

APPROVAL SHEET

Title of Thesis: The Metabolic Basis of Cystinosis

Name of Candidate: Susan Beth Marcus
Master of Science, 1981

Thesis and Abstract Approved:

Lewis Brown

May 12, 1981

Carol G. Smith, Ph.D.

May 12, 1981

Gou Moore

May 12, 1981

ABSTRACT

Title of Thesis: The Metabolic Basis of Cystinosis

Susan Beth Marcus, Master of Science, 1981

Thesis directed by: Lewis Aronow, Ph.D., Professor and Chairman,
Department of Pharmacology

Cystinosis is a rare, recessively inherited disorder characterized by an intralysosomal accumulation of the disulfide amino acid cystine (Schulman, et al, 1967). Its metabolic basis has eluded precise delineation since its discovery in 1903 by Abderhalden.

In its most severe form (nephropathic), it is present at birth, but symptomology does not appear until six months to one year of age. Affected children show glomerular damage usually progressing to death from uremia within the first decade of life. In the intermediate form, onset is by the second or third decade of life. The third type is completely benign, usually discovered only when a routine ophthalmologic examination reveals the characteristic cystine crystals in the cornea and conjunctiva.

Matsuo and Greenberg (1958) purified an enzyme, γ -cystathionase, isolated from rat liver which catalyzed both the deamination of homoserine and the cleavage of cystathionine. In the l-homocysteine-methionine cycle, cystathionase plays a key role in the conversion of cystathionine to cysteine with α -ketobutyric acid and ammonia being side products. We postulate that under normal conditions, increased cyst(e)ine results in inhibition of the enzyme, the cycle then favoring

formation of methionine. In cystinotic individuals, it is then postulated that there is a defect in this enzyme--there is excessive cystine accumulation because cystathionase is not inhibited by its normal regulator, cystine.

Activity of the enzyme was determined by the formation of α -ketobutyric acid (Friedemann and Haugen, 1943; Sayre and Greenberg, 1956), using either l-cystathionine or l-homoserine as substrate. In the presence of excess cystine, rat, monkey and human liver cystathionase was inhibited. Due to the rarity of cystinosis, it was not possible to obtain cystinotic liver samples for cystathionase determination at this time. However, on the basis of the results with normal enzyme, it is postulated that the metabolic basis of cystinosis is a defect in the regulatory control of the enzyme γ -cystathionase by its feedback inhibitor cystine.

THE METABOLIC BASIS

OF

CYSTINOSIS

by

Susan Beth Marcus

Thesis submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of
the Health Sciences in partial fulfillment of the
requirements for the degree of
Master of Science 1981

ACKNOWLEDGMENT

I wish to thank Dr. Lewis Aronow for his assistance and guidance during the course of this work.

The support from the faculty, staff and graduate students of the Department of Pharmacology is greatly appreciated.

Special thanks to Ms. Lemzie Clemmons and Ms. Elizabeth Dunn for the typing of this thesis.

TABLE OF CONTENTS

<u>Section</u>	<u>Page</u>
INTRODUCTION	1
I. Cystinosis: A short history	2
II. Clinical features of cystinosis	4
III. Pathology of cystinosis	8
IV. Cystine chemistry and its role in cystinosis	10
V. Treatment	16
VI. The metabolic basis of cystinosis	22
EXPERIMENTAL PROCEDURE	27
I. Chemicals and enzymes	27
II. Preparation of crude enzyme homogenate	28
III. Partial purification of crude enzyme homogenate	28
IV. Protein assay	29
V. Enzyme assay	29
RESULTS	32
DISCUSSION	44
REFERENCES	47
SUMMARY	57

LIST OF TABLES

<u>Table</u>	<u>Page</u>
I. Normal human liver homogenate; γ -cystathionase activity and inhibition by l-cystine	39
II. Comparison of km values between cystathionases of various species using l-homoserine as a substrate	40
III. Ability of l-cysteine to inhibit rat liver cystathionase	41
IV. Effect of l-cystine on normal and cystinotic spleen cystathionase	42
V. % Cystine inhibition under standard conditions	45

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. The l-homocysteine-methionine cycle	12
2. The l-homocysteine-methionine cycle; structural schematics	13
3. Final sequence in the transsulfuration pathway (l-homocysteine-methionine cycle); the cleavage of cystathionine by the enzyme γ -cystathionase	15
4. α -Ketobutyric acid standard curve	33
5. L-homoserine time course	34
6. Inhibition of rat liver cystathionase by l- cystine	36
7. Inhibition of monkey liver cystathionase by l-cystine	37
8. Inhibition of human liver cystathionase by l-cystine	38

INTRODUCTION

Cystinosis is a rare and fatal disease, found in only a few hundred children in this country (1). It is a recessively inherited metabolic disorder, transmitted to the child by both parents, who are carriers.

Biochemically it is characterized by an intracellular accumulation of the disulfide amino acid cystine in most body cells of these children. In many tissues such as the eye, spleen, kidney, liver and bone marrow, the cystine precipitates and is easily discerned as crystals under a phase microscope (2). In other tissues, such as cultured skin fibroblasts, crystallization is not evident, but the amino acid is present in 100-fold concentration higher than normal (3). The free non-protein cystine that accumulates is compartmentalized in lysosomes (4).

The disease is present at birth, but is not usually discernable until six to twelve months of age, when various symptoms begin to appear. Clinical features range widely between families. In its most severe form, known as nephropathic cystinosis symptoms result from an impairment of both tubular and glomerular functions. Also, renal abnormalities of the Fanconi syndrome with the usual deficiency in tubular reabsorption of water, phosphate, sodium, potassium, bicarbonate, glucose, amino acids and other organic acids appear. The presenting symptoms of the disease--polyuria, polydipsia and recurrent unexplained fevers are probably the result of dehydration, all stemming from a defect in water reabsorption. Renal loss of phosphate can be

related to later development of hypophosphatemic rickets, a type resistant to the usual antirachitic doses of vitamin D. The loss of potassium and bicarbonate results in chronic acidosis and hypokalemia. In addition, afflicted children show severe growth retardation and progressive glomerular damage usually progressing to death from uremia within the first decade of life.

Other children have a completely benign form of cystinosis and are only discovered when an ophthalmologic examination is done for other reasons and reveals characteristic crystalline opacities in the cornea and conjunctiva. In addition, some have an intermediate type of cystinosis characterized by later onset (second or third decade of life) of renal disease and renal failure.

Genetic heterogeneity of this disease is suggested by the variations in severity between families and the similar degrees of severity of clinical expression of the disease within a given family. However, the primary abnormal gene product leading to cystine accumulation remains an enigma to investigators today.

I. Cystinosis: A Short History

Since cystinosis is seldom encountered by a pediatrician, it presents a diagnostic problem--not in the difficulty of the diagnosis itself, but due to the fact that the possibility of the disease is overlooked. Even at postmortem, positive identification of such cases is difficult, unless the pathologist is aware of the possibility and has specially prepared the tissues. In the context of pediatric renal disease, cystinosis is much more common, being the single most frequent cause of Fanconi's syndrome (1). However, there are several different

clinical diseases that can produce Fanconi's syndrome, and a variety of disorders of sulfur metabolism, such as cystinuria, a disease completely different from cystinosis. Thus recognition of the various pathological and clinical characteristics of cystinosis and the Fanconi syndrome was shrouded in confusion for a long period of time.

The first recognition of the disease was in 1903 by Abderhalden (5). He described a child dying that had cystine crystals in many internal organs and attributed it to "inanition." Since there were excessive quantities of cystine in the urine of the child's father, paternal grandfather and two siblings, he called it familial cystine diathesis. This resulted in the primitive view that cystinosis was merely a more severe type of cystinuria.

Lignac (6), in 1924, reported three infant cases being the first to associate the cystine crystal deposition with a familial expression of recurrent fever, dwarfism, rickets, renal disease and wasting. Yet the finding of ureteral cystine stones in one of his cases only further confused the issue of cystinosis and cystinuria being the same.

In the 1930's additional features of the clinical symptoms of cystinosis emerged when Fanconi (7) associated rickets and stunted growth in a child with albuminuria and glycosuria. Similar reports by de Toni (8) and Debre (9) of vitamin D-resistant rickets with spontaneous fractures in dwarfed children also showing low phosphorus concentration in the serum, in addition to acidosis, glycosuria and albuminuria, led to the proposal of a syndrome by Fanconi (10) of "nephrotic-glycosuric dwarfism with hypophosphatemic rickets" in 1936.

The idea that cystinosis and Fanconi syndrome were the same in that cystinosis could cause the Fanconi syndrome was suggested by

Beumer and Wepler (11) and later proposed by Fanconi himself (12). In 1952, Bickel (13) and his associates with a larger series of patients (14 new cases) clearly distinguished between the generalized aminoaciduria of the Fanconi syndrome and the specific increase of excretion of cystine, lysine, and arginine indicative of cystinuria.

The association between cystinosis and Fanconi syndrome is not always a clearly cut one. Not all cases of the Fanconi syndrome show evidence of crystalline cystine deposits, such as in the case of adult onset of cystinosis. On the other hand, the presence of crystalline cystine deposits isn't necessarily indicative of Fanconi syndrome and progressive renal failure as found in the adult benign form of cystinosis.

Recent emphasis on studying cystinotic cells in vitro has allowed for direct diagnosis of the carrier state of cystinosis and the theory of intracellular compartmentalization of cystine in lysosomes (14). In the last decade different treatment approaches to cystinosis have been tried, without much success. It is quite possible that one of the answers lies in prevention, since recent work has suggested the capability of in utero diagnosis of cystinosis (15).

II. Clinical Features of Cystinosis

Clinical expression of cystinosis ranges widely and can be broken down into three categories--nephropathic, late-onset (intermediate) and benign cystinosis. Expression varies within the groups, however affected members in a given family exhibit the same type of disease, suggesting genetic heterogeneity.

Nephropathic cystinosis is the best known form and is the one referred to when one speaks of "cystinosis" or "cystine storage disease." Although these children have abnormal quantities of cystine in most of their internal organs, the major clinical symptomology can be related to the progressive impairment of tubular, then glomerular function of the kidney. The initial tubular impairment of function is congruous with the renal Fanconi syndrome and its characteristic deficiency in tubular reabsorption of water, phosphate, sodium, potassium, bicarbonate, glucose, amino acids and other organic acids. It is to be pointed out that unlike cystinuria, the aminoaciduria in cystinosis is generalized and the concentration of cystine in the urine is in the same range as that of other amino acids, therefore not being high enough for crystallization and formation of kidney stones.

Children with nephropathic cystinosis appear normal from birth to about six months of age. The first overt signs of the disease are usually produced by the renal tubular defect in water reabsorption. The polyuria and polydypsia which results make children with the disease extremely susceptible to dehydration. This vulnerability to dehydration explains the recurrent unexplained fevers, one of the disease's most common presenting symptoms. By one year of age, they usually show growth retardation, rickets, acidosis and other chemical evidence of renal tubular abnormalities revealed by an increased renal excretion of glucose, amino acids, phosphate and potassium. There is usually evidence of glomerular damage as well, and the clinical course that follows is determined by the rate at which glomerular insufficiency progresses (16).

Failure to thrive is one of the more prominent features of nephropathic cystinosis. Affected children show an increased rate of growth failure within the first twelve months of age and remain below the third percentile in both height and weight. Mental development, however, is normal. In most patients, rickets develops at an early age, even with the intake of the usual preventative doses of vitamin D (400 units/day).

The glomerular damage progresses sporadically, but unremittingly. Careful study of affected patients shows that glomerular dysfunction may remain steady for months or even years, followed by periods of rapid degeneration, appearing to be unrelated to any biochemical or environmental factors known to date.

Clinical features unrelated to the kidney are also present in these children. The majority have blonde hair and a fair complexion, often having lesser pigmentation than their parents. Despite their fair coloring, exposure to sunlight produces skin tanning with far less tendency for burning. Patients with cystinosis seem to tolerate infections well and show no unusual susceptibility to intercurrent illness.

There are many ocular complications of systemic disorders, but very few conditions can compare with those changes seen in the eyes of cystinotic patients. Corneal crystals are present and can be detected through the use of a slit lamp. In spite of such ocular changes, cystinotic patients show no consistent abnormality of vision, except for a severe photobia developing in children within the first few years of life.

The development of clinical symptoms of the Fanconi syndrome is paralleled by the appearance of laboratory evidence of renal tubular dysfunction (17,18,19). Proteinuria is often present, consisting primarily of the so-called tubular protein associated with the syndrome.

The clinical course of nephropathic cystinosis is one of continual progression of glomerular damage, eventually leading to death in uremia. The time course of this deterioration is not a consistent one with periods of apparent renal stability followed by times of rapid decline. During most of their lives, these children are able to maintain relatively normal degrees of activity. It is only in their last six months to year of life that they are severely incapacitated.

Some patients with cystinosis appear to have an attenuated form of the disease which permits them to survive well into the second decade of life. This type has been named late-onset, intermediate or adolescent cystinosis. These patients differ from those with the infantile nephropathic form in several ways. Primarily, renal dysfunction is not apparent until an older age (18 months-17 years) and is less severe. They usually don't have the complete Fanconi syndrome, and their glomerular insufficiency progresses more slowly. When more than one child within a family has this particular form, the age of onset and the symptomatology are similar. This suggests the possibility of this type actually encompassing a spectrum of different types of cystinosis, rather than a simply defined disease.

All of these children have shown crystalline deposits in their corneas and conjunctivas as well as having the typical cystine crystals in their bone marrow aspirates. Accordingly, they all show higher than

normal cystine concentrations in leukocytes and cultures of skin fibroblasts, but not as high as those seen with the nephropathic form (20). The presence of photobia and retinopathy has been variable, whereas skin pigmentation tends to be normal. Growth retardation, if present, is of less severity. Although limited information is available, this type of cystinosis, like its more severe form, appears to be transmitted as an autosomal recessive disease (21).

What appears to be a completely benign variant of cystinosis was first described by Cogan and associates in 1957 (22). Additional cases have been reported since then (20,23). The primary clinical difference between the other forms and this one is the failure of these patients to show either retinopathy or renal dysfunction. However, they do show crystalline deposits in the cornea, conjunctiva, bone marrow and leukocytes, though this causes no disability. As a result, these patients live to adult life. Their cystinosis is only identified incidentally during a routine ophthalmologic examination, revealing the crystalline deposits. This benign variant form of the disease, also having an autosomal recessive pattern of inheritance, seems to represent one end of the spectrum of the clinical expression of cystinosis.

III. Pathology of Cystinosis

The pathological findings of patients dying of renal failure due to cystinosis can be related to two different processes--the terminal uremia, and more importantly, those findings unique to the disease itself (24).

The specific finding of cystinosis is the deposition of cystine crystals, primarily in the reticuloendothelial cells of the bone marrow,

spleen, liver and lymphatic system. The extent of crystal deposition varies among patients.

These cystine crystals are best observed in their natural state and exist in several different forms--the more abundant rectangular shape and the less common hexagonal plates. Needle shaped crystals are also present; however their content has not yet been determined. The medium in which the crystallization occurs can affect the crystal form (13).

The corneal, conjunctival and retinal manifestations of cystinosis are unique and characteristic enough that a diagnosis can usually be made. Crystalline deposits in the cornea were first described by Bürki in 1941 (25). On gross examination, a diffuse haziness of the cornea can usually be made apparent by transverse illumination with a penlight. Slit-lamp observation discloses homogeneously distributed iridescent crystals in the cornea and conjunctiva. These tinsel like opacities are regularly seen in patients with cystinosis and their slit-lamp appearance is virtually diagnostic of all forms of cystinosis. One cannot preclude the existence of cystinosis in the absence of crystals. In some cases, it appears that the occurrence of crystals in the cornea is a postnatal development.

In addition to corneal and conjunctival changes, a peripheral retinopathy (26) is present, characteristic of the nephropathic form. The pathologic change consists of a generalized depigmentation in the form of clumps in irregular distribution. Such changes may be of diagnostic value since they can precede the corneal changes by months.

The major changes are found in the kidney, with the morphologic findings varying with the stage of the disease. Baar and Bickel (27)

classified the lesions of end stage kidneys into three stages of their development. Microdissection (28) reveals an abnormally thin first portion (neck) of the proximal convoluted tubule. This historic swan-neck deformity becomes apparent during the first years of life (29) and most likely coincides with the clinical onset of the Fanconi syndrome.

Cystine crystals, when observable, are mainly found intracellularly in the interstitial cells, but have also been seen in glomerular and tubular cells (27).

The first electron microscopic study of the kidney in cystinosis was that of Jackson and co-workers (20). They described crystals and crystalline spaces in unidentified interstitial cells. The interstitial tissue showed fibrosis, dark bodies and fibrils. They also noted that a properly oriented crystal gave a symmetrical hexagonal pattern very similar to a crystal of purified l-cystine, demonstrating that the crystals in a cystinotic kidney sample were indeed l-cystine. Electron probe studies revealed an elevated content of sulfur in the form of (di)sulfide in the dark cytoplasmic inclusions. Thus, the dark cells and inclusions were believed to contain abundant quantities of cystine.

Histologic findings in the benign adult form of cystinosis have not been reported. Kidneys from patients with the late-onset form are quite similar in type to those with the nephropathic form, and like them, exhibit polykarocytosis of the visceral glomerular cells (31).

IV. Cystine Chemistry and Its Role in Cystinosis

Sulfur amino acid intermediate metabolism is quite complicated, and our current understanding of the biochemical pathways is still

incomplete. In order to get a better picture of cystine and its role in cystinosis, a selective review of sulfur metabolism would be in order.

The cystathionine pathway (Figs. 1 and 2) refers to the steps in which l-methionine, is converted by way of S-adenosyl-l-methionine, S-adenosyl-l-homocysteine, l-homocysteine and l-cystathionine to l-cysteine. Under normal conditions, the principal intracellular sulfur containing amino acid is cysteine, which can be synthesized from methionine as described above (32).

In normal human beings, methionine is an essential amino acid, although cystine, which is not, can replace 80-90% of the human requirement for methionine. Children can grow normally on a diet containing adequate methionine but no cystine (33). In contrast, cells in culture generally require cyst(e)ine in addition to methionine. Cell lines, such as human fibroblasts, have an absolute requirement for l-cystine (34).

The first step in the conversion of methionine to cysteine is the formation of S-adenosylmethionine from methionine and ATP, catalyzed by methionine adenosyltransferase (EC 2.5.1.6). Methionine has other metabolic uses in addition to conversion to its adenosyl derivative, such as its direct utilization in protein synthesis and into a variety of sulfur compounds (33,35).

S-adenosylmethionine, because of its sulfonium bond can be considered a high-energy compound (36). It is widely employed as a methyl group donor in many biochemical reactions requiring transmethylation. All of these reactions produce a common sulfur containing product, S-adenosylhomocysteine, resulting from the loss of the methyl group of its

THE L-HOMOCYSTEINE-METHIONINE CYCLE

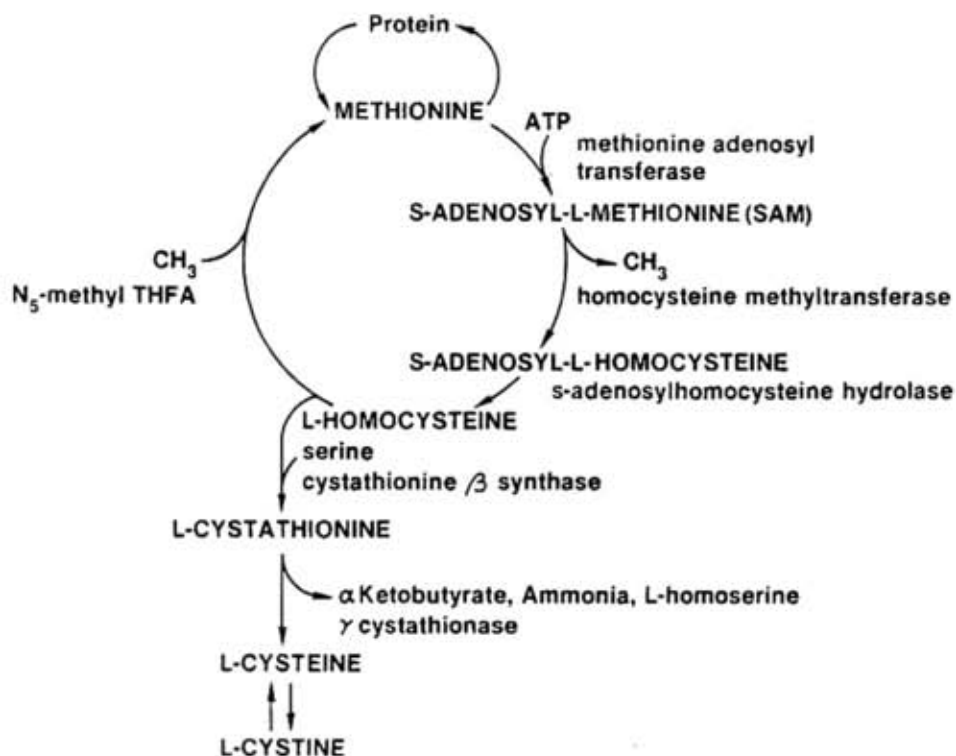


FIGURE 1 The l-homocysteine-methionine cycle; the process by which methionine undergoes several changes to form the amino acid cyst(e)ine.

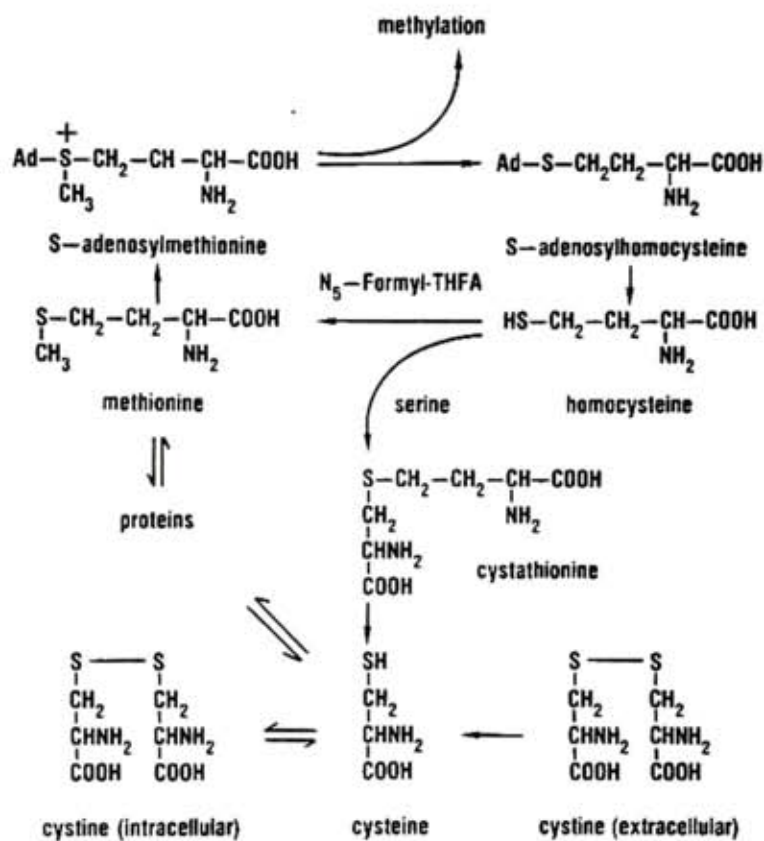


FIGURE 2 The 1-homocysteine-methionine cycle; structural schematics.

precursor. S-adenosylhomocysteine is (reversibly) converted to adenosine and L-homocysteine by a liver enzyme, S-adenosylhomocysteine hydrolase (EC 3.3.1.1). The equilibrium of the reaction favors S-adenosylhomocysteine accumulation (37).

Homocysteine lies at an important metabolic branch point, either being converted to cystathionine through the transsulfuration pathway or being remethylated to form methionine. Homocysteine remethylation can be carried out by several well characterized reactions. Two such alternative mechanisms existing in humans are transfers of methyl groups from choline via betaine and the utilization of 5-methyltetrahydrofolic acid as a methyl donor (38).

The major alternative to remethylation is an addition reaction of homocysteine with serine to form the thioether, cystathionine. The reaction is catalyzed by cystathionine β -synthase (EC 4.2.1.22). The enzyme, purified from rat liver, is firmly bound with and requires pyridoxal phosphate for activity. It has a molecular weight of approximately 250,000 and is composed of two non-identical subunits, each represented twice (39). Human liver cystathionine β -synthase has a molecular weight similar to that found in rats and also contains bound pyridoxal phosphate. Gradient elution using DEAE cellulose by Tudball and Reed (40) led to separation of two active forms of the enzyme. Mammalian cystathionine β -synthase, can also catalyze an alternative reaction, known as cysteine sulphydration, in which sulfide, instead of homocysteine, is combined with serine. Under physiologic conditions, the formation of cystathionine is thermodynamically favored.

The final sequence in this transsulfuration pathway (Fig. 3) is the cleavage of cystathionine to cysteine, α -ketobutyrate, and ammonia

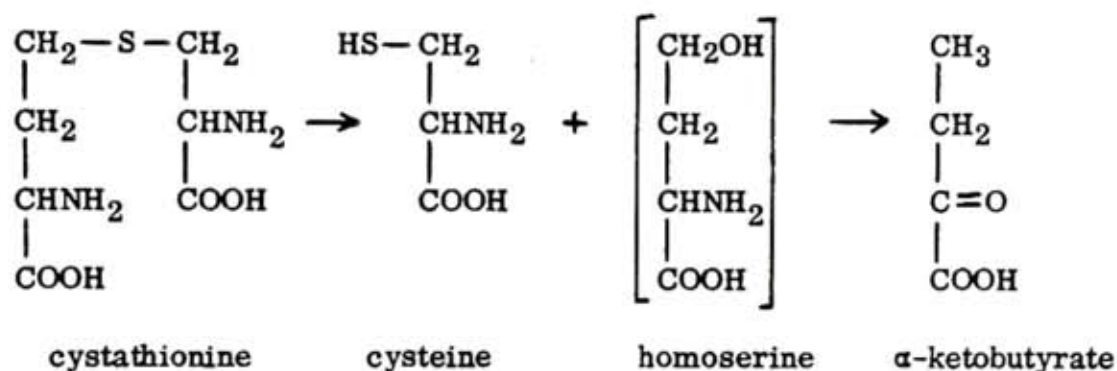


FIGURE 3 Final sequence in the transsulfuration pathway (1-homocysteine-methionine cycle); the cleavage of cystathionine by the enzyme γ -cystathionase to form cysteine, α -ketobutyrate and ammonia, with homoserine being a possible intermediate in the reaction.

by the enzyme, γ -cystathionase (EC 4.4.1.1). The enzyme contains a bound pyridoxal phosphate and has been isolated in pure form (41). It can also deaminate homoserine to form α -ketobutyrate. No direct evidence exists for the production of l-homoserine as an intermediate during the cleavage of cystathionine by cystathionase. However, homoserine is required in combination with cysteine for cystathionine synthesis, with cystathionase acting in the reverse direction (42). Alternative pathways for cysteine synthesis have not been elucidated in man.

Cystine is the disulfide or oxidized form of cysteine. When it is passed into the cell, it is reduced to cysteine (thiol form), via a cytoplasmic system that involves the cysteine-containing compound reduced glutathione. Glutathione is present in large amounts and must be present to keep cysteine reduced and very soluble instead of being oxidized in the extremely insoluble form cystine (43).

V. Treatment

The symptomatic treatment of the earlier stages of nephropathic cystinosis consists of providing an adequate fluid intake, healing the rickets and correcting the metabolic acidosis and potassium deficit. In most patients, this is readily accomplished, resulting in a marked improvement in the patient's personality, eating, activity and "general well being." As the disease progresses, treatment becomes one for renal insufficiency. Patients with intermediate cystinosis usually require similar treatment as given to those with the nephropathic form. Patients with benign cystinosis are essentially normal and require no therapy.

The acidosis and hypokalemia are most easily corrected by the use of a potassium-containing alkalizing mixture such as a solution of sodium and potassium citrate (18). With this therapy, the serum potassium concentration usually remains at a low normal level or slightly less, but no signs of hypokalemia, such as EKG changes, are present.

With most patients, the rachitic changes are corrected with doses of 10,000 to 15,000 units per day of vitamin D. Since there is the possibility of glomerular damage (44) secondary to large doses of vitamin D, the lowest effective dose of vitamin D should be used. Phosphate salts are effective, but must be used with care. The simultaneous use of alkalizing solutions and phosphate salts may produce tetany. The diuretic, hydrochlorothiazide, has been successful in the treatment of cystinotic rickets (45).

Increased appetite, strength and weight, and a decreased aminoaciduria have been claimed as a result of the anabolic agent, 1-methyl-androstenedione acetate. The response, however, is not consistent (46), and it is felt by some (47) that patients whose rickets and electrolyte imbalance are controlled do well for several years without additional agents, although their growth is usually lower than normal.

The treatment of cystinosis with a specific diet or drug therapy is based on the idea that cystine deposition causes the other symptoms, being toxic to cells (48). Dietary therapy revolves around the belief that in restricting the intake of cystine and its precursor methionine, cystine deposition would be limited. The chemical basis behind drug therapy is that the accumulated cystine must be transformed into a more soluble substance, which can be excreted, reducing the body burden of cystine.

Efforts to influence the course of cystinosis by implementing diets low in cystine and methionine content have not been successful. It has been shown that the intracellular storage of cystine was reduced in cultured cystinotic cells when grown in a reduced cystine medium (49). Special diets for cystinotic children are either derived from natural proteins low in cystine and methionine, such as lentil preparations, or from amino acid mixtures free of cystine and methionine supplemented with natural proteins up to the allowance of sulfur-containing amino acids. The diets are not palatable, and compliance is a problem. Although the idea behind such diets was validated in studies with cystinotic cells, in cystinotic patients it has been proven otherwise (50). The diets had no demonstrable effect in improving growth, retarding the rate of deterioration of renal function and reducing corneal crystal deposits. Thus, dietary therapy is not a recommended method of treatment.

Specific drug therapy has focused on coupling cystine or reducing it to form a more soluble substance. Treatment with the thiol compounds (51), dimercaprol (BAL) and D-penicillamine (β,β -dimethyl cysteine) was used in an attempt to reactivate or maintain the thiol-dependent enzyme systems. It had been thought that the coupling of stored cystine with penicillamine to form a mixed disulfide would solve the storage problem. The drug did reduce fasting plasma concentration of free cystine by approximately fifty percent, but had no effect on the free cystine in leukocytes (52). Using ion exchange chromatography (53,54), both penicillamine and its mixed disulfide are easily detectable; however neither was detected in the leukocytes of penicillamine-treated patients. In effect, one cannot even be certain that

penicillamine enters body cells, and it has not yet been shown to be effective therapy for cystinosis.

Another approach, using a sulphydryl reducing agent, would result in the reduction of its more soluble form cysteine. It has been reported that dithiothreitol (55) produces a selective decrease in the intracellular content of cystinotic cells in culture. Clinical trials showed a progressive decrease in the free cystine content of peripheral leukocytes with stable kidney function and no harm to the intestine or alteration in hemoglobin and in insulin, both disulfide-containing proteins. This approach is promising; however due to its limited trials, we do not yet have a conclusive demonstration that it ameliorates the course of the disease (56).

The observation that in a cystinotic fetus most organs contained excessive amounts of cystine except for the adrenal gland where there was abundant ascorbic acid, sparked an interest in Vitamin C as a therapeutic agent (57). Cultured cystinotic fibroblasts treated with vitamin C resulted in a decreased cystine content, stabilizing at about one half of that of the control cells within three days (58). The mechanism of this effect has yet to be explained. The next step was the implementation of a controlled, double-blind clinical trial (59). After 28 months, the study was discontinued based on the observation that only those in the vitamin C group had an incidence of increasing serum creatinine with progression to renal failure. This investigation demonstrated that vitamin C is not likely to be of benefit in the management of nephropathic cystinosis.

Thoene and associates (60) showed that intracellular cystine could be brought to normal levels in cystinotic children with the administration of cysteamine. This drug was tested on a patient who was in end-stage renal failure. Shortly after therapy was started, the patient developed grand mal seizures. On the basis of this experience, therapy was discontinued. More recently, it was theorized that cysteamine, unlike the thiol compounds used previously, was able to enter the lysosomal pool and react with cystine to form cysteine and the mixed disulfide of cysteine and cysteamine, which could easily diffuse from the lysosome (61). Cysteamine therapy has been reconsidered (62,63). The taste of the medicine precludes its testing in a double-blind manner. Unlike vitamin C, upon oral or intravenous administration, cysteamine produces a drop in leukocyte cystine content to close to or within the normal range. Lower doses have been used, possibly precluding unwanted side effects such as allergic reactions. The efficacy of this therapy may not be established for some time, as many cystinotic patients only improve temporarily. However, it is a mode of therapy which will receive more attention in the near future.

More recently, phosphocysteamine (WR 638), the phosphorothio-ester of cysteamine, having about one-half the toxicity of its parent compound and lacking the disagreeable odor, has been demonstrated to be equally effective as cysteamine itself (64). Since marked cystine depletion is both an in vivo and in vitro process which occurs without significant increases in the extracellular free-thiol concentration, it is possible that the conversion of phosphocysteamine to free cysteamine occurs only intracellularly. Thus, the compound may deliver the needed

agent directly to the site of cystine storage via cytoplasmic or lysosomal phosphatase action. The compound, phosphocysteamine, may therefore be a superior agent in treating nephropathic cystinosis.

Within the past year (65), it was demonstrated that cystine, from the degradation of extracellular cystine-containing proteins, could accumulate in cystinotic fibroblasts. Comparison of the rates of pinocytosis and proteolysis of extracellular proteins by normal and cystinotic cells does not indicate that these processes are directly involved in the accumulation of cystine. However, in this study, it was shown that the rate of cystine accumulation by cystinotic cells could be modulated by the concentration of bovine serum albumin in the culture medium, possibly providing an effective tool for further investigation.

Since the primary abnormality leading to renal failure in cystinosis seems to be a recessive genetic defect of the intracellular environment, it would seem that normal cells transplanted into a cystinotic patient would not accumulate cystine. Thus, in theory, a cystinotic patient would be as good a candidate for a renal transplant as any other child with chronic renal disease. Experience with renal transplantation in children is now extensive; age and size are no longer barriers to success (66).

With the recent development of amniocentesis, antenatal diagnosis of cystinosis becomes possible. The demonstration of a characteristic biochemical defect in cultured cystinotic cells raised the possibility of a prenatal diagnosis by examination of cultured amniotic cells, providing the defect was expressed in amniotic cells (15). Abortion of affected fetuses would constitute a form of prevention.

VI. The Metabolic Basis of Cystinosis

Since Abderhalden (1903) (5) first detected widespread storage of crystalline cystine in the tissues of an infant at autopsy, noting the familial incidence of the disorder, diagnosis of cystinosis has been made by postmortem and antemortem examination in increasing numbers. The nature of cystinosis and its underlying biochemical basis remains a puzzle despite joint efforts by investigators. Over the past years, several hypotheses have been put forth seeking to explain cystine storage and its relation to the Fanconi syndrome.

Since histologic examination of cystinotic tissues revealed cystine crystals primarily in reticuloendothelial cells, it would have seemed a likely explanation that the crystals were first formed extracellularly and were then phagocytized. However, over twenty-five years ago, it was concluded that crystal formation was within the cells and the crystals were not trapped by phagocytes (67). The fact that cystine solubility in cystinotic plasma is about 50 times greater than the fasting plasma cystine concentration in patients with cystinosis also precludes the idea of the extracellular crystallization of cystine in this disease (68).

Bickel and associates (13) observed increased concentrations of a number of plasma amino acids in cystinosis, suggesting a generalized failure of their incorporation into proteins resulting in intracellular deposition of cystine and renal anomalies. In the plasma and in the extracellular compartment, cystine exists mainly in its oxidized form. It was at first thought that the fasting plasma concentration of cystine was slightly elevated (69); however later studies

could find no consistent abnormality in cystinotic patients, the plasma amino acid concentrations being essentially normal (20).

From another viewpoint, it was proposed that there was a specific enzyme defect in cysteine-cystine metabolism, leading to accumulation of the excess cystine in the tissues with consequent nephrotoxic effects. The reduction of cystine to cysteine by cystine reductase was believed to be the site of such a defect (44). It was later concluded that either cystine reductase (EC 1.6.4.1) was deficient (70) or the system catalyzing the transfer of hydrogen from reduced glutathione to cystine was low in activity (71). Later, Patrick (1962) (72) found the reduction of cystine to cysteine by cystine reductase and by glutathione-cysteine transhydrogenase to be normal in cystinotic liver. Also, no abnormality was found in the cystine-reductase activity of the kidney.

Presently the only evidence of an abnormality of cystine metabolism in cystinosis lies in the presence of cystine crystals. The free-cystine content of leukocyte preparations of cystinotic children was found to be 80 times greater than normal, even though the white cells were assayed free of crystals (73). Leukocytes from parents contained six times the normal cystine content, providing the first biochemical identification of the heterozygote. Following subcellular fractionation of cystinotic leucocytes, three-quarters of the intracellular cystine was found in the granular (acid phosphatase-rich) fraction.

Schneider et al (14) found that skin fibroblasts maintained in culture from individuals who were homozygous and heterozygous for cystinosis could be distinguished from normal on the basis of their free cystine content. Their study provided evidence that cystine is compartmentalized in a subcellular location in cystinotic cells, the basis of

the present most widely accepted theory of cystinosis. It was agreed that the very growth of cystinotic fibroblasts in the presence of more than 100 times the usual content of free-cystine was proof that the accumulated cystine was not freely dispersed throughout the cell; otherwise it would have inhibited enzymes requiring free sulphydryl groups for activity (74). As in cystinotic leukocytes, most of the intracellular cystine of cystinotic fibroblasts was found in the "granular" fraction of the cell.

Cystinotic cells contain large amounts of free cystine in spite of normal activities of the soluble enzyme system involved in its further reductive metabolism. An explanation for this would be an intracellular compartmentalization of cystine in such an area making it inaccessible to these enzymes.

Compartmentalization of cystine appears to be a major symptom of cystinosis, rather than the result of cystine crystallization within the cytoplasm. Evidence has been gathered depicting the lysosome as the site of cystine accumulation. Electron microscopic examination of a cystinotic lymph node (75) showed crystals located within membrane bound organelles. These organelles had the appearance of lysosomes and contained the enzyme acid phosphatase, a lysosomal marker. Hummeler and coworkers (1970) (76) have also shown that rectal biopsies from cystinotic patients contained crystals which appeared to be intralysosomal in lamina propria cells. Lysosomal location of cystine was also found in cystinotic conjunctiva (77), cornea (78), kidney (79) and lymphocytes (76).

Due to the question of the specificity of acid phosphatase as a lysosomal marker, confirmation by a different method was needed to show that the crystalline outlines were within lysosomes. Cystinotic conjunctival cells were incubated in vitro in medium containing added ferritin, an electron-dense protein which via phagocytosis enters lysosomes. A piece of conjunctiva from the same biopsy was incubated in the absence of ferritin as a control. Electron microscopy revealed ferritin particles concentrated at the same intracellular sites where crystalline images were seen, confirming lysosomal cystine crystal storage (77).

Experiments on human cells (49, 80) suggest that the lysosomal membrane is relatively impermeable to nonmetabolized peptides and amino acids with a molecular weight greater than 200 or so. It would follow that normal lysosomes would have a mechanism which permitted the efflux or metabolism of cystine with molecular weight 240 and that in cystinosis the function may be impaired.

Thus, there is a reasonable body of evidence to indicate that cystine crystals are compartmentalized within the lysosomes of both the nephropathic and benign forms of the disease. It is suggested, therefore, that cystinosis be considered a lysosomal storage disease, although differing from other such disorders in the small molecular size of its stored substance cystine.

New insight into the problem behind cystinosis has caused us to take a closer look at the transsulfuration pathway, in which the sulfur atom of methionine is converted into the sulfur atom of cysteine.

As previously mentioned, homocysteine lies at an important metabolic branch point. It may be either converted to cystathionine or be

remethylated to form methionine, completing the sulfur conservation cycle. Although the condensation of homocysteine and serine catalyzed by cystathionine β -synthase can be reversed if homocysteine is rapidly removed (81), under physiologic conditions the equilibrium favors cystathionine synthesis. The formation of cystathionine also serves to remove sulfur from the pathway.

In the cleavage of cystathionine, it appears that γ -cystathionase normally functions almost entirely in the direction of the formation of cysteine. Thus γ -cystathionase, in its active form will cleave cystathionine, draining it off from the l-homocysteine-methionine cycle. Inhibition of γ -cystathionase would allow for the branch point to favor remethylation of homocysteine to form methionine.

We will show that l-cystine functions as an effective inhibitor of the enzyme γ -cystathionase. Thus a feedback inhibition cycle is established. Cystathionase generates cysteine; it is converted to cystine; intracellular levels of cystine regulate the activity of cystathionase.

The metabolic basis of cystinosis may reside in a defect in this autoregulatory mechanism, such that the enzyme is no longer subject to inhibition by cystine. The regulatory inhibition of enzyme activity no longer takes place. Cystathionase would remain in the active form, l-homocysteine would continuously be condensed with serine to form cystathionine, feeding into overproduction of cyst(e)ine.

EXPERIMENTAL

Over 30 years ago, it was observed that an extract of rat liver catalyzed the cleavage of cystathionine, producing cysteine and α -ketobutyric acid (82). At the same time, it was also demonstrated that the same extract decomposed homoserine to yield the same keto-acid. Later, a considerable purification from rat liver was achieved which catalyzed homoserine oxidative deamination.

Matuso and Greenberg (41) purified an enzyme (γ -cystathionase) isolated from rat liver which catalyzed both the deamination of homoserine and the cleavage of cystathionine. Matsuo and Greenberg showed over many stages of purification that the relative activity of the preparation for cleavage of cystathionine and homoserine deamination stayed constant, and it is universally accepted that a single enzyme catalyzes both reactions. It is quite likely that homoserine is an intermediate in the cystathionine cleavage reaction, but it has not been established with certainty. The enzyme is found in several organs, and liver, particularly that of rats, had the greatest activity. For this reason, rat liver cystathionase was used as a prototype for our experiments.

I. Chemicals and Enzymes

L-homoserine (A grade, M.W. 119.1), l-cystine (A grade, M.W. 240.3) and l-cystathionine (A grade, M. D. 222.3) were obtained from Calbiochem-Behring Corporation. All other chemicals were purchased from chemical sources. Rat liver samples (Sprague-Dawley Derived) containing cystathionase, were obtained from within the Department of Pharmacology, USUHS. Monkey liver cystathionase (African green) was a

gift of Dr. James H. Vickers, Pathobiology and Primatology Branch, Bureau of Biologics, FDA. Samples of normal human liver and spleen were donated by Dr. Michael Smith, Department of Pathology, USUHS. Normal human liver was donated by Dr. John Martin, Laboratory of Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke, NIH. Cystinotic spleen was obtained from Dr. Joseph Schulman, Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development, NIH.

II. Preparation of Crude Enzyme Homogenate

The sample was weighed and then homogenized in twice its weight in volume of 0.2 M KPO_4 buffer. The homogenate was centrifuged at 12,000 RPM for 60 minutes. After centrifugation, the scum was aspirated off the top and the supernatant was saved. The pellet was resuspended in one-half the original weight in volume of 0.2 M KPO_4 buffer, and centrifuged for an additional half hour. The supernatant was collected and recombined with the original supernatant. Pyridoxal phosphate was added to a final concentration of 1×10^{-4} M in order to ensure enzyme activity. Without this addition, it was found that activity of the enzyme was lost. The homogenate was kept on ice at all times.

III. Partial Purification of Crude Enzyme Homogenate

Controlled Heat Denaturation--The crude homogenate was transferred into a 250 ml. beaker, equipped with a thermometer, and was agitated in a water bath until the temperature of the homogenate reached 60° . This temperature was maintained for 5 minutes. The homogenate was centrifuged at 12,000 RPM for one hour. The supernatant was saved and the pellet was discarded.

Ammonium sulfate fractionation—This was carried out according to the method outlined by Iglehart et al (83). Basically, the homogenate was carried through from 0 to 50% NH_4SO_4 saturation by slowly adding the salt over a period of one hour with constant stirring. Stirring was continued for an additional three hours at 0° , and the precipitate discarded after centrifugation. The solution was then brought to 75% NH_4SO_4 saturation in a similar manner and the precipitate collected and dissolved in buffer containing a final concentration of $1 \times 10^{-4} \text{ M}$ pyridoxal phosphate.

IV. Protein

Protein determinations were carried out according to the method of Lowry as modified by Shatkin (84) using bovine serum albumin (Sigma Chemical Company) as a standard. Samples were prepared for protein assay by serial dilutions of 1:10 and 1:100 with distilled water.

V. Enzyme Assay

Homoserine deaminase-cystathionase activity was assayed. One-tenth ml of enzyme preparation was incubated at 37°C in a 1.0 ml reaction mixture containing 0.1 ml of each of the following: 0.1 M potassium phosphate buffer pH 8.0, 10^{-4} M pyridoxal phosphate and 10^{-2} M dithiothreitol. Final substrate concentrations of added l-homoserine, l-cystine and l-cystathionine ranged from $1 \times 10^{-2} \text{ M}$ to $5 \times 10^{-2} \text{ M}$. L-cystine (2×10^{-5} , $5 \times 10^{-5} \text{ M}$ final concentrations) was added where indicated in inhibition studies. In order to keep pH optimal for the enzyme (pH 7.8-8.0) the pH of the amino acid solutions l-cystine and l-cystathionine, when in substrate concentrations, was adjusted with 1 N KOH to $\text{pH} \approx 7.8$

before adding to the assay system. Inhibitor concentrations of l-cystine were dilute enough in that they did not affect the optimal pH of the system. The final volume was brought to 1 ml with the addition of glass distilled water. The incubation was carried out in test tubes of about 5 ml capacity, at 37°C for 30 minutes. At the end of the incubation, the reaction was terminated with the addition of 1 ml of 12% TCA. The tubes were left in an ice bath for 10 minutes. They were then centrifuged at 3500 RPM for 10 minutes at 4°C, for the removal of the protein precipitate.

Enzyme activity was determined by the formation of α -ketobutyric acid, using a modification of the techniques of Friedemann and Haugen (85) and Sayre and Greenberg (86). One ml aliquots of the supernatant solution were assayed for α -ketobutyric acid. To the aliquot, 0.5 ml 2,4 dinitrophenylhydrazine 1mg/ml (Eastman #1866) was added, using 12 ml stoppered centrifuge tubes. The tubes were agitated and then incubated at room temperature for 5 minutes. After 5 minutes, 1 ml ethyl alcohol (absolute) and 8 ml of ethylacetate were added. The tubes were stoppered and shaken vigorously for 2 minutes, and then centrifuged at 3,500 RPM for 4 minutes to ensure separation of the aqueous and organic phases. Using a pipette, 7 ml of the ethylacetate phase (upper layer) were transferred to another stoppered centrifuge tube containing 4.0 ml 10% sodium carbonate. These tubes were shaken and centrifuged as before. Following centrifugation, the upper phase was removed by aspirating with a Pasteur pipette. Three ml of the sodium carbonate phase were transferred to a test tube containing 2 ml of 2.5 N sodium hydroxide. The tubes were inverted to ensure mixing and were then read on a Gilford spectrophotometer at 425 m μ .

The α -ketobutyric acid standard curve was set up as follows: In duplicate, α -ketobutyric acid (0.01 ml - 0.1 ml of a 0.01M solution) was placed in stoppered centrifuge tubes, with the addition of 6% TCA to make a total volume of 1ml with final concentrations of γ -ketobutyric acid ranging from 0.1 to 1.0 μ moles. At this point, the extraction procedure was carried out as outlined above, reading the tubes at 425 m μ .

RESULTS

The α -ketobutyric acid standard curve (Fig. 4) was satisfactorily linear over the range of 0.1 to 1.0 μmol . Control experiments (not shown) showed that incubating α -ketobutyric acid for 30' at 37° with or without the enzyme preparations did not lead to α -ketobutyric acid loss. Freezing the enzyme samples over a period of weeks had no obvious effect on enzyme activity.

Rat homogenate had substantial enzyme activity using l-homoserine as a substrate, so the homogenate was therefore diluted 1:10 with the phosphate buffer at the onset of the experiment. Since there was good activity in the rat samples, it was not necessary to carry them through partial purification by NH_4SO_4 fractionation as was done with the monkey and human samples. During the ammonium sulfate fractionation purification process it was necessary to resuspend the final precipitate in buffer containing pyridoxal phosphate ($1 \times 10^{-4}\text{M}$). We found that unless pyridoxal phosphate was added, enzyme activity would rapidly decrease, most likely due to the fact that cystathionase is a pyridoxal phosphate bound enzyme. Due to the high cost of l-cystathionine, l-homoserine was used as the substrate. Since homoserine was employed as the usual substrate, we confirmed in a single experiment that with l-cystathionine as the substrate, cystathionase activity was also inhibited upon the addition of l-cystine. Enzyme activity increased in a linear fashion for at least one hour (Fig. 5).

Appropriate controls were included in each enzyme assay, with tubes containing reagents only, substrate but no enzyme, enzyme but no substrate, complete system and complete system plus inhibitor (l-cystine). Cystine was tested at two concentrations. The data was analyzed

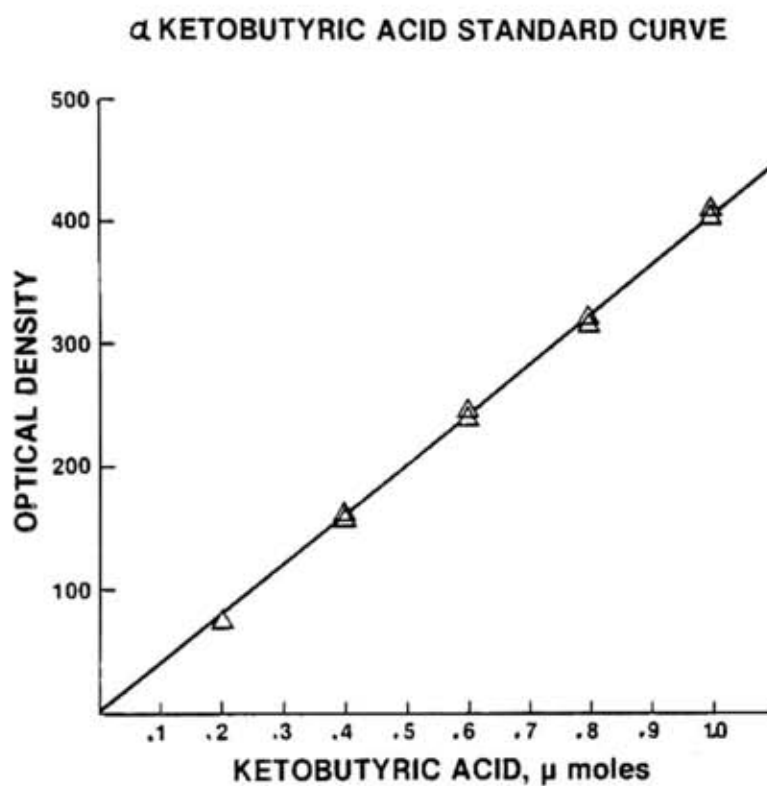


FIGURE 4 Incubation of α -ketobutyric acid over the concentration range of 0.1 to 1.0 μ moles for 30' at 37°.

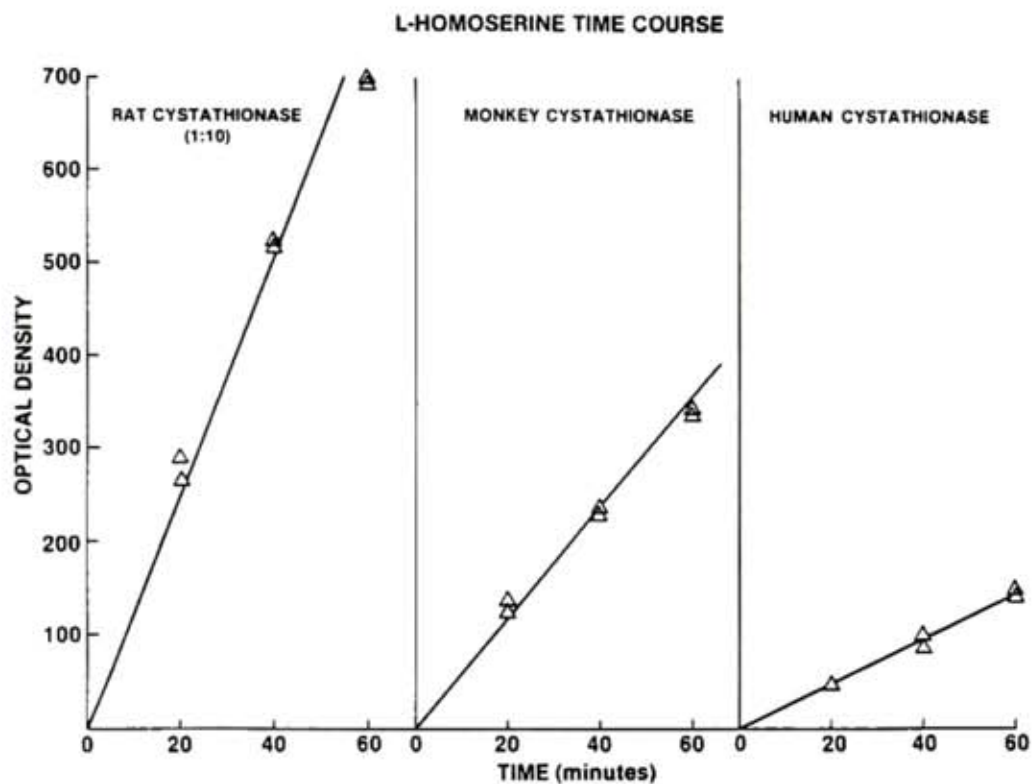


FIGURE 5 Incubation of the enzyme, γ -cystathionase, for each of the species at 37° over a period of 1 hour. The l-homoserine substrate concentration employed was $2 \times 10^{-2}M$.

by construction of Lineweaver-Burke plots (Figs. 6,7,8) and in each of the species, enzyme activity was inhibited by the two l-cystine concentrations employed ($2 \times 10^{-5} \text{M}$, $5 \times 10^{-5} \text{M}$) as shown by the decreased optical densities read at 425 mμ. Data obtained from a representative experiment generated by the normal human liver homogenate is given in Table I. Assuming simple first order kinetics as delineated by Matsuo and Greenberg (40) in their pilot studies, inhibition by l-cystine was competitive. The K_m calculated for each of the species was comparable to those reported by others (Table II).

In a previous experiment using rat liver as the enzyme, the ability of l-cysteine ($1 \times 10^{-3} \text{M}$ final concentration) to inhibit cystathionase was investigated. Enzyme activity was calculated (Table III) before and after the addition of cysteine. It was found that cysteine, in addition to cystine was inhibitory to cystathionase.

It was possible to obtain normal and cystinotic spleen. These samples were subjected to the same purification procedures and experimental conditions as the liver samples, using dl-homoserine and l-homoserine as the substrates. Only one inhibiting concentration of l-cystine was employed, $5 \times 10^{-5} \text{M}$. Protein determinations for normal and cystinotic spleen gave values of 6.84 mg protein/ml and 6.51 mg protein/ml respectively. The enzyme activities were determined for both normal and cystinotic samples before and after the addition of the inhibiting concentration of cystine as shown in Table IV. It was found that while enzyme activity from normal spleen was inhibited with the addition of cystine, cystinotic spleen showed little to no inhibition when incubated with cystine. This series of experiments provided some

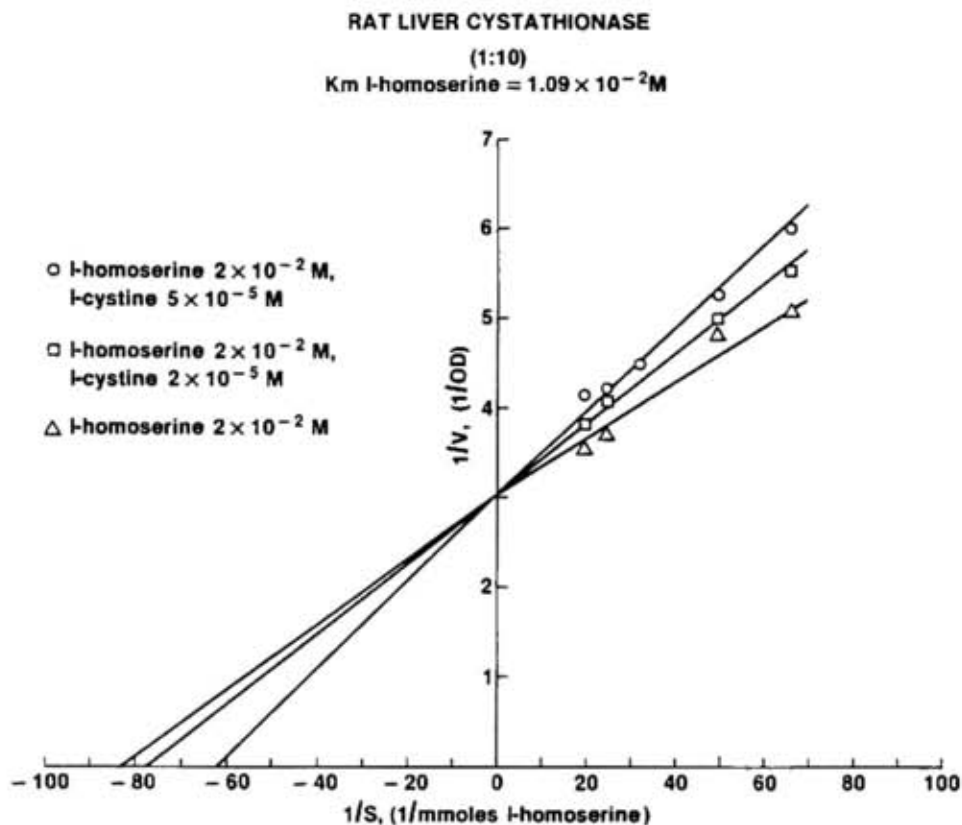


FIGURE 6 Inhibition of Rat Liver Cystathionase by L-Cystine

The enzyme was incubated for 30' at 37° without the inhibitor (L-homoserine only) and with $2 \times 10^{-5} M$ and $5 \times 10^{-5} M$ L-cystine. Rat homogenates were diluted 1:10 with phosphate buffer (pH 7.8) due to high activity of the enzyme.

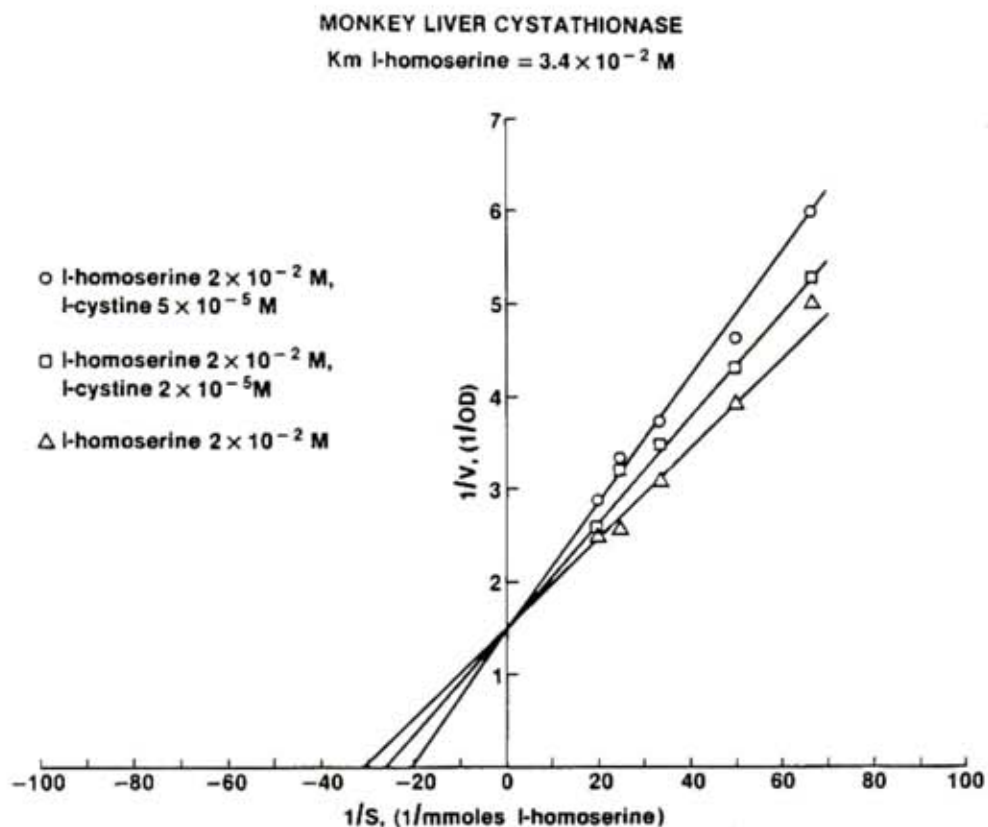


FIGURE 7 Inhibition of Monkey Liver Cystathionase by L-Cystine

Incubation of the enzyme was for 30' at 37° containing substrate only (l-homoserine) and substrate plus inhibitor (l-homoserine and l-cystine, 2×10^{-5} M or 5×10^{-5} M).

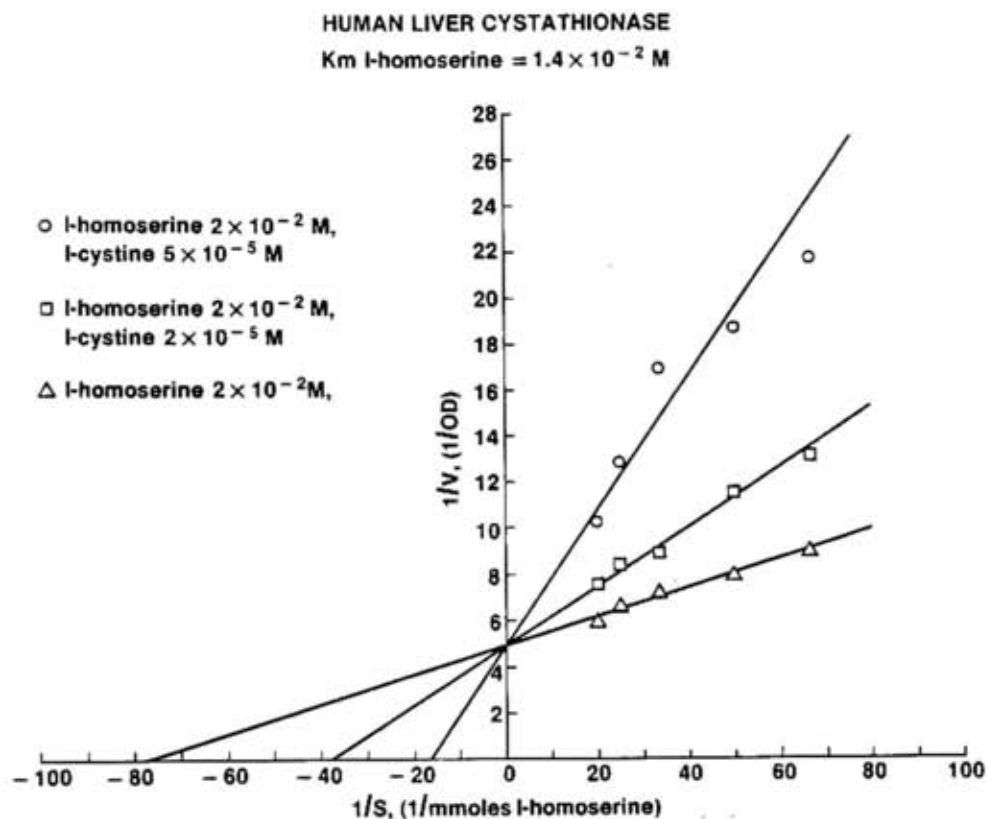


FIGURE 8 Inhibition of Human Liver Cystathionase by L-Cystine

Human cystathionase was incubated with the substrate l-homoserine and with the inhibitor l-cystine (2×10^{-5} M, 5×10^{-5} M) in addition to the substrate. Incubation was for 30' at 37°.

l-homoserine (mmole/ml)	l-cystine (mmole/ml)	Optical Density	Percent Inhibition (Average)
15	--	.104, .107	--
20	--	.108, .115	--
30	--	.133, .131	--
40	--	.150, .148	--
50	--	.156, .161	--
15	0.02	.080, .078	25
20	0.02	.093, .091	18
30	0.02	.102, .104	22
40	0.02	.114, .109	25
50	0.02	.126, .126	21
15	0.05	.056, .055	47
20	0.05	.063, .064	44
30	0.05	.076, .073	44
40	0.05	.095, .093	37
50	0.05	.116, .108	30

TABLE I Normal Human Liver Homogenate; γ -Cystathionase Activity and Inhibition by L-Cystine

Data obtained from the Gilford spectrophotometer read at 425 m μ . In duplicate, activity of the enzyme was assayed by formation of α -ketobutyric acid following a 30' incubation at 37°, with and without the inhibitor, l-cystine.

**COMPARISON OF K_m VALUES BETWEEN CYSTATHIONASES OF
VARIOUS SPECIES USING L-HOMOSERINE AS A SUBSTRATE**

SPECIE	K_m (mM)	SOURCE
Rat	20	Matsuo and Greenberg, 1957
	30	Kato et al, 1966
	20	Yao et al, 1979
	11	Experimental
Cat	100	Yao et al, 1979
Monkey	34	Experimental
Human	29	Experimental

TABLE II

l-homoserine (mmole/ml)	Enzyme Activity (OD units/mg protein)		Percent Inhibition
	Substrate Only	Substrate Plus Inhibitor	
10	0.091	0.044	52
20	0.115	0.074	36
40	0.130	0.114	12

TABLE III Ability of L-Cysteine to Inhibit Rat Liver Cystathionase

Experimental conditions consisted of a 30' incubation at 37° using 1.0 ml of the enzyme preparation, l-homoserine concentrations as shown, and an inhibitor concentration of l-cysteine of 1×10^{-3} M. Enzyme activities calculated before (substrate only) and after (substrate plus inhibitor) addition of l-cysteine show a loss of activity with the inhibitor, l-cysteine. Protein content for the rat liver was determined to be 29.6 mg protein/ml.

Enzyme	Enzyme Activity (OD units/mg protein)		
	Substrate Only	Substrate Plus Inhibitor	Percent Inhibition
Normal spleen cystathionase	0.012*	0.002*	83*
	0.021*	0.013*	38*
	0.018	0.007	61
	0.022	0.013	41
	0.021	0.011	48
Cystinotic spleen cystathionase	0.030*	0.028*	7*
	0.028*	0.021*	25*
	0.023	0.020	13
	0.037	0.028	24
	0.011	0.011	0

TABLE IV Effect of L-Cystine on Normal and Cystinotic Spleen Cystathionase

Experimental conditions consisted of a 30' incubation at 37° using 10 ml of the spleen homogenate, with l-homoserine as the substrate except where indicated by an (*) in which case dl-homoserine was the substrate, and a cystine concentration of 5×10^{-5} M in inhibition studies. Enzyme activities for normal spleen calculated after l-cystine addition show a loss of enzyme activity when compared to substrate only (homoserine 2×10^{-2} M, no cystine) values. Cystinotic spleen samples showed little to no loss of enzyme activity upon addition of cystine. Protein determinations gave values of 6.89 mg protein/ml and 6.51 mg protein/ml for normal and cystinotic spleen samples, respectively.

basis on which to propose our hypothesis on the metabolic basis of cystinosis. However, the specific activities of the enzyme from these samples was very low, and the experimental results cannot be used to definitively validate the hypothesis.

DISCUSSION

We have shown that γ -cystathionase prepared from normal rat, monkey, and human liver is markedly inhibited by l-cystine. The inhibition is of a competitive type (Figs. 6,7,8, Table V). In addition to l-cystine, we found l-cysteine to be an inhibitor of γ -cystathionase as well (Table II). However, it was noted that inhibition of the enzyme by cysteine necessitated at least a 100-fold higher concentration than that of cystine.

These results suggest that γ -cystathionase may be a key enzyme in the l-homocysteine-methionine pathway, although it is not at a branch point itself (Fig. 1). In normal individuals, the enzymatic mechanism regulating cystine accumulation appears to be increased intracellular cystine content. With increasing levels of intracellular cystine, cystathionase through feedback inhibition becomes inactive, conversion of homocysteine to cystathionine reaches equilibrium, and the pathway then favors the formation of methionine.

In cystinotic individuals, it is theorized that the metabolic defect lies within cystathionase itself. Under normal conditions, as the cystine concentration increases, cystathionase is inhibited. However, the cystathionase in patients with cystinosis is postulated to remain active and there is a resulting over-accumulation of cystine in the cell. We therefore postulate that the genetic basis of cystinosis is a variant γ -cystathionase enzyme protein, such that the enzyme is not inhibited by concentrations of cystine that inhibit the normal enzyme. Due to the rarity of the disease itself, obtaining cystinotic liver samples has not yet been possible. Thus, at this time, we have

% CYSTINE INHIBITION UNDER STD. CONDITIONS *

ENZYME	% INHIBITION
RAT LIVER CYSTATHIONASE	16.9 ± 0.8
MONKEY LIVER CYSTATHIONASE	21.0 ± 2.5
HUMAN LIVER CYSTATHIONASE	48.2 ± 2.8

* STD. CONDITIONS: 30' INCUBATION, $\bar{x}$ FINAL CONCENTRATIONS:

L-HOMOSERINE, 2×10^{-2} M

L-CYSTINE, 5×10^{-5} M

USING STD. ASSAY PROCEDURES

TABLE V

not been able to study the enzyme in liver samples of affected individuals. There is some experimental data with spleen samples, however. Enzyme activity in spleen is low, but detectable (Table IV). In normal human spleen, cystathionase is inhibited by l-cystine, as expected. Cystinotic spleen homogenates had low activity, but the degree of inhibition by l-cystine was very low. The data is too scanty to prove the hypothesis, but seems to indicate an enzyme with altered properties. At some future time, it is hoped that experimental evidence will confirm our hypothesis concerning the metabolic basis of cystinosis.

REFERENCES

1. Schulman J.D. Cystinosis: a diagnostic problem. *Ped. Ann.* 8:462-3, 1979.
2. Schulman J.D., moderator. Genetic disorders of glutathione and sulfur amino-acid metabolism. *Ann. Inter. Med.* 93:330-46, 1980.
3. Schneider J.A., Rosenblum F.M., Bradley K.H. and Seegmiller J.E. Increased free cystine content of fibroblasts cultured from patients with cystinosis. *Biochem. Biophys. Comm.* 29:527-31, 1967.
4. Schulman J.D., Bradley K.H. and Seegmiller J.E. Cystine: compartmentalization with lysosomes in cystinotic leucocytes. *Science.* 166:1152-4, 1969.
5. Abderhalden E. Familiäre cystindiatheese. *Z. Physiol. Chem.* 38: 557-61, 1903.
6. Lignac G.O.E. Über störung des cystinostoffwechsels bei kindern. In: Stanbury J.B., Wyngaarden J.B. and Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The McGraw-Hill Book Company; 1974:1581.
7. Fanconi G. Dienicht diabetischen glykosurien und hyperglykämien des altern Kindes. In: Stanbury J.B., Wyngaarden J.B. and Frederickson D.S., eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The McGraw Hill Book Company; 1974:1581.
8. de Toni G. Remarks on the relations between renal rickets (renal dwarfism) and renal diabetes. In: Stanbury J.Wyngaarden J.B., Frederickson D.S., eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The McGraw-Hill Book Company; 1974:1581.

9. Debré R., Marie J., Clézet F. and Messimy R. Rachitisme tardif coexistent avec une nephrite chronique et une glycosurie. In: Stanbury J.B., Wyngaarden J.B. and Fredrickson D.S. eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The McGraw-Hill Book Company; 1974:1581.
10. Fanconi G. Der fruhinfantile nephrotisch-glykosurische zwergwuchs mit hypophosphatamischer rachitis. In: Stanbury J.B., Wyngaarden J.B. and Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The Mc-Graw Hill Book Company; 1974: 1581.
11. Beumer H. and Wepler W. Über die cystinkrankheit der ersten Lebenszeit. In: Stanbury J.B., Wyngaarden J.B. and Frederickson D.S., eds. The Metabolic Basis of Inherited Disease. 3rd ed. New York: The McGraw-Hill Book Company; 1974:1581.
12. Fanconi G. Weitere beiträge zur cystinkrankheit (der amindiabetes). In: Schulman J.D., ed. Cystinosis. DHEW publication (NIH); 1973: 1-3.
13. Bickel H., Baar H.S., Astley R., Douglass A.A., Finch E., Harris H. Harvey C.C., Hickmans E.M., Philpott M.G., Smallwood W.C., Smellie J.M. and Teal C.G. Cystine storage disease with aminoaciduria and dwarfism (Lignac-Fanconi disease). Acta. Paediat. 42 (Suppl. 90): 1, 1952.
14. Schneider J.A., Rosenbloom F.M., Bradley K.H. and Seegmiller J.E. Increased free-cystine content of fibroblasts cultured from patients with cystinosis. Biochem. Biophys. Res. Comm. 29:527-31, 1967.

15. Schulman J.D., Fujimoto W.Y., Bradley K.H. and Seegmiller J.E.
Identification of heterozygous genotype for cystinosis in utero
using a new pulse labeling technique. J. Pediat. 77:468-70, 1970.
16. Schulman J.D., Wong V.G., Bradley K.H. and Seegmiller J.E. A
simple technique for the biochemical diagnosis of cystinosis.
J. Pediat. 76:289-92, 1970.
17. Bickel H. Entwicklung der biochemischen Läsion bei der Lignac-
Fanconischen Krankheit. In: Schulman J.D., ed. Cystinosis.
DHEW Publication (NIH); 1973:13.
18. Harrison H.E. The Fanconi syndrome. J. Chronic Dis. 74:346-55.
19. Bickel H. Proximal tubular defects. In: Black D.A.K., ed. Renal
Disease. Oxford: Blackwell Scientific Publications; 1962:347.
20. Schneider J.A., Wong V., Bradley K.H. and Seegmiller J.E. Bio-
chemical comparisons of the adult and childhood forms of cystinosis.
New Engl. J. Med. 279:1253-7, 1968.
21. Goldman H., Seriver C., Aaron K., Delvin K. and Canlas Z. Adoles-
cent cystinosis: comparisons with infantile and adult forms.
Pediatrics. 47:979-88, 1971.
22. Cogan D.G., Kuwabara T., Kinoshita J., Sheehan L. and Merola L.
Cystinosis in an adult. J.A.M.A. 164:394-6, 1957.
23. Lietman P.S., Frazier P.D., Wong V.G., Shotton D. and Seegmiller
J.E. Adult cystinosis - a benign disorder. Am. J. Med. 40:511-
17, 1966.
24. Stanbury J.B., Wyngaarden J.B. and Fredrickson D.S., eds. The
Metabolic Basis of Inherited Disease. 3rd ed. New York: McGraw-
Hill Book Company; 1974:1585.

25. Bürki E. Über die Cystinkrankheit in Kleinkindersalter unter besonderer Berücksichtigung des Augenbefundes. In: Schulman J.D., ed. Cystinosis. DHEW Publication (NIH); 1973:23.
26. Wong V.G., Lietman P.S. and Seegmiller J.E. Alterations of pigment epithelium in cystinosis. Arch. Path. 77:361-9, 1967.
27. Baar H.S. and Bickel H. Part 8: Morbid anatomy, histology and pathogenesis of Lignac-Fanconi disease (cystine storage disease with aminoaciduria) In: Schulman J.D., ed. Cystinosis. DHEW Publication (NIH); 1973:37.
28. Clay R.D., Darmady E.M. and Hawkins M. The nature of the renal lesion in Fanconi syndrome. J. Pathol. and Bacteriol. 65:551-8, 1953.
29. Teree T.M., Friedman A.B., Kest L.M. and Fetterman G.H. Cystinosis and proximal tubular nephropathy in siblings: Progressive development of the physiological and anatomical lesion. Am. J. Dis. Child. 117:481-7, 1970.
30. Jackson J.D., Smith F.G., Litman N.N., Yile C.L. and LaHa H. The Fanconi syndrome with cystinosis. Electron microscopy of renal biopsy specimens from five patients. Amer. J. Med. 33:893-910, 1962.
31. Zimmerman T.J., Hood C.I. and Gasset A.R. "Adolescent" cystinosis: A case presentation and review of the recent literatures. In: Stanbury J.D., Wyngaarden J.D., Frederickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: McGraw-Hill Book Company; 1978:1665.
32. Schulman J.D., ed. Cystinosis. DHEW publication (NIH); 1973:67-8.

33. Brenton D.P., Cusworth D.C., Dent C.E. and Jones E.E. Homocystinuria, clinical and dietary studies. *Quart. J. Med.* 35:325-46, 1966.
34. Eagle H., Washington C., and Friedman S.M. The synthesis of homocystine cystathionine, and cystine by cultured diploid and heteroploid human cells. *Proc. Natl. Acad. Sci. U.S.* 56:156-63, 1966.
35. Laster L., Mudd S.H., Finkelstein J.D. and Irreverre F. Homocystinuria due to cystathionine synthase deficiency. *J. Clin. Invest.* 44:1708-19, 1965.
36. Mudd S.H., Klee W.A. and Ross P.D. Enthalpy changes accompanying the transfer of a methyl group from S-adenosylmethionine and other sulfonium compounds to homocysteine. *Biochemistry.* 5:1653-60, 1966.
37. Shapiro S.K. and Yphantis D.A. Assay of S-methyl methionine and S-adenosyl methionine homocysteine transmethylases. *Biochim. Biophys. Acta.* 36:241-4, 1959.
38. Stanbury J.D., Wyngaarden J.D. and Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: McGraw-Hill Book Company; 1978:459.
39. Kashiwamata S., Kotakey and Greenberg D.M. Studies of cystathionine synthase of rat liver: Dissociation into two components by sodium dodecyl sulfate disc electrophoresis. *Biochim. Biophysics. Res. Comm. Acta.* 212:501-3, 1970.
40. Tudball N. and Reed M.A. Purification and properties of cystathionine synthase of human liver. *Biochem. Biophys. Res. Comm.* 67: 550-5, 1975.

41. Matsuo Y. and Greenberg D.M. A crystalline enzyme that cleaves homoserine and cystathionine: I. Isolation procedure and some physiochemical properties. *J. Biol. Chem.* 230:545-60, 1958.
42. Sturman J.A., Beratis L.G. and Gaull G.E. Transsulfuration by human long term lymphoid lines. *J. Biol. Chem.* 255:4763-5, 1980.
43. Segal S. Cystinosis and its therapy. *N. Eng. Jour. Med.* 300: 789-90, 1979.
44. Worthen H.G. and Good R.A. The de Toni - Fanconi syndrome with cystinosis. *Amer. J. Dis. Child.* 95:653-88, 1958.
45. Callis L., Castello F., Fortuny G., Vallo A. and Ballabriga A. In: Schulman J.D., ed. Cystinosis. DHEW publication (NIH); 1973:20-1.
46. Bickel H., Lutz P. and Schmidt H. The treatment of cystinosis with diet or drugs. In: Schulman J.D., ed. Cystinosis. DHEW publication (NIH); 1973:199.
47. Stanbury J.B., Wyngaarden J.B. and Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: The McGraw-Hill Book Company; 1978:1675.
48. van Creveld S. and Grunbaum A. Renal rickets and cystinuria. *Acta Paediat.* 29:183-210, 1942.
49. Schulman J.D. and Bradley K.H. Cystinosis: Selective induction of vacuolation in fibroblasts by L-cysteine-D-penicillamine disulfide. *Science*. 169:595-7, 1970.
50. Schmidt H., Lutz P. and Bickel H. Werbeurteilung der diätetischen Behandlung der Cystinose. In: Stanbury J.B., Wyngaarden J.B. and Frederickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: McGraw-Hill Book Company; 1978:1677.

51. Clayton B.F. and Patrick A.D. Use of dimercaprol or penicillamine in the treatment of cystinosis. *Lancet*. 2:909-10, 1961.
52. Seegmiller J.E., Friedman T., Harrison H.E., Wong V. and Schneider J.A. Cystinosis. *Ann. Intern. Med.* 69:883-905, 1968.
53. Crawhall J.C. and Segal S. The intracellular cysteine/cystine ratio in kidney cortex. *Biochem. J.* 99:19C-20C, 1966.
54. Crawhall J.C., Thompson C.J. and Bradley K.H. Separation of cystine, penicillamine disulphide, and cysteine-penicillamine mixed disulphide by automatic amino acid analysis. *Anal. Biochem.* 14: 405-13, 1966.
55. Goldman H., Scriver C.R., Aaron K. and Pinsky L. Use of dithiothreitol to correct cystine storage in cultured cystinotic fibroblasts. *Lancet* 1:811-2, 1970.
56. DePape-Brigger D., Goldman M., Scriver C.R., et al. The in vivo use of dithiothreitol in cystinosis. *Pediatric Res.* 11:124-31, 1977.
57. Schneider J.A., Verroust F.M., Kroll W.A., et al. Prenatal diagnosis of cystinosis. *New Engl. J. Med.* 290:878-82, 1974.
58. Kroll W.A. and Schneider J.A. Decrease in free cystine content of cultured cystinotic fibroblasts by ascorbic acid. *Science*. 186: 1040-2, 1974.
59. Schneider J.A., Schlesselman J.J., Mendoza S.A., Orloff S. Thoene J.G., Kroll W.A., Godfrey A.D. and Schulman J.D. Ineffectiveness of ascorbic acid therapy in nephropathic cystinosis. *New Engl. J. Med.* 300:756-9, 1979.

60. Thoene J.G., Crawhall J., Oshima R.G., Rhead W. and Schneider J.A. Cystine depletion of cystinotic fibroblasts by cysteamine (Mercaptoethylamine, MEA), *Pediatr. Res.* 9:356, 1975.
61. Thoene J.G., Oshima R.G., Crawhall J.C. and Schneider J.A. Cystine depletion of cystinotic cells by aminothiols. *Proc. Soc. Med.* 70 (Suppl. 3):37, 1977.
62. Schulman, J.D. Approaches to the treatment of inborn errors of sulphur amino acid and peptide metabolism. In: The Management of Genetic Disorders. New York: Alan R. Liss, Inc.; 1979;206-8.
63. Girardin E.P., DeWolfe M.S. and Crocker J.F.S. Treatment of cystinosis with cysteamine. *J. Pediat.* 94:838-40, 1979.
64. Thoene J.G. and Lemons R. Cystine depletion of cystinotic tissues by phosphocysteamine (WR 638). *J. Pediat.* 96:1043-4, 1980.
65. Thoene J.G. and Lemons R. Modulation of the intracellular cystine content of cystinotic fibroblasts by extracellular albumin. *Pediatr. Res.* 14:785-7, 1980.
66. Broyer M., Kleinknecht C, Loirat C., Marti-Henneberg C. and Roy M.P. Growth in children treated with long-term hemodialysis. *J. Pediatr.* 84:642-9, 1974.
67. Barr H.S. Pathologic des Anmosäuren-Diabetes. In; Stanbury J.B., Wyngaarden J.B., Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: McGraw-Hill Book Company; 1978:1670.
68. Schneider J.A. and Seegmiller J.E. Unpublished observation. In: Stanbury J.B., Wyngaarden J.B., Fredrickson D.S., eds. The Metabolic Basis of Inherited Disease. 4th ed. New York: McGraw-Hill Book Company; 1978:1669.

69. Brigham M.P., Stein W.H. and Moore S. The concentrations of cysteine and cystine in human blood plasma. *J. Clin. Invest.* 39: 1633-8, 1960.
70. Worthen H.G. and Good R.A. Pathogenesis of cystinosis. *Amer. J. Dis. Child.* 102:494-5, 1961.
71. Seegmiller J.E. and Howell R.R. Cystine metabolism in de Toni-Fanconi Syndrome with cystinosis. *Clin. Res.* 9:189, 1961.
72. Patrick, A.D. The degradative metabolism of l-cysteine and l-cystine in vitro by liver in cystinosis. *Biochem. J.* 83:248-56, 1962.
73. Schneider J.A., Bradley K.H. and Seegmiller J.E. Increased cystine in leukocytes from individuals homozygous and heterozygous for cystinosis. *Science.* 157:1321-2, 1967.
74. Patrick A.D. Deficiencies of SH-dependent enzymes in cystinosis. *Clin. Sci.* 28:427-43, 1965.
75. Patrick A.D. and Lake B.D. Cystinosis: Electron microscopic evidence of lysosomal storage of cystine in lymph node. *J. Clin. Pathol.* 21:571-5, 1968.
76. Hummeler K., Zajac B.A., Genel M., Holtzapple P.G. and Segal S. Human cystinosis: Intracellular deposition of cystine. *Science.* 168:859-60, 1970.
77. Schulman J.D., Bradley K.H. and Seegmiller J.E. Lysosomal sites of crystalline deposits in cystinosis as shown by ferritin uptake. *Arch. Pathol.* 90:259-64, 1970.
78. Kenyon K.R. and Sensenbrenner J.A. Electron microscopy of cornea and conjunctiva in childhood cystinosis. *Am. J. Ophthalmol.* 78: 68-76, 1974.

79. Spear G.S. Pathology of the kidney in cystinosis. In: Sommers S.C. Pathology Annual New York: Appleton-Century-Crofts; 1974:81.
80. Schulman J.D. and Bradley K.H. Metabolism of amino acids, peptides, and disulfides in the lysosomes of fibroblasts cultured from normal individuals and those with cystinosis. J. Exp. Med. 132:1090-1104, 1970.
81. Brown F.C. and Gordon P.H. Cystathionine synthase from rat liver: Partial purification and properties. Can. J. Biochem. 49:484-91, 1971.
82. Carroll W.R., Stacy G.W. and du Vigneaud V. α -Ketobutyric acid as a product in the enzymatic cleavage of cystathionine. J. Biol. Chem. 180:375-82, 1949.
83. Iglehart J.D., York R.M., Modest A.P., Lazarus H. and Livingston D.M. Cystine requirement of continuous human lymphoid cell lines of normal and leukemic origin. J. Biol. Chem. 252:7184-91, 1977.
84. Shatkin A.J. Colorimetric reactions for DNA, RNA and protein determinations. In: Habel K. and Salzman N.P., eds. Fundamental Techniques in Virology. New York: Academic Press; 1969:231-7.
85. Friedemann T.E. and Haugen G.E. Pyruvic acid; determination of keto acids in blood and urine. J. Biol. Chem. 147:415-42, 1943.
86. Sayre F.W. and Greenberg D.W. Purification and properties of serine and threonine dehydrases. J. Biol. Chem. 220:787-99, 1956.

SUMMARY

Cystinosis is a rare, recessively inherited disorder. It is characterized by an intralysosomal accumulation of the disulfide amino acid cystine.

In its most severe form (nephropathic), it is present at birth, but is usually not apparent until 6 months to one year of age. Those affected suffer from progressive glomerular damage, with death occurring from uremia within the first decade of life. An intermediate form of the disease is evident, onset usually by the second or third decade of life. The third type is completely benign, only discovered when a routine ophthalmologic examination reveals the characteristic cystine crystals in the cornea and conjunctiva.

In the transsulfuration pathway, the enzyme γ -cystathionase plays a key role in the conversion of cystathionine to cysteine which is oxidized to cystine. Under normal conditions, increased cystine concentration results in inhibition of cystathionase. The result is that homocysteine is remethylated to methionine, rather than shifted to cystine formation. It can be considered that intracellular cystine levels control the two pathways. If cystine is low, its formation is favored. When cystine is high, the cystathionine pathway is shut down and remethylation to methionine is favored.

On the basis of the work done, it is postulated that the metabolic basis of cystinosis is a defect in the regulatory mechanism of the enzyme γ -cystathionase such that the enzyme is no longer inhibited by increased cystine levels.