 12.7×10^{-2} M and that for arginine was estimated as 4.60×10^{-2} M.

Thus, the hydrolysis of homoarginine to urea apparently is an enzyme catalyzed reaction.



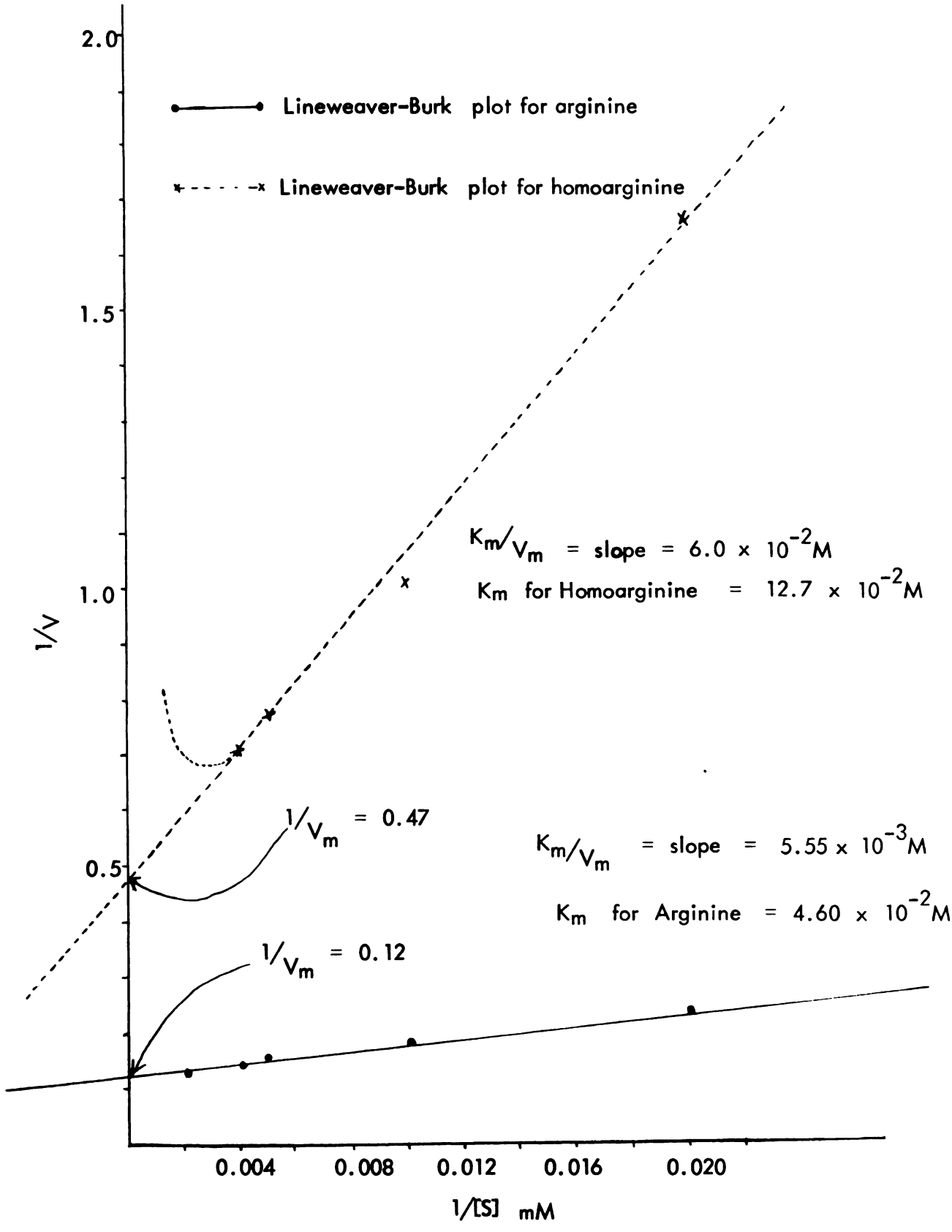


Fig. 17: Lineweaver-Burk plots for arginine and for homoarginine

DISCUSSION

This research was undertaken to determine the nature of the anomaly of a particular mentally retarded patient, R.D. The patient was first recognized as biochemically unusual because of the large amount of citrulline detected by chromatography in his urine, and further studies indicated that he also excretes unusually large amounts of ornithine, homocitrulline, and homoarginine in his urine and lysine, citrulline, and homoarginine in his blood. Excessive arginine was occasionally excreted by the patient. He appeared to have a unique syndrome because none of the two carefully described cases of citrullinemia had this large variety of amino acid abnormalities. The other described cases did not seem to represent instances of inadequate analysis because they had been described as having large amounts of ammonia in their blood and as suffering from ammonia intoxication, which is not true for this patient.

It could be assumed that all of the unusual characteristics of the patient should be ascribed to a primary defect in his metabolism. The most probable defect appears to be a deficit or complete absence of argininosuccinic acid synthetase (condensing enzyme), which would explain the large amounts of citrulline in his blood and urine. Analysis of both of the patient's parents, his only sib (a brother), and his two nieces and a nephew has failed to indicate any similar defects within the family. The patient shows normal creatinine and urea clearances, which are indicative of a normal kidney function.

HYPOTHESIS

This analysis of the patient leads to the hypothesis that his major defect is the deficit or complete absence of ASA synthetase. This results in citrullinemia and the citrullinemia causes the other amino acid defects that have been observed in him.

The following constitute an explanation of the biochemical and physiological relationships of all of the patient's defects (see Fig. 1):

(1) As a result of the enzyme defect, citrulline accumulates in the serum to a level of over 60 times normal. This citrulline is cleared by the kidney, giving rise to the profound citrullinuria noticed in the patient (always many thousandfold above normal).

(2) Because of citrulline accumulation in the serum, the synthesis of more citrulline from ornithine is greatly inhibited. The level of serum ornithine remains approximately normal, which suggests that this excess ornithine is rapidly cleared by the kidney to produce the patient's hyperornithinuria and some ornithine may also be metabolised through other pathways (Roloff et al., 1940).

(3) Ornithine competes with lysine (which is homoornithine) for renal clearance (Scriver, 1967). The apparent hyperlysinemia observed in the patient could therefore be due to the excessive amounts of ornithine he clears at the expense of lysine.

(4) Ryan and Wells (1964) have shown that when normal subjects are given a lysine load, excessive homocitrulline and homoarginine are excreted in the urine. In the series of experiments reported here, it has been shown that lysine can be enzymatically converted first to homocitrulline and further to homoarginine. These enzymatic reactions have been found to be dependent on the presence of carbamyl phosphate, ATP and aspartate. Ryan et al. (1969) have shown that there is a direct transamidation of lysine to homoarginine. Thus, the large amounts of homoarginine in the patient's serum probably can be accounted for as the result of two separate processes. Apparently, serum homocitrulline levels are maintained near normal by a combination of clearance by the kidneys and metabolism to homoarginine.

(5) The occasional hyperargininuria could be due to the competition for arginase by homoarginine at the expense of arginine. The resulting excess serum arginine is thus rapidly cleared by the kidney to give rise to hyperargininuria.

THE BACK-UP UREA CYCLE

In this study, experiments with rat liver have suggested that an alternate cycle for urea synthesis exists, adding evidence to an hypothesis that has been in the literature for several years. The proposed cycle (Fig. 18), lysine-homocitrulline-homoargininosuccinic acid-homoarginine-lysine, would be parallel to the well-known ornithine-urea cycle (Fig. 1).

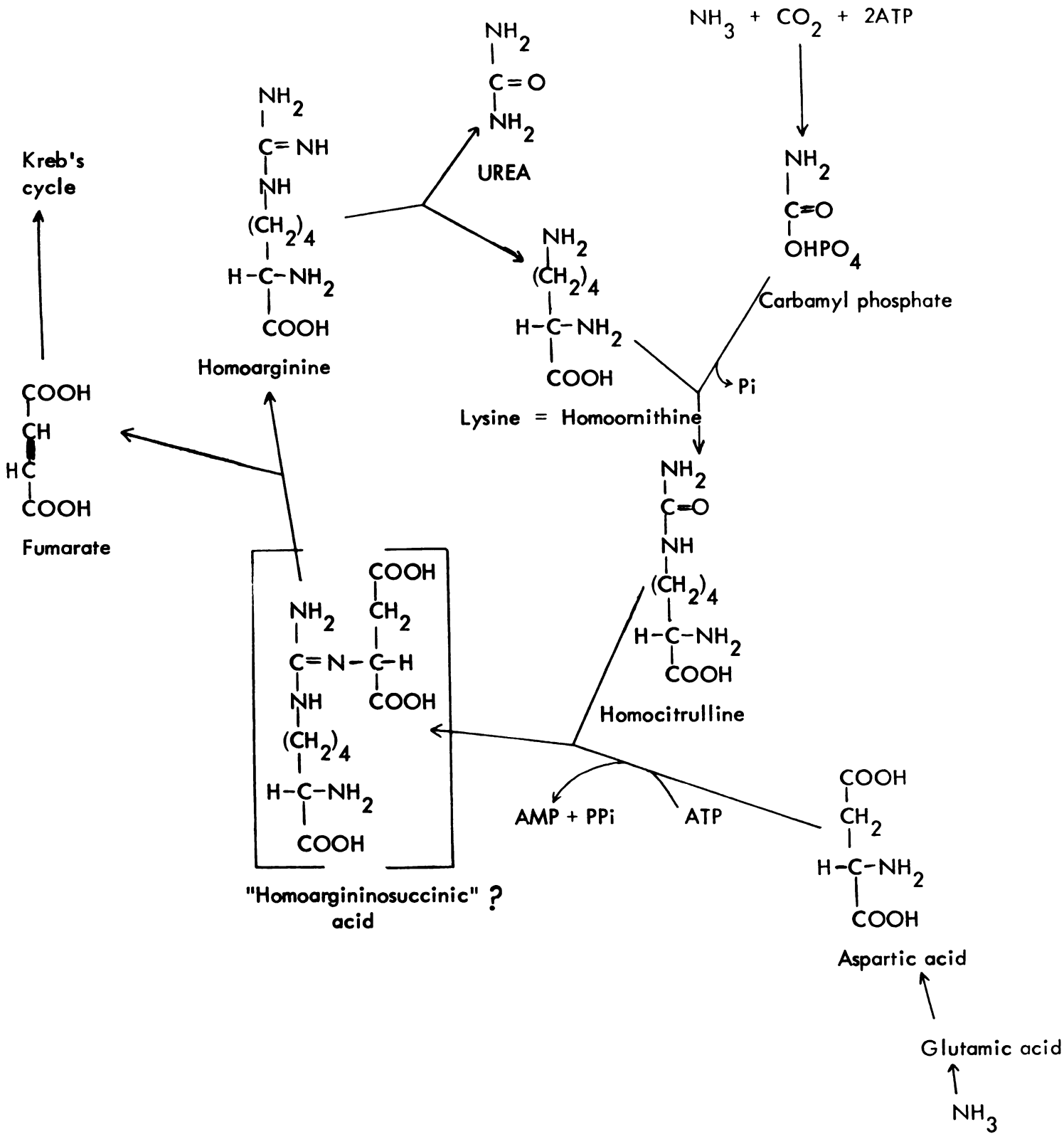


Fig : 18 THE BACK-UP UREA CYCLE

It has been shown in these experiments that lysine can be converted to homocitrulline in an enzymatic reaction that is dependent on carbamyl phosphate concentration. Thus,

$$\text{Lysine} + \text{Carbamyl phosphate} \longrightarrow \text{Homocitrulline}$$

Studies with radio-isotopes have suggested that lysine, in the presence of carbamyl phosphate, ATP and aspartate, can be converted to homoarginine. This reaction is distinct from the previously described transamidation of lysine to homoarginine (Ryan et al., 1969). Thus,

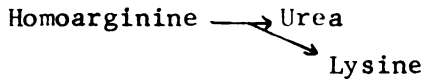
$$\text{Lysine} + \text{Carbamyl phosphate} + \text{ATP} + \text{Aspartate} \longrightarrow \begin{matrix} \text{Homoarginine} \\ \searrow \\ \text{Fumarate} \end{matrix}$$

The fact that aspartate is required for this reaction implies that an intermediary in addition to homocitrulline exists before homoarginine is reached. This intermediary would presumably be homoargininosuccinic acid (HASA), a compound that would be similar to argininosuccinic acid but with one extra CH_2 group. The assumption that fumarate is produced in the process is based on our knowledge of the chemical behavior of argininosuccinic acid.

Assays carried out to determine the metabolic fate of homocitrulline have suggested the possibility of its conversion to urea in what appears to be an enzymatic reaction that is dependent on ATP.

It has been known for some time (and confirmed here) that the hydrolysis of homoarginine by the enzyme arginase yields urea. Following the hydrolysis of arginine, urea and ornithine are produced. By the same token, it would be proper to assume that when homoarginine is hydrolized,

lysine (which is homoornithine) is produced as well as urea. Thus,



Thus, the experiments reported here appear to confirm the existence of the complete cycle, lysine to homocitrulline to HASA to homoarginine and back to lysine. Since the synthesis of carbamyl phosphate is the major first reaction in the disposal of excess blood ammonia, this cycle can serve in ammonia removal from the system.

This hypothesized alternate pathway implies that homoornithine (lysine) is a secondary substrate in place of ornithine, homocitrulline in place of citrulline, HASA in place of argininosuccinic acid, and homoarginine in place of arginine. By comparing reactions of the ornithine-urea cycle to those of this lysine-urea cycle, it appears that the efficiency of the latter is at most 5 per cent that of the former. This creates serious doubts as to the physiological significance of the lysine-urea cycle.

RELATIONSHIP OF THE LYSINE-UREA CYCLE TO THE PATIENT'S DISORDER

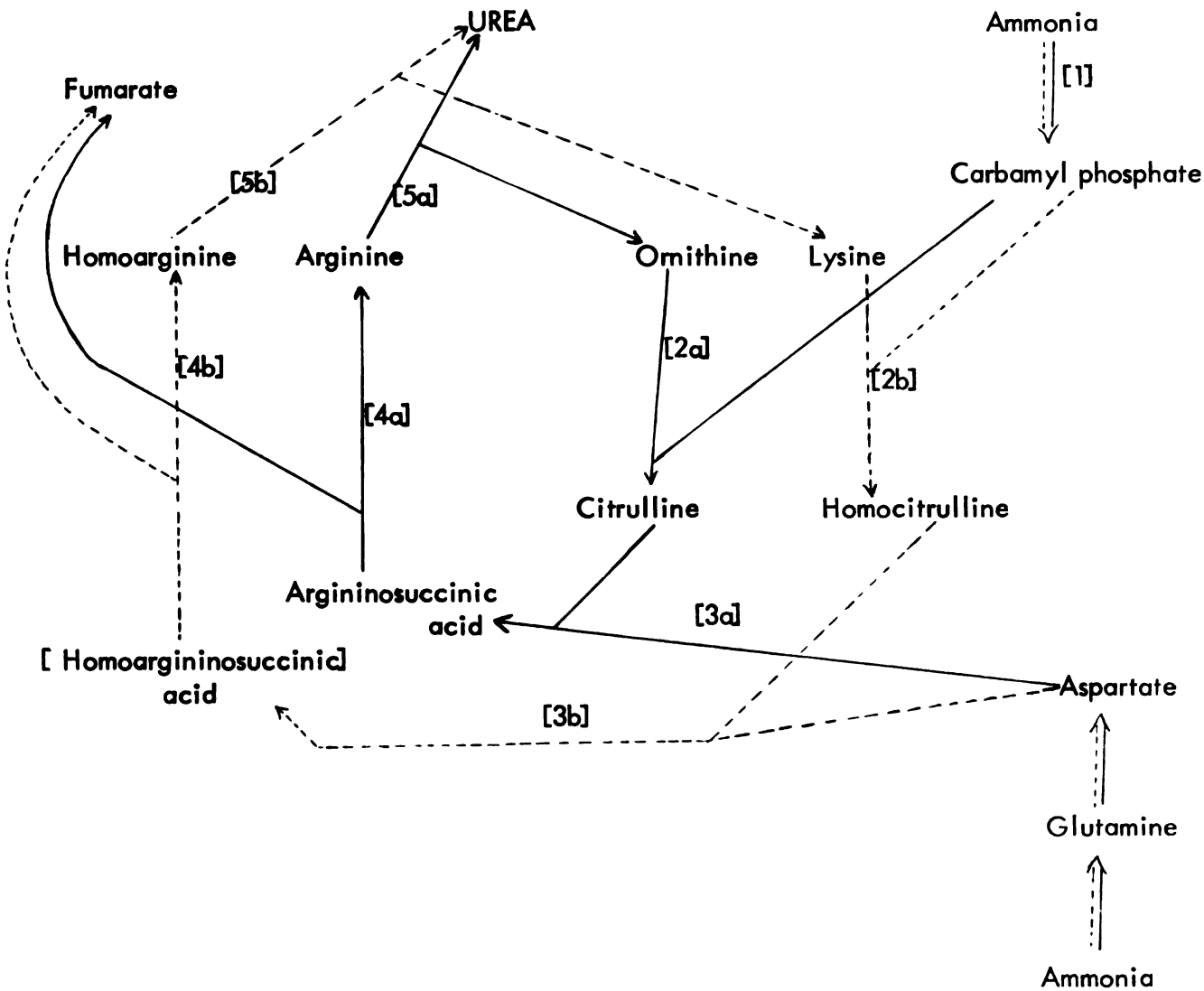
The interrelationships of the various aminoacidopathies reported in the patient have been explained. There is evidence from his mother's report, that this patient may have suffered from ammonia intoxication as a child. The absence of excessive amounts of ammonia in the patient's blood suggests that he became adapted to his condition by developing ways to regulate his ammonia levels. With the data at our disposal, it is possible to advance some theories about the ammonia regulatory mechanism

of this patient (see Fig. 19).

(1) The data suggest that the defective enzyme is ASA synthetase (3a in Fig. 19). It is possible that the secondary substrates are acted upon by the same enzymes that act upon the primary substrates. If this is the case, then the reaction catalyzed by enzyme 3b should also be defective. This means that we cannot hypothesize that this patient is getting rid of his ammonia via the lysine-urea cycle, but rather by spilling large amounts of citrulline and homocitrulline in his urine. Indeed, this patient excretes more citrulline in his urine than any previously described citrullinemic.

(2) Another possibility is that, although substrates of both cycles are acted upon by the same enzymes, the inability to utilize one substrate may not directly inhibit the ability to utilize the other substrate. In this case, it would be the lysine-urea cycle that has carried the major burden of regulating his ammonia levels. This would imply that the same enzyme has different reaction sites or properties for the two substrates, and that although the enzyme is defective for one substrate, it continues to function for the other.

(3) A third possibility is to postulate different sets of enzymes for the ornithine-urea cycle and the lysine-urea cycle, although the enzymes of each cycle may be capable of some activity on the substrates of the other. If this is true,



Lysine-Urea cycle (Back-up cycle)

Ornithine-Urea cycle

Fig. 19: The lysine-urea and the ornithine-urea cycles

then we can hypothesize that this patient is using the lysine-urea cycle which has become efficient because of a new steady state for the substrates, and that the early problems experienced by the patient occurred before he acquired this steady state. The experiments on rat liver indicated very little activity for the enzymes of the postulated secondary cycle. It is possible that they do occur or are induced in appreciable amounts in human liver, or that they exist in some other tissue, such as muscle.

REASONS FOR NORMAL UREA PRODUCTION

The normal levels of urea could be gotten from a combination of the following:

(1) Direct transamidation of lysine to homoarginine and the subsequent hydrolysis of homoarginine to urea.

(2) The very high concentration of citrulline could lead to the conversion of some citrulline to arginine and the subsequent hydrolysis of arginine to urea.

(3) Conversion of lysine to homoarginine via homocitrulline and HASA, and the subsequent hydrolysis of the homoarginine to urea.

EVIDENCE CONCERNING THE TWO-CYCLE HYPOTHESIS

Several lines of evidence suggest that there are two separate cycles for the production of urea and the disposal of ammonia.

(1) Defects of the ornithine-urea cycle usually do not lower urea production in affected individuals. Alternate hypotheses can explain this fact, so that it has been hypothesized that the defective enzymes

are functional because of the higher concentrations of substrates, that some non-enzymatic conversion has carried the substrate through the blocked path, or that transamidation of lysine to homoarginine has led by a separate path to urea. These hypotheses are tenable, and may either be correct or may supplement the lysine-urea cycle. However, it is difficult to assume that any one of these hypothesized sources of urea is a good substitute for the normal ornithine-urea cycle, and thus, it is questionable that they explain the large amounts of urea in most of the persons with ornithine-urea cycle defects.

(2) Defects that increase the concentration of a substance in the ornithine-urea cycle may also cause increases in the concentration of substances in the hypothesized lysine-urea cycle. Thus, hyperornithinemia is accompanied by homocitrullinuria (Shih et al., 1969). Citrullinemia, as in the patient reported here, is accompanied by hyperlysinemia, homocitrullinuria, and high blood and urine levels of homoarginine.

(3) What may be an enzyme defect of the lysine-urea cycle leads to ammonia intoxication. Thus, a hyperlysinemic patient without homocitrullinuria (suggesting a block in the enzyme that converts lysine to homocitrulline, was found to have ammonia intoxication (Colombo et al., 1967). On the other hand, hyperlysinemia with homocitrullinuria (suggesting a block in lysine metabolism other than lysine to homocitrulline) is not associated with ammonia intoxication (Ghadimi et al., 1967). These facts suggest that the conversion of lysine to homocitrulline may be a step in the removal of ammonia from the body, as one would expect if the lysine-urea cycle actually exists.

If the two cycles do exist, there is evidence that they are independent in part but, in one step, share an enzyme.

(1) There appear to be separate defects for the enzymes controlling the step from ornithine to citrulline and that from lysine to homocitrulline. Ornithine transcarbamylase deficiency (Russell et al., 1962) has been reported in some persons, while an apparent deficiency in the transcarbamylation of lysine has been reported in one individual (Colombo et al., 1967). However, experiments reported here indicated strong competition between lysine and ornithine for the utilization of OCT, but there is no evidence of whether the lysine was actually being converted, or was merely interfering with the conversion of ornithine.

(2) Arginase is known to be able to hydrolyse both arginine and homoarginine, suggesting that only one enzyme functions for both cycles.

(3) The only defect of the ornithine-urea cycle that has not been reported in man is a deficiency of arginase. With the above assumption that arginase catalyzes the homologous reactions in both cycles, it could be assumed that a serious deficiency of this enzyme would be lethal, as it would block both the ornithine-urea and the lysine-urea cycles. This then strengthens the suspicion that in all of the other steps, separate enzymes are needed to catalyze homologous reactions of the two cycles.

THE POSSIBLE ROLE OF THE LYSINE-UREA CYCLE

One would assume that the ornithine-urea cycle could adequately account for the disposal of ammonia by the body and that a second cycle

is not needed. However, as noted above, an apparent defect of the lysine-urea cycle leads to severe impairment of the affected individual, suggesting that the two functioning cycles are utilized by all normal persons. Despite the fact that both cycles appear to be needed, there is no obvious reason for this.

From an evolutionary standpoint, one might suppose that a single cycle would eventually assume the entire function of ammonia disposal and that most species would possess only one of these cycles. The evidence and data presented here is that both cycles are functional both in man and in the rat. In our present state of knowledge, there is at least one other example of an alternate genetic pathway for an apparently adequate system: the delta chain of hemoglobin has no known function not adequately performed by the beta chain in those primates in which it is found (new world monkeys and anthropoids, including man). Either mutation and selection have not efficiently destroyed the delta chain, except in old world monkeys, or our knowledge of primate hemoglobins is still incomplete. These same considerations may apply to the two urea cycles.

THE COURSE OF CITRULLINEMIA

There are two citrullinemic patients adequately described in the literature plus the individual who is described here, and each presents a distinct set of biochemical abnormalities. Because they have different biochemical characteristics, they may represent separate types of mutations of the gene for ASA synthetase. However, they might all have similar (complete or leaky) defects of the enzyme, but may represent

the stages of a series in which the patient of Morrow et al. (1967) presents the earliest stage, with ammonia intoxication and low urea production. The patient of McMurray et al. (1962) has ammonia intoxication but produces normal amounts of urea. The patient described here has no ammonia intoxication (but a history suggestive of it in the past) and normal urea production. The hypothesis that is put forth is that the primary defect is in the production of ASA synthetase, which blocks the ornithine-urea cycle. Early in life, ammonia builds up in the blood to toxic levels and urea production is negligible. As the lysine-urea cycle becomes more efficient (presumably because its high efficiency occurs only if there is low efficiency of the ornithine-urea cycle) first urea production proceeds to the normal range and then serum ammonia levels fall below the toxic threshhold. The damage wrought by early ammonia intoxication apparently is not fully repaired. Perhaps, if diagnosed early enough in life, a low protein diet would minimize the damage to the brain. The assumption of efficient urea production by the lysine-urea cycle would depend on whether its late efficiency appears naturally in all persons or is dependent on induction by its substrates in those with ornithine-urea cycle defects.

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APPENDICES

THE PEDIGREE (See Appendix 1)

I. (1) Wilomina P, died age 60 of cancer; Polish extraction

(2) Gusta D, died age 60 of pneumonia; German and Austrian extraction

(3) Louise S, born 1876, died 'natural' death; Swiss extraction

II. (1) Died at age 4 of diphtheria

(2) Died at age 43; was married but had no children

(3) Died at age 16 of peritonitis

(4) Died at age 28 of Diabetes mellitus

(5) Adopted

(7) Was married and has no children

(9) Father of propositus

(10) Mother of propositus

(11) Was married but has no children

(13) Died at age 9 of peritonitis

(16) Was married but has no children

III. (1) Very mildly retarded (was reported as slow)

(2) Same as 1; also has peritonitis

(3) Same as 1

(7) Same as 1

(8) Same as 1; very nervous and never married

(9) Same as 1; has speech impediment; never completed grade school; complains of headaches

(10) Suffers from TB; had a spleenolectomy and has ulcers of the stomach

(11) Only sib of propositus; tested biochemically and found to be normal

(12) PROPOSITUS

(38) Has congenital hip dislocation

IV. (6) Has crossed eyes and other eye lesions

(14) Has crossed eyes

(18) Very short in stature; much less than 5 ft at 18

(19) Same as 18

(22) 5 normal children (their sexes were not given)

(23) 3 normal children (their sexes were not given)

(26) Has brain tumor

(27) 2 normal children (their sexes were not given)

(39) Very mildly retarded; in special school for reading problems

(42) 3 normal children (no sexes were given)

(44) 1 normal child (no sex was given)

(47) Tested biochemically and was normal

(48) Same as 47

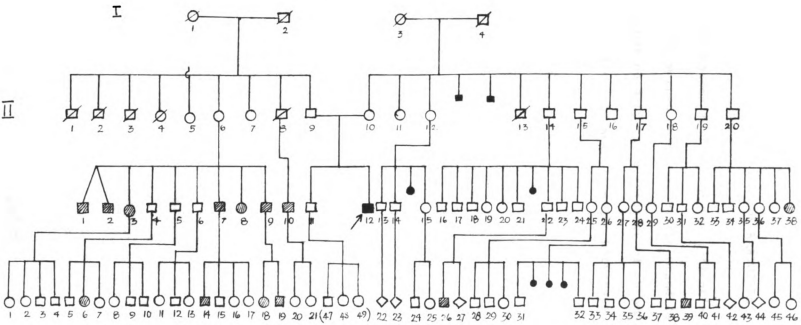
(49) Same as 47

NOTE:

(1) The number of miscarriages in the maternal part of the propositus. There were 7 out of 69 births for a frequency of about 10 per cent.

(2) The number of recognizable anomalies in the paternal half of the propositus. There are 11 out of 45 births for a frequency of about 25 per cent.

App.1 THE PEDIGREE



KEY

PROPOSITUS

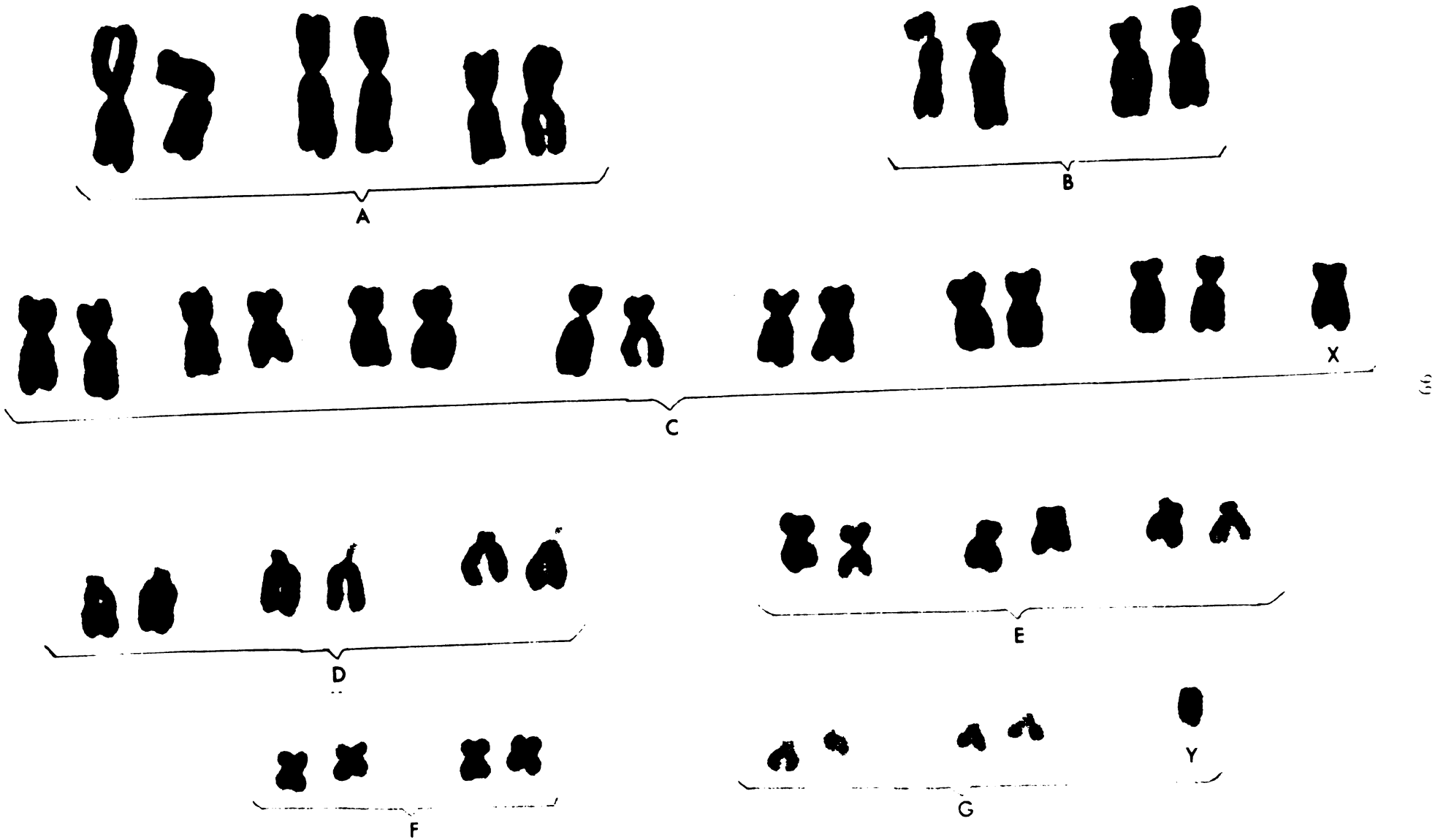
or Defects other than Citrullinemia

or Miscarriages

Sexes were not given

THE KARYOTYPE

Chromosome analysis of the patient's leukocytes was done (Appendix 2) using the method described by the Grand Island Biological Company (Gibco). There was no detectable evidence of chromosomal abnormality in the patient as can be seen from the karyotype in Appendix 2.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