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CYSTATHIONINE SYNTHASE: A STUDY OF THE ENZYME IN NORMAL AND DEFICIENT HUMAN CELLS

by

LYNN DALE FLEISHER

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1974

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Abstract

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by

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The most extensively studied of the diseases of sulfur amino acid metabolism is homocystinuria due to a deficiency of cystathionine synthase activity, which is inherited in an autosomal recessive manner. An absence of synthase activity in affected individuals has been demonstrated in liver and brain, and more recently in cultured skin fibroblasts and amniotic fluid cells, and in phytohemagglutinin-stimulated lymphocytes. Distinguishing heterozygotes on the basis of loading tests has not proved reliable, and thus it is generally attempted by assay of synthase activity in liver biopsy material. However, tissue culture provides a readily available source of material for enzyme assay and this has prompted workers to seek an *in vitro* method for heterozygote detection. Phytohemagglutinin-

stimulated lymphocytes and cultured skin fibroblasts have been used, but overlap between heterozygotes and controls, or between heterozygotes and homozygous affected individuals, has cast doubt upon the reliability of the procedure. Without this critical differentiation, and other pertinent enzymatic data, prenatal diagnosis of cystathionine synthase deficiency has not been feasible.

This dissertation describes an improved assay system, developed to measure cystathionine synthase activity in cultured cells, which allows the distinction of obligate heterozygotes from clinically typical homozygotes for synthase deficiency, and from controls. The method developed involved several modifications of the conditions utilized in previous assays. The production of L-cystathionine by the cell extract was linear with protein concentration and with time. Enzymatic activity was greatest at pH 8.4. Activity was maximal upon the addition of 15 μmoles of L-homocysteine and 10 μmoles of serine. The omission of pyridoxal phosphate from the reaction mixture did not greatly alter the activity from that obtained with the standard amount of 0.015 μmoles. However, when the pyridoxal phosphate was increased to 0.5 μmoles a significant increase in product formation was seen.

Utilizing this improved system, cystathionine synthase activity was detected and quantified in cultured long-term lymphoid cell lines. Mean ($\pm$ SEM) control enzymatic activity was found to be 9.49 $\pm$ 0.98 nmoles/mg protein/hour. Obligate heterozygotes for synthase deficiency had activity of 3.21 $\pm$ 0.37, and a clinically typical patient had activity of 0.88. These data suggest that utilization of cultured lymphocytes for the in vitro detection of carriers and patients with synthase deficiency is feasible.

Enzymatic activity in cultured skin fibroblasts was highest in controls (mean $\pm$ SEM = 20.97 $\pm$ 1.81), intermediate in obligate heterozygotes (4.40 $\pm$ 0.92), and lowest in patients (0.77 $\pm$ 0.42). Of the five homozygotes studied, activity was detectable in three, two of whom demonstrated values of 0.74 and 0.83. The third, documented to be clinically and biochemically atypical, had an enzymatic activity of 2.29, which fell at the low extreme of the heterozygote group and was the only overlapping value detected. Cultured skin fibroblasts from two sibs of a patient were also investigated. The first had a synthase activity of 21.60 and was classified as normal, while the second showed an activity of 6.76, suggesting a heterozygous condition.

Enzymatic activity in cultured normal fetal fibroblasts was found to be 32.89 $\pm$ 5.06 and cultured normal amniotic fluid cells had activity of 40.70 $\pm$ 4.58. There was no significant difference between the

means of these two groups, but both differed significantly ($p < .001$) from the mean found in control adult fibroblasts. Amniotic fluid cells obtained during the sixteenth week of pregnancy from an obligate heterozygote mother were cultured, and lysates from the cell cultures were analyzed for synthase activity. Activity was found to be extremely high (73.79), and the diagnosis was made, and later confirmed, that the woman carried a normal fetus.

Appropriate kinetics were established for cystathionine synthase in all of the above cell types, and the dissertation directs itself to a discussion of the importance of this type of investigation in the study of human biochemical genetics.

to Lonnie . . . . . for everything . . . . . and for always

ACKNOWLEDGEMENTS

At first thought, "acknowledgement" seems to be a word too formal, too often used, too rarely reflected upon. But, according to Webster*, to acknowledge means "to own with gratitude or as a benefit or as imposing obligation; to express thanks for". With this in mind, I would like to take this opportunity to express my deepest appreciation to the following who, whatever else our relationship may be, I count as very special friends:

To Drs. Nicholas Beratis and Harris Tallan, who answered many inane questions, many times, and who taught me to turn laboratory techniques into research tools.

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And, to my parents, Sylvia and Irving Friedman, a most private and special "thank you", for the innumerable ways in which they express the love and understanding that unites us.

*Webster's New Collegiate Dictionary, G. and C. Merrian Co., publishers, Springfield, Massachusetts, 1961, p. 8.

CONTENTS

	Page
Abstract	iv
Acknowledgements	ix
List of Tables	xi
List of Illustrations	xii
Part I - Introduction	1
Part II - Materials and Methods	48
Part III - Results	54
Part IV - Discussion	85
Literature Cited	132

TABLES

		Page
Table 1.	Cystathionine synthase activity in cultured adult skin fibroblasts from controls and from heterozygotes and homozygotes for synthase deficiency.	77
Table 2.	Comparison of cystathionine synthase activity (Mean $\pm$ SEM) in cultured adult skin fibroblasts from controls and from heterozygotes and homozygotes for synthase deficiency.	79
Table 3.	Cystathionine synthase activity in cultured fetal fibroblasts and amniotic fluid cells.	82
Table 4.	Comparison of mean cystathionine synthase activity in cultured control cells.	83
Table 5.	Cystathionine synthase activity in long-term lymphoid cell lines from controls and from heterozygotes and homozygotes for synthase deficiency.	84

ILLUSTRATIONS

		Page
Figure 1.	The metabolism of the sulfur-containing amino acids.	6
Figure 2.	Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of protein concentration.	55
Figure 3.	Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of time of incubation.	56
Figure 4.	Effect of pH on cystathionine synthase from cultured human adult skin fibroblasts.	57
Figure 5.	Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of L-homocysteine concentration.	59
Figure 6.	Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of L-serine concentration.	60
Figure 7.	Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of pyridoxal phosphate concentration.	61
Figure 8.	Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of L-serine concentration.	62
Figure 9.	Rate of reaction of cystathionine synthase from cultured human fetal skin fibroblasts as a function of L-serine concentration.	63
Figure 10.	Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of L-homocysteine concentration.	64
Figure 11.	Rate of reaction of cystathionine synthase from cultured human fetal skin fibroblasts as a function of L-homocysteine concentration.	65

		Page
Figure 12.	Effect of pH on cystathionine synthase from cultured human amniotic fluid cells.	67
Figure 13.	Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of protein concentration.	68
Figure 14.	Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of time of incubation.	69
Figure 15.	Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of pyridoxal phosphate concentration.	70
Figure 16.	Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of L-serine concentration.	71
Figure 17.	Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of L-homocysteine concentration.	72
Figure 18.	Effect on pH on cystathionine synthase from human long-term lymphoid cell lines.	73
Figure 19.	Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of time of incubation.	74
Figure 20.	Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of pyridoxal phosphate concentration.	75
Figure 21.	Cystathionine synthase activity in cultured skin fibroblasts.	80
Figure 22.	Simple gene-dose relationship for monomeric protein.	110

Page

Figure 23. Formation of a dimeric enzyme protein in a normal individual. 112

Figure 24. Formation of a dimeric enzyme protein in an individual homozygous for a mutant synthase gene. 113

Figure 25. Formation of a dimeric enzyme protein in an individual heterozygous for a mutant synthase gene. 114

I. INTRODUCTION

Aminoaciduria

In 1810 cystine, the first amino acid to be isolated, was prepared from a urinary calculus by W. H. Wollaston (172). Almost a century passed before the classic description of A. E. Garrod (50), which suggested that several genetic diseases were caused by inborn errors of amino acid metabolism. Still another forty years were to elapse before the development of simple laboratory methods for the clinical investigation of aminoacidopathies; that is, techniques suitable for the detection and identification of urinary amino acids. The critical contribution was the development of two-dimensional paper chromatography (71), which allowed the identification and quantitative estimation of each amino acid in a mixture. Applications of this method soon followed (31, 32) and still continue to increase our understanding of both aberrant and normal amino acid metabolism. Systems utilizing ion-exchange chromatography (109, 110), electrophoresis (108), and microbiological assay of single amino acids (173) have greatly expanded our knowledge in this area.

Numerous diseases, both acquired and hereditary, have been found to be associated with aminoaciduria (41). The number of hereditary disorders of amino acid metabolism discovered has increased from about seven in 1948 to more than forty in 1970. Aminoaciduria, however, is not always a pathologic condition. Normal urine contains small amounts of many amino acids. The situation is especially difficult to evaluate during the first few months of life, when several amino acids are excreted in increased amounts into the urine. This is presumably because renal tubular reabsorptive mechanisms are not fully developed or because relevant enzymes are not fully active at birth. Even when a specific amino acid is excreted as a result of a defective enzyme there may be no disease associated with the biochemical abnormality. Beta-aminoisobutyric aciduria is a well-known example of such a "harmless" inborn error of metabolism (41).

Diseases associated with aminoaciduria may be heritable or acquired. There are a large number of acquired disorders which result in blood and urine amino acid aberrations, including protein and vitamin deficiencies and liver necrosis. The heritable aminoacidurias may be secondary manifestations of other diseases such as Fanconi's syndrome, which involves generalized and nonspecific proximal tubular damage, or galactosemia, in which excess galactose 1-phosphate interferes with the transport mechanism in the ranal tubules (28). Heritable aminoacidopathies may also be primary, resulting from an inborn error of amino acid metabolism or transport. One or more amino acids are

seen to accumulate in the urine and often also in the blood and other body fluids.

Three types of primary aminoaciduria can be distinguished. The "overflow" pattern of aminoaciduria involves an increase in the plasma level of one or more amino acids, presumably resulting from a metabolic block produced by deficient enzymatic activity, with a consequent overflow into the urine. Phenylketonuria is the most common example of this type of aminoaciduria, in which the amino acids involved undergo normal renal tubular reabsorption, but overflow when the absorption site is saturated. These disorders are best detected by analyzing the blood serum or plasma. Low-threshhold (or no-threshhold) aminoacidurias are also due to deficiencies of enzymes involved in amino acid metabolism. However, the unmetabolized substrates collecting behind the metabolic block do not accumulate to a very high concentration in the blood. They are excreted in large quantities into the urine, with a rate approximating that of inulin, presumably because the kidneys lack the mechanism for reabsorbing them. This lack of active renal tubular reabsorption is common among substances that serve as intermediates in intracellular metabolic pathways and thus are not normally found in extracellular fluid. Homocystine and cystathionine are two such substances, and disorders in their metabolism are most easily detected by analysis of urine samples. Renal, or transport aminoacidurias result from a defective protein involved in the transport, rather than the metabolism,

of one or more amino acids. These amino acids leak into the urine, in the presence of normal or low plasma concentrations, because their transport across the renal tubular membranes is blocked. Hartnup's disease and cystinuria are examples of renal aminoacidurias. Aminoaciduria may also involve a mixture of amino acids, one or more of which are in excess due to an overflow mechanism, and one or more of which are no-threshhold substances.

Thus, hereditary aminoaciduria is intriguing to those involved in the study of biochemical genetics, as it often represents the direct result of an altered metabolic pathway and may lead to a better understanding of normal metabolism. Furthermore, defective activity of enzymes involved in amino acid metabolism may result in mental retardation. The study of aminoaciduria has thus been of clinical significance in the diagnosis and treatment of mentally defective individuals and more recently, in the prevention of their birth. Several types of sulfur aminoacidurias have been reported, including homocystinuria, cystathioninuria, and sulfite oxidase deficiency, which were found in mentally deficient patients. Striking advances in the understanding of organic sulfur metabolism are occuring, many via discoveries of inborn errors of methionine metabolism.

Methionine Metabolism

Much of the early information regarding the transsulfuration pathway came from feeding experiments with rats which showed that

methionine was essential for growth and in this capacity could substitute for cystine (131). Homocysteine was later found to be an adequate alternate for methionine, providing that a source of preformed methyl groups was available. These observations in laboratory animals were later confirmed in patients with cystinuria by the demonstration that feeding either methionine or homocysteine could increase cystine excretion (35). Furthermore, the sulfur moiety of [^{35}S]methionine, and later cystathionine, was found to appear in cystine. This information confirmed the existence of a transsulfuration pathway and established both homocysteine and cystathionine as intermediate compounds. More recently, enzymatic studies have contributed to the delineation of the entire pathway.

The metabolism of the sulfur-containing amino acids is illustrated in Figure 1. Fundamentally, methionine has four metabolic roles in man. It is an essential amino acid, supplying its important sulfur atom, and thus a necessary precursor in the synthesis of protein in the body. Via conversion to S-adenosylmethionine, methionine serves as the major biological methyl group donor, and also as a source of the aminopropyl group transferred in the formation of the polyamines spermidine and spermine. Methionine is also converted by means of the transsulfuration pathway to cystathionine, cysteine, and derivatives of cysteine.

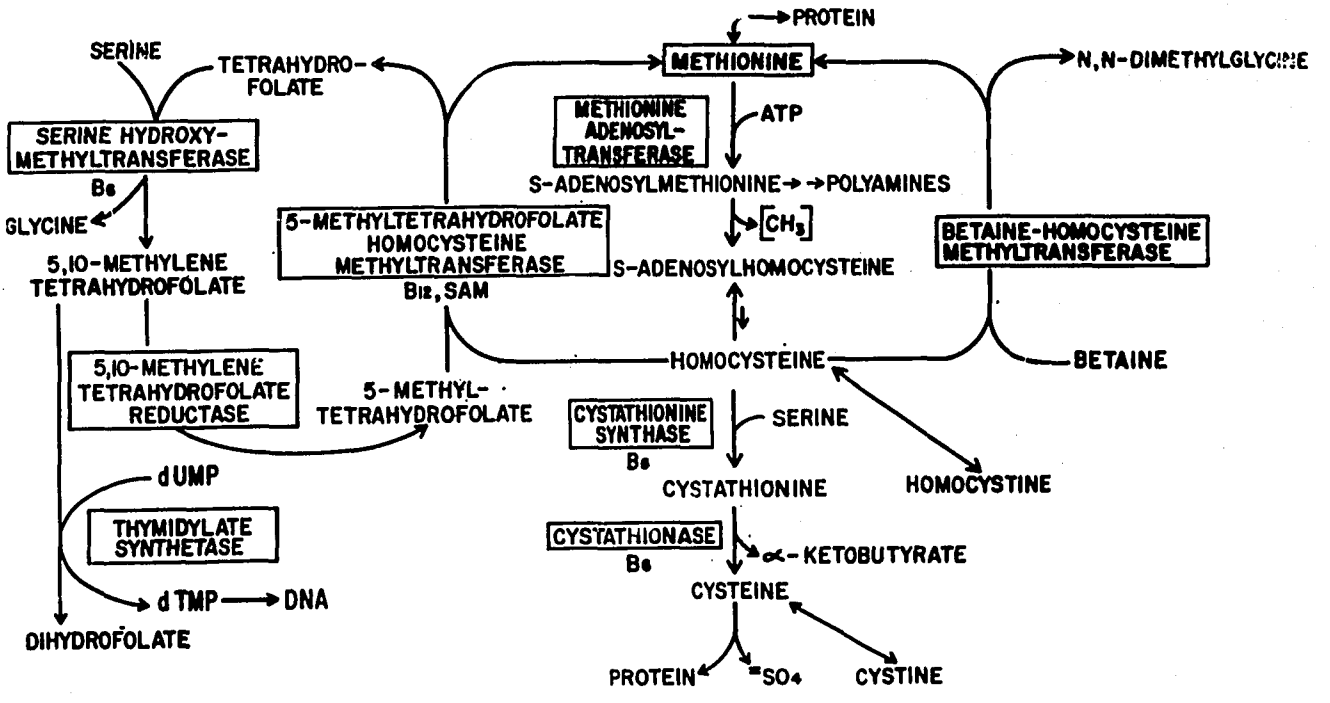
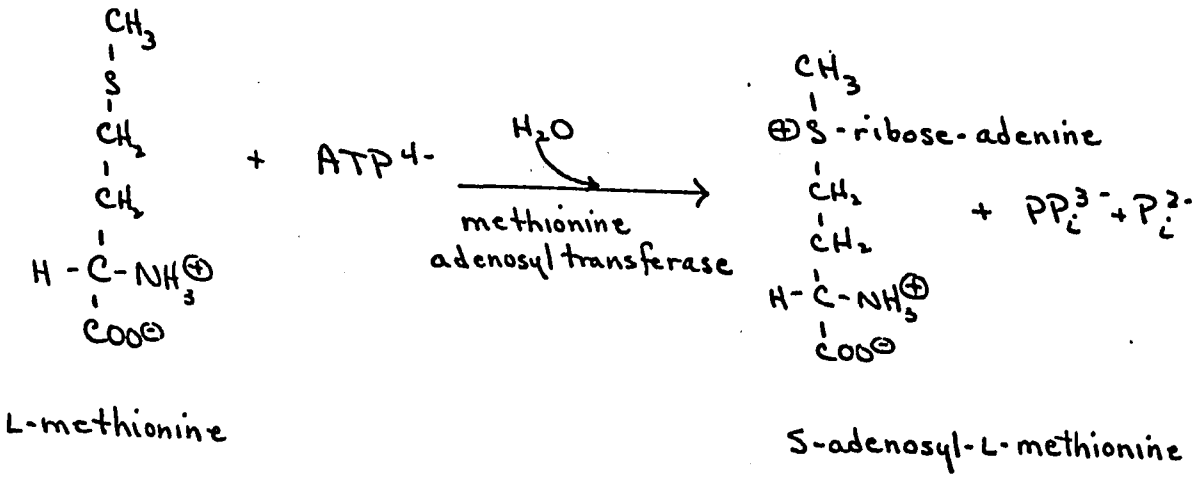


Figure 1. The metabolism of the sulfur-containing amino acids.

Methionine enters the amino acid pool from dietary intake and from the breakdown of body protein. The amino acid is first activated by conversion to S-adenosylmethionine ("active methionine") in a reaction which is catalyzed by methionine-activating enzyme and which results in the transfer of an adenosyl group to the sulfur atom of methionine. (Scheme a).

-a-

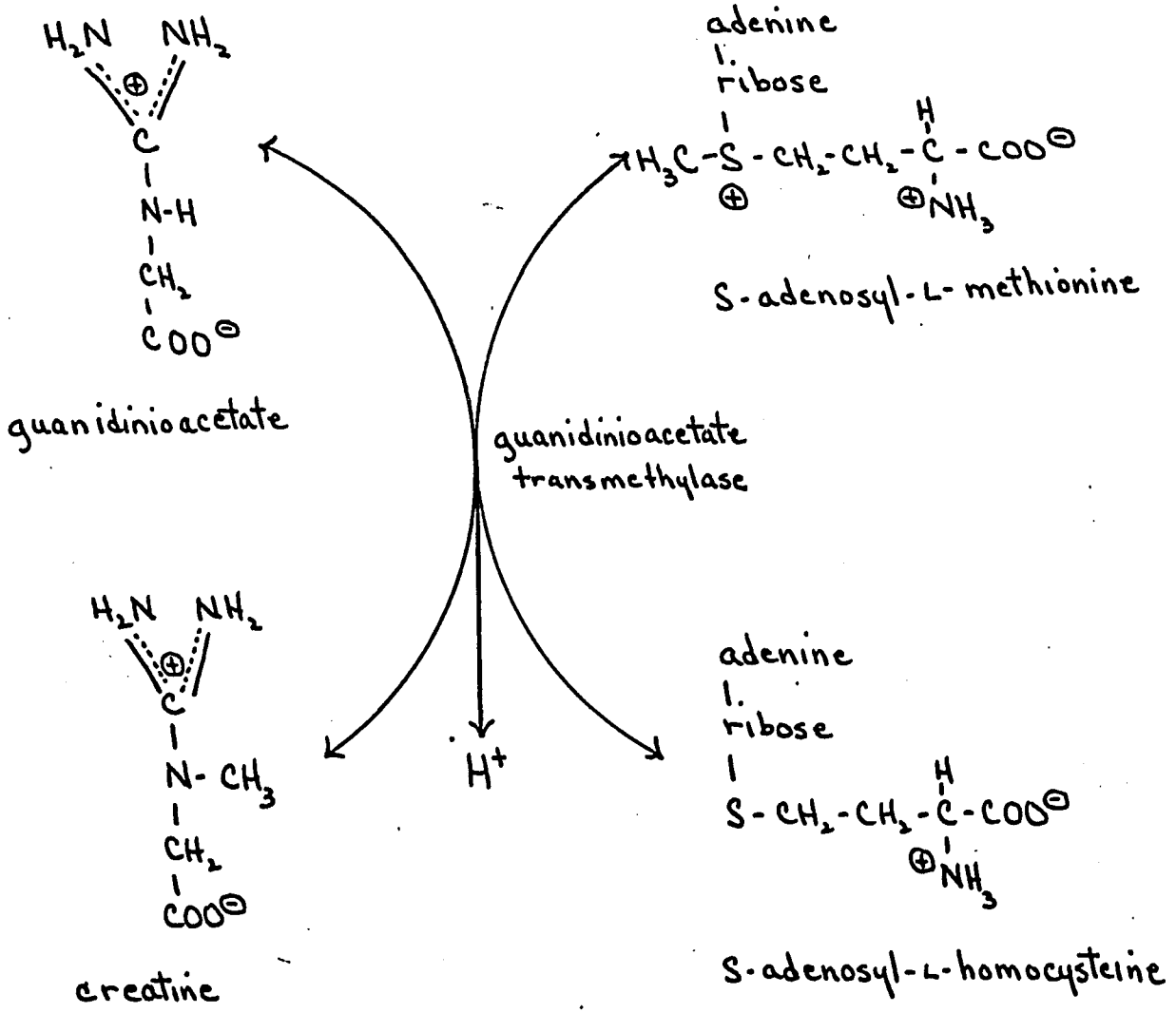


The formation of S-adenosylmethionine, a high energy compound due to the presence of the methyl-sulfonium bond, is an unusual reaction. During the reaction the three phosphate groups of ATP are bound to the enzyme as PPPi which is cleaved to pyrophosphate and orthophosphate before release. Thus, the energy of cleavage of the ester bond between adenosine and PPPi, and the energy of cleavage of one pyrophosphate linkage is the driving force of the reaction. In addition, the energy of cleavage of the resulting pyrophosphate to orthophosphate

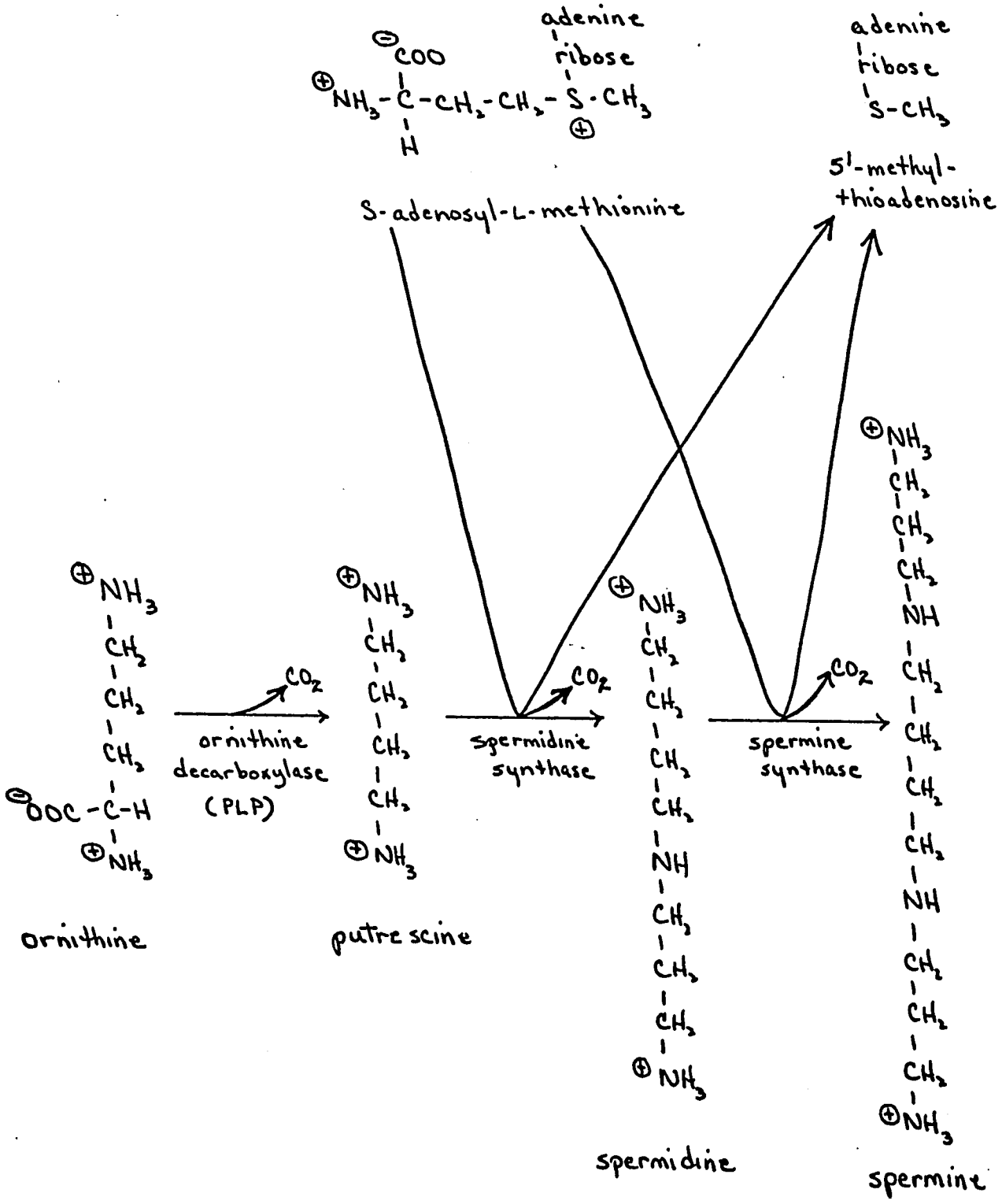
by intracellular pyrophosphatase adds to the driving energy.

The sulfonium compound immediately donates its methyl group to appropriate acceptor molecules and the transmethylation results in the formation of S-adenosylhomocysteine. In the presence of suitable transmethylases the methyl group from S-adenosylmethionine is utilized in the formation of many important biological compounds. One significant function is in the formation of creatine, which is necessary for the storage of high energy phosphate in skeletal muscle (Scheme b).

-b-



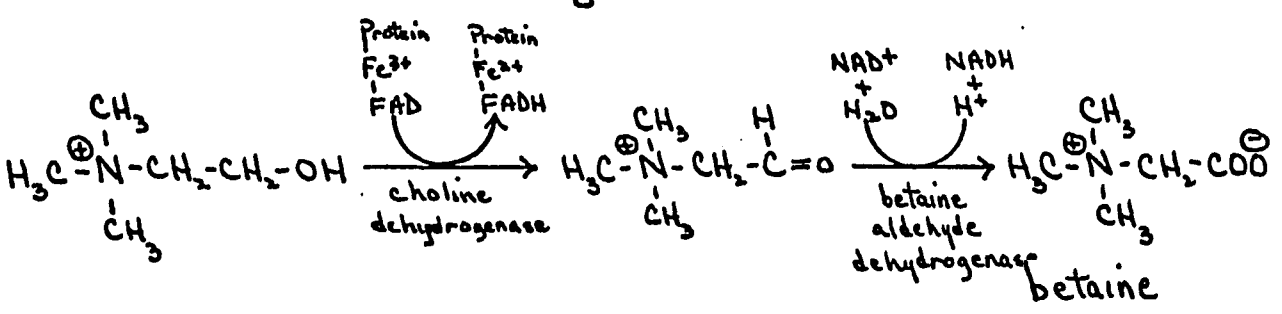
At rest, creatine is in the form of phosphocreatine, which spontaneously cyclizes to form creatinine. Creatinine is excreted into the urine and thus is a constant drain on the supply of methyl groups. S-adenosylmethionine also donates the three methyl groups needed for the formation of phosphatidyl choline, the phospholipid involved in membrane structure and triglyceride transport, which is hydrolyzed intracellularly to form choline. This occurs in three successive transfers. Methyl groups are also enzymatically transferred from S-adenosylmethionine to ribonucleotides assembled on DNA, to form the unusual methylated derivatives of the common bases. Other roles for the methyl group derived from S-adenosylmethionine include the formation of epinephrine from norepinephrine, and its further metabolism, the formation of carnosine from anserine, and the degradation of histamine. The transfer of the aminopropyl group of S-adenosylmethionine, rather than its methyl group, is involved in the synthesis of the polyamines spermidine and spermine. These polycationic compounds seem to occur in association with nucleic acids and may be important in control mechanisms involved in protein synthesis and/or cell division. The diamine putrescine is formed by the decarboxylation of ornithine. The amino acid skeleton of S-adenosylmethionine is transferred, after decarboxylation, to condense with putrescine to form spermidine and spermine. A single enzyme seems to be involved in the transfer and decarboxylation in animals (Scheme c).


adenine
ribose
S-CH3
COO
NH3-C-CH2-CH2-S-CH3
H
S-adenosyl-L-methionine
adenine
ribose
S-CH3
5'-methyl-thioadenosine
NH3
CH2
CH2
CH2
CH2
NH
CH2
CH2
CH2
CH2
NH
CH2
CH2
CH2
CH2
NH3
CO2
ornithine decarboxylase (PLP)
CO2
spermidine synthase
CO2
spermine synthase
ornithine
putrescine
spermidine
spermine

S-adenosylhomocysteine is a thioether, similar to methionine, and its formation during transmethylation liberates free energy due to the loss of the sulfonium structure. The compound is enzymatically hydrolyzed to adenosine and homocysteine. The homocysteine can then follow one of two metabolic pathways. It may be remethylated to form methionine by one of at least two enzyme systems, one involving betaine and the other, N-5-methyltetrahydrofolate. In either case a cycle would be completed and methionine would be conserved for utilization in protein synthesis or in the formation of S-adenosylmethionine. It is likely that both the betaine and folate pathways contribute to the remethylation function.

One of the primary sources of methyl groups for the regeneration of methionine is choline. To make the reaction energetically feasible choline is oxidized at its alcohol carbon to form betaine (Scheme d). This occurs in two oxidation steps, within the mitochondria, and the reactions are linked to the electron transport chain, generating five moles of high energy phosphate.

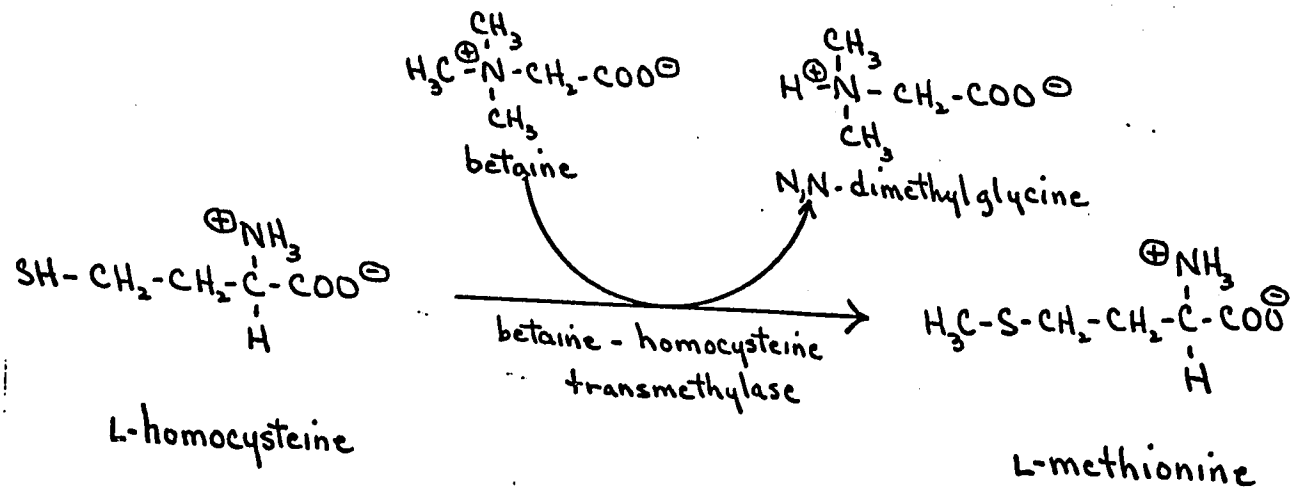
-d-



choline betaine aldehyde betaine

Betaine, or N,N,N-trimethylglycine, then donates one of its methyl groups to form methionine (Scheme e).

-e-

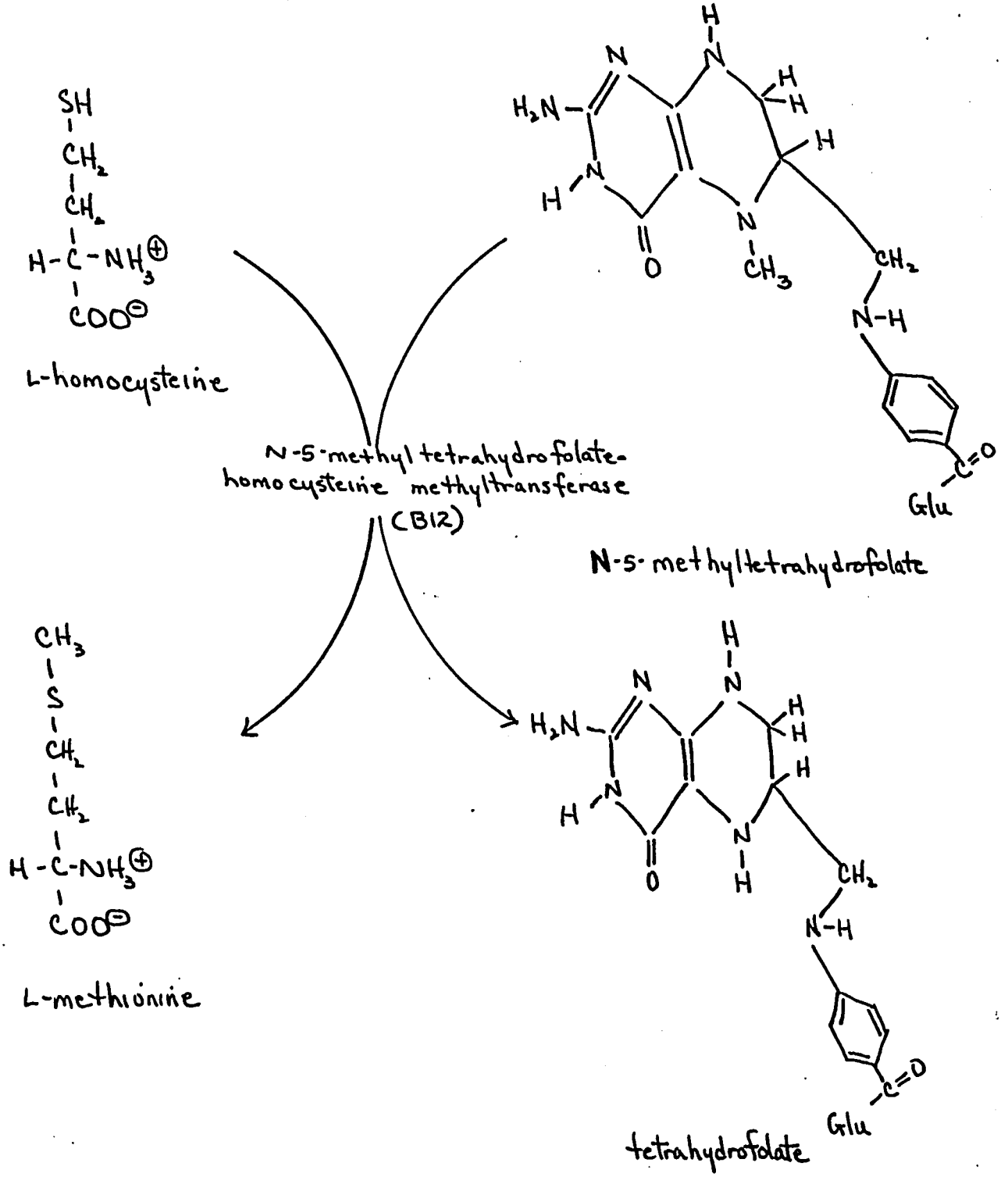


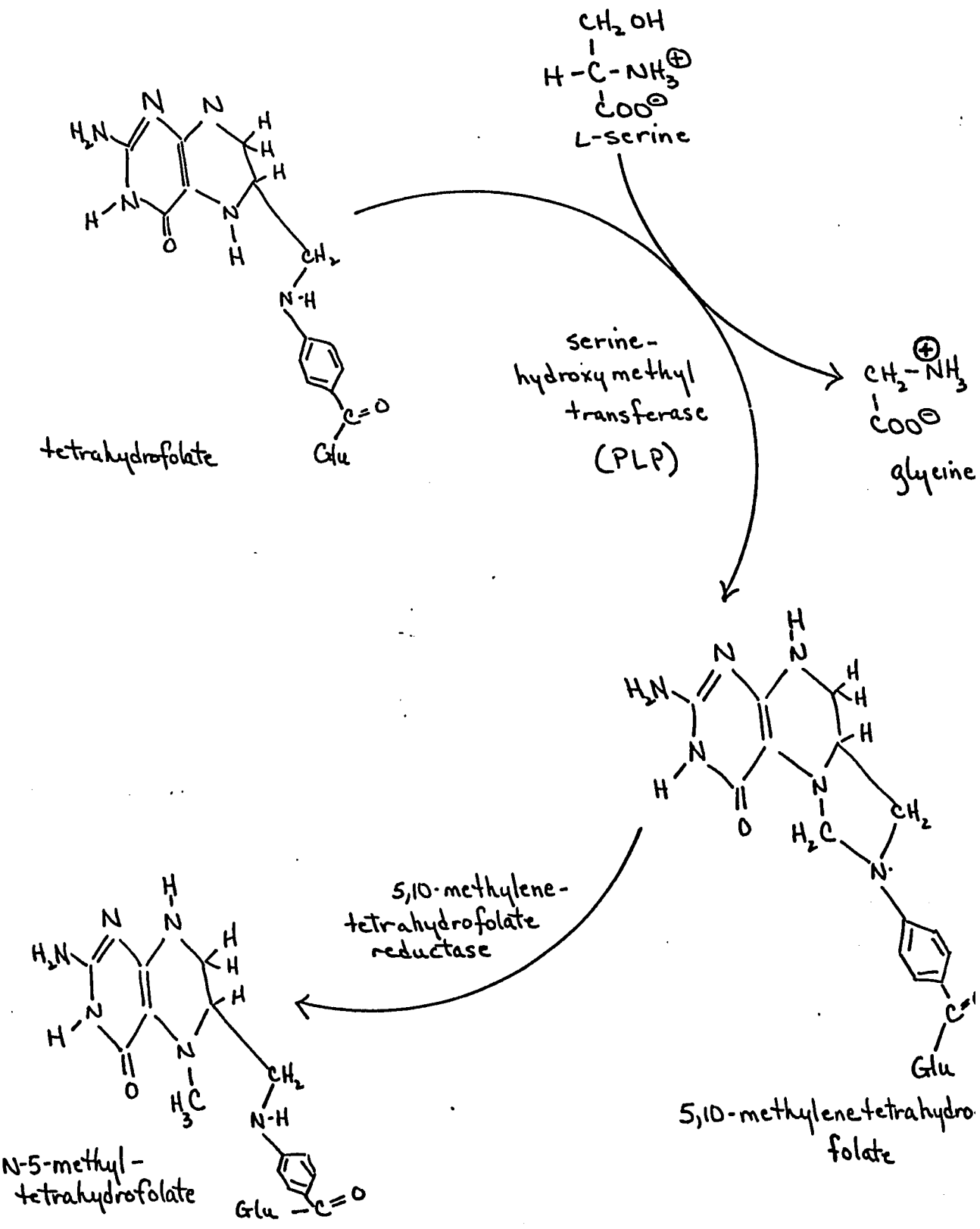
Flavoprotein oxidases within mitochondria can remove the remaining methyl groups, which no longer have a high energy nature due to loss of the quarternary nitrogen structure, from N,N-dimethylglycine. The methyl groups are oxidized to formaldehyde, and these oxidations are also linked to the electron transport chain, generating two additional moles of high energy phosphate for each pair of electrons transferred. The formaldehyde can be further oxidized within the mitochondria to formate and on to carbon dioxide. Alternately, it can add to the one-carbon pool by combining with tetrahydrofolate. Thus, the

methyl group donated by methionine to the choline residue of the phospholipids is not lost. When excess choline accumulates from degradation of phosphatidylcholine, the methyl groups can be recovered via direct remethylation of homocysteine to methionine or by incorporation into the one-carbon pool.

The methyl group necessary for the regeneration of methionine can also be obtained directly from the one-carbon pool. Transfer of a methyl group from N-5-methyltetrahydrofolate to homocysteine occurs via a reaction catalyzed by an enzyme requiring a B12 (cobamide) derivative as a coenzyme. (Scheme f). The resulting tetrahydrofolate can receive a methylene group from serine in a reaction catalyzed by serine hydroxymethyltransferase, and requiring pyridoxal phosphate (PLP) as cofactor. Methylene tetrahydrofolate reductase catalyzes the next reaction, to complete the cycle, forming N-5-methyltetrahydrofolate. (Scheme g). Impairment of the remethylation of homocysteine by N-5-methyltetrahydrofolate methyltransferase, due to defective metabolism of the necessary cobamide coenzyme, results in a syndrome characterized by homocystinemia, cystathioninemia, and hypomethioninemia (70, 97, 99, 118, 119, 121). Methylmalonicaciduria is also a feature, due to the requirement of a B12 coenzyme by methylmalonyl-CoA mutase, which catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA. Homocystinuria has also been associated with a deficiency of methylene-tetrahydrofolate reductase activity (120).

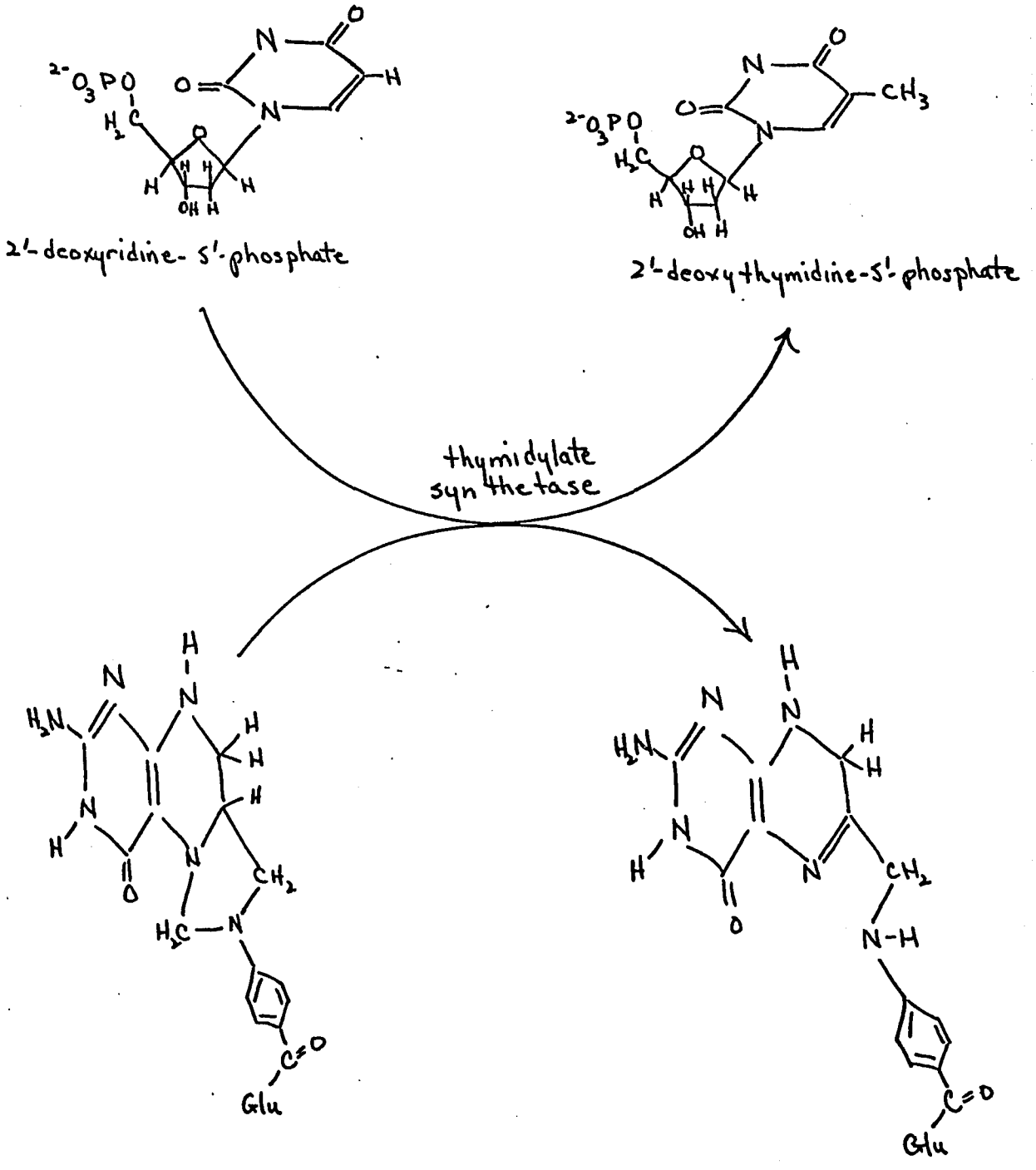
-f-


L-homocysteine
N-5-methyl tetrahydrofolate-homocysteine methyltransferase (B12)
N-5-methyltetrahydrofolate
Glu
L-methionine
tetrahydrofolate
Glu


L-serine
tetrahydrofolate
Glu
serine-hydroxymethyl transferase (PLP)
glycine
5,10-methylene-tetrahydrofolate reductase
5,10-methylenetetrahydrofolate
N-5-methyl-tetrahydrofolate

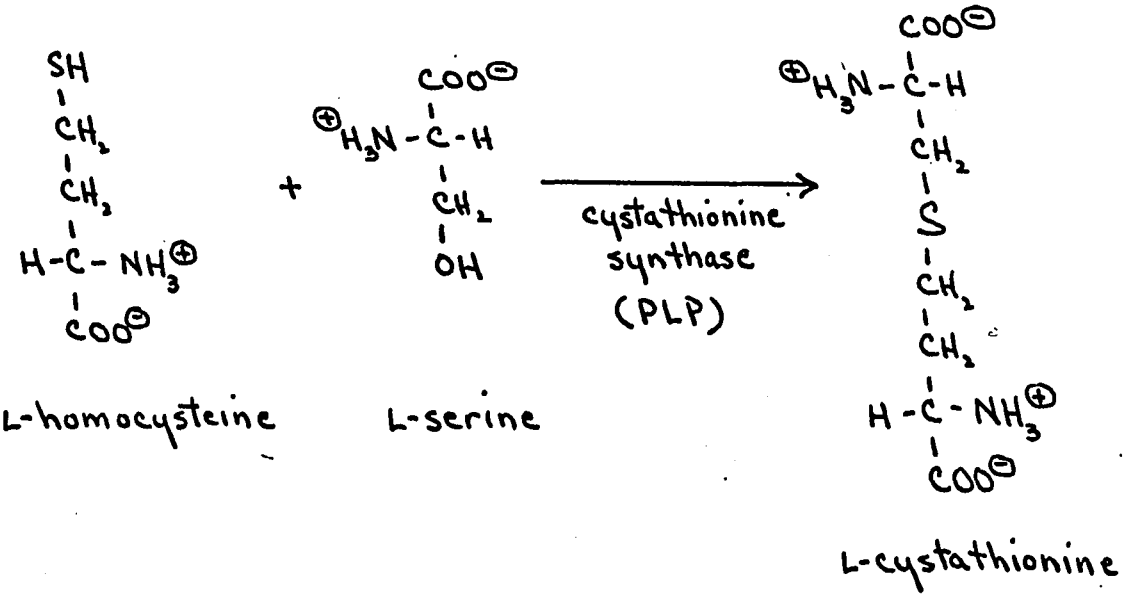
5,10-methylene tetrahydrofolate is also important in the synthesis of thymidylate and thus, DNA (Scheme h).

-h-



The second metabolic pathway available to homocysteine provides an exit from the transmethylation-remethylation cycle. In the presence of the enzyme cystathionine synthase, which appears to require pyridoxal phosphate (PLP) as its coenzyme (88, 111), serine and homocysteine undergo condensation to form cystathionine (Scheme i).

-i-



Cystathionine is then hydrolytically cleaved to yield cysteine and α-ketobutyrate in the presence of the enzyme cystathionase and the coenzyme pyridoxal phosphate. A deficiency of cystathionase activity

may result in the clinical syndrome of cystathioninuria. The cysteine formed can then participate in synthetic reactions, or be oxidized to cystine, taurine, and inorganic sulfate. Similarly, excess homocysteine can be oxidized to homocystine. In the normal adult 80-90% of dietary methionine intake is balanced by the excretion of inorganic sulfate and taurine. It has been established (95) that this transsulfuration pathway is the dominant one for the conversion of the sulfur atom of methionine to inorganic sulfate. Normally, dietary methionine is essential for growth and development in mammals (136, 170), while cysteine is a "non-essential" amino acid in the adult. However, cysteine or cystine can lower the methionine requirement, thus "sparing" methionine (135, 138, 171), although methionine cannot be totally replaced (136, 139, 170).

The equilibrium of the reaction S-adenosylhomocysteine $\rightleftharpoons$ homocysteine and adenosine is strongly in the direction of synthesis, while the condensation of serine and homocysteine to cystathionine is generally considered irreversible in vivo. Free homocyst(e)ine is not normally present in tissues or body fluids. Thus, the detection of homocystine in the urine of an infant with congenital anomalies and failure to thrive (64), and in two mentally retarded sibs whose clinical features were thought to suggest a possible metabolic defect (23), indicated that the problem could involve the metabolism of methionine. The original hypothesis (21) suggested that the immediate cause of the pathology in homocystinuria might be a cystine deficiency, due to an

error in the conversion of methionine to cysteine, or a problem in methionine transport. However, in 1964, Mudd and his colleagues (114) demonstrated a deficiency of cystathionine synthase activity in liver obtained from a child with homocystinuria, and proposed that this enzyme deficiency caused the disorder. Although there are other biochemical lesions resulting in the excretion of homocystine (70, 97, 99, 118-121) by far the majority of cases represent cystathionine synthase deficiency, and all references below to homocystinuria will pertain to this type unless otherwise noted.

Homocystinuria

Although only recognized as a clinical entity since 1962 (23, 64), homocystinuria has since been reported in more than one hundred patients and represents the most extensively studied of the diseases of sulfur amino acid metabolism. In full development, the clinical picture is quite distinctive (22, 56, 143), and resembles Marfan's syndrome. The patients appear normal at birth and the average developmental milestones are generally achieved during the first two years of life. However, the clinical signs that develop are often so well-defined as to be recognizable merely upon inspection. They include: ectopia lentis, resulting from a failure in development of the suspensory ligament of the lens; fine, fair, somewhat sparse hair; a malar flush which may become cyanotic; a peculiar "shuffling" gait; progressive osteoporosis and skeletal abnormalities; and severe

thromboembolic disease. The syndrome is also characterized by pathological changes in the tissues (22,56,67,143,169), including: intimal fibrosis and degeneration of the medium and large-sized arteries, fatty changes of the liver, and non-specific pathological changes in the brain, especially in the grey matter. Approximately two-thirds of the patients are mentally retarded, although the deficiency is usually not as severe as that seen in phenylketonuria. A sizeable minority of older patients have been found to be of normal intelligence, and it has been suggested (143) that those who are not retarded may be less severely affected and thus may live longer.

Cystathionine synthase deficiency is inherited in an autosomal recessive manner. Pedigree analyses demonstrate multiple affected sibs in an approximately equal sex ratio, segregation data compatible with this mode of inheritance, and occasionally, consanguinity. An autosomal recessive pattern of transmission was originally suggested by Carson et al. (21), based on the observation of this extensive and identical metabolic disorder occurring in two sibs, while the remainder of the family appeared normal. However, the conclusive evidence was presented by Finkelstein et al. (43), who demonstrated hepatic cystathionine synthase activity in the parents of a patient with homocystinuria that was 40% of the mean control value. Thus, the clinically normal parents are heterozygous for a mutant gene which, in the homozygous state, results in a complete absence (or a very low level) of enzyme activity.

The first reported cases of homocystinuria (23, 64) were of northern European extraction. However, although the majority of patients are of this origin (106), a review of the literature indicates a world-wide distribution (15, 19, 34, 51, 90, 153, 154, 167). This suggests that the gene involved may be "relatively common", as most "rare recessive" genes show a non-homogeneous distribution. Furthermore, definitely established parental consanguinity has not been frequently reported. Since there appears to be an inverse relationship between the degree of consanguinity among the parents of patients and the incidence of a rare recessive disorder, the suggestion is strengthened that the gene determining a deficiency of cystathionine synthase activity occurs "relatively" frequently. At present, this disorder is second in frequency only to phenylketonuria as an inborn error of amino acid metabolism associated with mental retardation.

The Metabolic Defect

In their original paper on homocystinuria, Carson et al. (21) proposed an inborn error of methionine metabolism, or transport, as the basis of the disorder. They considered the possibility that the enzyme cystathionine synthase might be deficient, but did not pursue this course, turning instead to investigate a possible defect in methionine transport. Speculation about the known pathways of methionine metabolism, and the observed accumulation of both

methionine and homocystine in homocystinuria, led Mudd et al, (114) to suggest deficient activity of cystathionine synthase as the basic metabolic error. Utilizing micromethods based on the differential adsorption of labelled product (114), and sufficiently sensitive to make use of the tissue obtained from needle biopsies of liver, they showed cystathionine synthase activity to be deficient or absent in a patient's liver, although methionine-activating enzyme and cystathionase were comparable in activity to controls. Obligate heterozygotes exhibited synthase activities that were approximately 40% of control values (43). Brenton, Cusworth and Gaull (12, 14) demonstrated an absence of cystathionine in the brains of two patients with homocystinuria; thus suggesting an absence of the synthase. The absence of cystathionine from all areas of the brain of affected individuals was confirmed by Gerritsen and Waisman (66). It had been known for some time that the concentration of cystathionine in the occipital lobe of normal human brain is very high (160). Gerritsen and Waisman (66) attributed its absence in the brain of homocystinurics to the absence of cystathionine synthase activity in the liver, suggesting that cystathionine would normally be transferred from the liver to the brain, and recommending treatment with cystathionine. However, this explanation has been refuted by in vivo and in vitro evidence (49, 56) that the transsulfuration of methionine to cysteine and thus, the production of cystathionine, takes place in the brain itself. Later, Mudd and his coworkers (96) demonstrated the absence of cystathionine synthase

activity in autopsy brain from Waisman's patients (65) noting that methionine-activating enzyme and cystathionase were present. All three enzymatic activities were present in normal human autopsy brain.

Interestingly, Gaull and Gaitonde (57) demonstrated a normal ability to convert methionine to cysteine in the optic lens from a patient with homocystinuria. Assuming no other pathways for the conversion of methionine to cysteine in the lens, it would appear that cystathionine synthase activity was present in that organ, although absent from liver and brain. This suggests that the genetic control of homocysteine metabolism is different in the lens from that in liver and brain.

More recently, Uhlendorf and Mudd (165) have determined cystathionine synthase activity in fibroblasts grown in tissue culture, derived from small skin biopsies of homocystinuric patients and controls. The fibroblasts of the patients demonstrated absent or extremely low levels of cystathionine synthase, although activity of methionine-activating enzyme appeared normal. Absence of synthase activity in patients with homocystinuria has also been demonstrated in phytohemagglutinin-stimulated lymphocytes (68). Thus, it has been established that deficient activity of the enzyme cystathionine synthase is the basic metabolic error responsible for the clinical syndrome associated with homocystinuria.

Cystathionine synthase is the condensing enzyme responsible for the synthesis of the thioether cystathionine from homocysteine and serine,

and requires pyridoxal phosphate as a coenzyme. The suggestion has been made that cystathionine synthase, serine dehydratase, and threonine dehydratase (threonine deaminase) are enzymic activities of one protein (148). However, cystathionine synthase and serine dehydratase activities have been resolved into two separate fractions after chromatography on hydroxylapatite columns (16). Furthermore, Mudd et al. (115) have shown that human livers which have deficient activity of cystathionine synthase do not have abnormally low activity of threonine dehydratase. This was followed by demonstrations, using classical separation procedures (16, 17), that the synthase is a separate enzyme.

Laster et al. (95) investigated the metabolic consequences of cystathionine synthase deficiency and demonstrated that cystathionine synthesis is an obligatory reaction in the conversion of methionine to inorganic sulfate in man. They also concluded that the deficiency of enzymatic activity detected in the liver reflects a generalized reduction in the body's capacity to convert homocysteine to cystathionine. This suggestion is supported by evidence from other studies (116, 117) which indicate that: a) the liver is the major site of cystathionine synthesis in mammals, and b) the enzyme defect is present in other tissues, such as brain.

Thus, patients who are deficient in synthase activity, and excrete abnormal amounts of homocystine into their urine, have a reduced capacity to form inorganic sulfate from methionine. Methionine accumulates in the blood and cerebrospinal fluid and overflows into the

urine. Loading tests involving either oral or intravenous loads of 100mg. methionine/kg. of body weight can clearly delineate patients with homocystinuria, although heterozygotes cannot be distinguished from normals by this method. Interestingly, after loading with L-methionine, the concentration of homocystine in the plasma and urine of a patient remains constant, although the methionine concentration in the plasma remains elevated above resting levels for three to seven days (13). It had been observed that the excretion of methionine, homocystine, and the mixed disulfide cystine-homocystine could account for only a small part of the total sulfur derived from ingested methionine (approximately 2gm/day) in these patients (13). Laster et al. (95) showed that the bulk of the sulfur excreted was in the form of unidentified neutral sulfur.

Pathogenesis

Homocystinuria is one of a number of clinical syndromes that are known to result from an inherited deficiency of a particular enzyme. However, the mechanism by which this deficiency results in the phenotypical disease has not been established for most of these. In homocystinuria, as in the other inborn errors of metabolism, compounds prior to the metabolic lesion accumulate to varying extents depending on the activity of other enzymes in the same and/or alternate pathways. Another consequence of the block is a deficiency of the products subsequent to the affected reaction, particularly if

these substances cannot be synthesized by any other means. It has been generally accepted that accumulations proximal to the block may be toxic, while distal deficiencies,with a few exceptions, are not physiologically important because these products are available in the diet. Assumptions regarding this hypothesis have been reviewed (52-55). Although there is evidence, from nutritional studies, of the toxicity of feeding high levels of methionine and homocysteine to animals (92,156), it remains clear that a simple excess of these amino acids cannot duplicate the distinctive pathological changes observed in the disease. Plainly, feeding exhorbitant amounts of an amino acid is quite different from blocking the metabolism of usual quantities of it, a fact which has been documented by the inefficacy of attempts to recreate the pathological findings of phenylketonuria by feeding large amounts of phenylalanine (55).

It is feasible that certain functional proteins may be particularly susceptible to the formation of mixed disulfides, formed by excess homocysteine, and thus be severly affected; as may also be thiolated nucleic acids (18). Also, it is possible that excess homocyst(e)ine may be incorporated into protein in place of cyst(e)ine, although it is improbable that the amino acid activating systems lack the specificity with which to differentiate between the two substrates. In addition, homocyst(e)ine has not been found in the proteins of either hair or brain from homocystinuric patients (12, 14), although incorporation into plasma proteins of mixed disulfides has been

reported (10).

The subcutaneous administration of homocysteine thiolactone to rabbits was reported to produce homocyst(e)inemia, and the resulting vascular lesions were found to reproduce the distinctive pathological blood vessel changes found in patients with homocystinuria (67, 102, 105), and those found in individuals with early arteriosclerosis. Furthermore, the same authors also noted the production of accelerated arteriosclerosis in a homocysteinemic individual (102). Cultured fibroblasts derived from the skin of this patient were seen to produce an unusual granular proteoglycan substance, while the addition of homocysteine to normal cultured skin fibroblasts was associated with the replacement of a portion of the normal fibrillar proteoglycans by the same granular material (103). They suggested that homocyst(e)inemia resulted in accelerated arteriosclerosis in cystathionine synthase deficiency by altering the usual fibrillar structure of the arterial wall proteoglycan molecules (104, 105). This alteration was thought to be due to increased sulfation of the carbohydrate envelope of the molecule (104). However, it is not certain that administration of the thiolactone will result in homocyst(e)inemia, and when some of the animal experiments were repeated (33), neither abnormal vascular lesions, nor homocyst(e)inemia, could be detected.

Consideration of possible pathological effects of the deficiencies of products distal to the synthase reaction immediately suggests that

inadequate amounts of cystathionine may result in serious consequences. Normally present in very high concentrations in human brain (12, 116, 160), cystathionine is especially concentrated in white matter (14), and has been implicated as a putative transmitter substance in the spinal interneurons of the cat (168). The phylogenetic distribution of this amino acid is especially provoking. The concentration of cystathionine in the brain of man is higher than in the brains of other primates and notably higher than that of lower mammals and birds. An absence of brain cystathionine is associated with homocystinuria (12, 14) and a supernormal concentration of the amino acid may be present in patients with cystathioninuria (12, 77). Both of these disorders may be characterized by mental deficiency. However, the significance of the absence of cystathionine from the brains of patients with homocystinuria remains unknown. Perhaps it serves an essential function in the development of the brain, although the amino acid is absent from human brain until late fetal life. The probability that cystathionine may play a special role in the functioning of the central nervous system notwithstanding, the diverse and numerous manifestations of homocystinuria are not easily explained by a deficiency of this compound alone, since it is only barely measurable in liver and connective tissue.

A block in the transsulfuration of methionine will obviously result in cysteine becoming an "essential" amino acid. This conclusion has been substantiated by nitrogen balance studies (11) which

demonstrated that positive nitrogen balance could be maintained in homocystinuric patients by administration of a diet in which half of the dietary sulfur was supplied as methionine and half as cysteine. When methionine was decreased with respect to cysteine this positive balance increased. However, when the entire dietary source was methionine, profound changes in the overall rate of protein synthesis, as demonstrated by a large negative nitrogen balance, occurred. The plasma cysteine concentration in patients with homocystinuria is generally barely measurable. The possibility that a cysteine deficiency may be a major factor in the pathogenesis of homocystinuria was recognized early (21). A generalized decrease in protein synthesis in the body, due to a cysteine deficiency, could have multiple effects. The defective synthesis of mucopolysaccharides and the accumulation of neutral lipid in the liver of these patients may result from this overall reduction. Obviously, a slowdown in protein synthesis during differentiation of the developing brain may generate numerous pathological phenomena.

Treatment

Although it is likely that the changes in plasma and tissue concentrations of the sulfur-containing amino acids in homocystinuria are associated with the characteristic clinical features, the mechanism for the pathophysiology of the disease has not been established. However, it has been suggested (134) that patients with high plasma homocystine

concentrations are likely to show more severe clinical manifestations. than patients who have relatively low homocystine levels. The second group tends to have higher plasma methionine concentrations. Thus, homocystine, homocysteine, or their derivatives may be more harmful than high methionine levels. In this case, an important goal of treatment would be the maintenance of the lowest possible plasma homocystine concentrations.

Another therapeutic consideration is the age of the patient. If the condition is detected early in infancy, or ideally, prenatally, the aim of therapy would be to prevent the development of mental and visual impairment, skeletal abnormalities and vascular problems. If the problem is discovered in an older child or an adult, in whom much of the damage to intelligence, lenses and bones has already been done, the prevention of possibly fatal intravascular thromboses is important. Whether the reduction of homocystine concentrations, either by dietary manipulation or by megavitamin therapy, in the plasma of an already damaged patient will prolong life and increase the chance for good health is an unanswered question at this time.

The therapeutic approach to homocystinuria, as in other metabolic disorders, has been twofold. Potentially toxic products accumulating proximal to the block must be eliminated, while those substances that are deficient as a result of the block must be supplied (52). Thus, the first form of treatment attempted in these patients consisted of diets restricted in methionine and supplemented with

cysteine (74, 93, 133, 134, 142). Diets such as these are difficult to dispense and, as methionine is normally present in plasma at very low levels, biochemical control over any long period of time is difficult and dangerous.

However, Perry and his coworkers have reported successful treatment of homocystinuria with a low-methionine diet, supplemental cystine, and choline as a methyl donor (134). They found that plasma homocystine levels were significantly reduced in three children with homocystinuria, who were unresponsive to massive doses of pyridoxine, through the use of a diet providing only 10mg of methionine/kg of body weight/day. This was supplemented with L-cystine and large doses of choline which may act by facilitating the remethylation of homocysteine to methionine. They also reported a three year old child who had been treated for homocystinuria since early infancy by dietary manipulation, and who remained mentally and physically normal, although both of his sibs were severely affected by six months of age.

In 1967 Barber and Spaeth (4) reported that some cases of homocystinuria responded to large doses of vitamin B6 in the form of pyridoxine-hydrochloride. Although a number of patients have since been shown to respond to the vitamin (5, 20, 27, 59, 85, 174), others have responded only incompletely or not at all (20, 27, 60, 74, 75, 134, 149, 174). Even in the patients who do respond, that is, whose plasma concentration and urinary excretion of homocystine

return to normal, the characteristic clinical abnormalities that have previously developed remain. Methionine-loading tests administered before and after successful treatment with pyridoxine have shown that methionine metabolism, although improved, is still abnormal, demonstrating that the metabolic block persists (59,60). But again, the response may hinge on the time of administration of the vitamin. Perhaps prenatal or early postnatal treatment is essential if a true "response" is to be achieved.

Homocystinuria due to cystathionine synthase deficiency is actually but one of a number of diseases in which the clinical and/or chemical abnormalities remain on a normal intake of a particular vitamin, but can be alleviated with pharmacological doses of the same vitamin. There are in fact, over one dozen "vitamin-dependent" genetic diseases, involving at least six different vitamins. They are characterized by being inherited, by involving one or another specific biochemical abnormality, and by responding not to physiologic replacement therapy, but only to massive doses of the vitamin in question. In the three B6-responsive genetic diseases in which the enzymatic defect is known, the affected enzyme normally requires pyridoxal phosphate as a coenzyme.

The precise mechanism of action of vitamin B6 in homocystinuria is as yet unclear. However, an important concept has emerged from studies of the response to B6. It is evident that genetic heterogeneity exists among patients with homocystinuria and is manifest in

their response to the vitamin (62). Patients who show the least residual enzymatic activity appear unresponsive or only slightly responsive to vitamin therapy. These patients generally show no stimulation of cystathionine synthase activity by B_6 either *in vivo* or *in vitro*. Patients who evidence a "complete" biochemical response to the vitamin *in vivo* appear to have higher residual activity. Some of these patients show increased enzymatic activity in response to B_6 *in vitro*. Seashore *et al.* (146) found that when large concentrations of pyridoxal phosphate were added to the assay medium of lysates from cultured skin fibroblasts, enzymatic activity was seen to increase in one, although not in another, patient who was responsive to B_6 *in vivo*. A recent study by Uhlendorf *et al.* (164) involved the measurement of cystathionine synthase activity in extracts of fibroblasts from synthase-deficient patients at varying concentrations of pyridoxal phosphate. Of 25 synthase-deficient, clinically responsive patients, 24 had detectable synthase activity (0.1 to 10% of mean control value) in fibroblast extracts assayed without addition of pyridoxal phosphate. In addition, their fibroblast synthase activities were not stimulated by the addition of pyridoxal phosphate any more than were extracts of control cells (30-50% stimulation was obtained with increasing concentration of pyridoxal phosphate). Of 10 synthase-deficient, non-responsive patients, 9 had no detectable synthase activity in fibroblast extracts. Interestingly, there was one non-responsive patient who did demonstrate synthase activity, and

this activity was unusual in that it was enhanced ten-fold by the in vitro addition of pyridoxal phosphate. Thus, there appears to be a strong correlation between the presence of detectable synthase activity in fibroblast extracts from synthase-deficient patients, and the responsiveness of the patients to treatment with vitamin B_6. When extracts of liver were assayed with large concentrations of pyridoxal phosphate no increased cystathionine synthase activity was seen by two groups (62, 113), although a third group did see higher activity (174). These two tissues may respond differently to in vitro addition of pyridoxal phosphate for reasons unrelated to the enzymatic lesion under study; e.g., the effects of culture conditions on skin fibroblast cells.

Thus, it would appear that at least three classes of cystathionine synthase deficiency can be distinguished. These include a B_6-unresponsive type, and two B_6-responsive types; one in which enzymatic activity is increased with vitamin therapy and one in which no stimulation is observed. However, in none of the cases has the precise molecular mechanism for the vitamin-mediated response been identified. There are a number of possible explanations for the response to very large amounts of vitamin cofactors in genetic diseases (139). It is possible that the vitamin cannot get into the cell or that there is a problem in the conversion of the vitamin into its coenzymatically active form. Neither of these mechanisms could explain the situation with regard to homocystinuria, since pyridoxal phosphate is the major

form of pyridoxine acting as a coenzyme in man and no other pyridoxine-dependent reactions are upset. Another feasible explanation would involve the synthesis of a mutant apoenzyme that shows an altered association with its coenzyme. Large amounts of the cofactor might enhance enzymatic activity. This type of situation could explain the increased activity of cystathionine synthase seen in some patients who respond to B_6 with greater enzymatic activity. Alternately, the coenzyme may act to stabilize a mutant synthase apoenzyme. In the rat, pyridoxine has been shown to increase enzymatic activities by decreasing the degradation rate of the enzyme protein (72). Pyridoxine is also known to stabilize enzymes during purification procedures. If pyridoxine acts by stabilizing the synthase apoenzyme for which it serves as a cofactor, it would also explain the increased activities of cystathionase and serine hydroxymethyltransferase, two other B_6-linked enzymes which increase in activity after pyridoxine treatment (62). This hypothesis would also account for the response to B_6 in patients who show increased cystathionine synthase activity, but not those whose enzyme levels do not rise. The B_6 response of this latter group may be the result of the stimulation of an alternate pathway of sulfur amino acid metabolism, although no positive evidence for this hypothesis has been noted (95). The defect in those patients who do not respond to massive doses of vitamin B_6 may involve the production of a mutant enzyme whose lesion lies in an area unrelated to its coenzyme, such as its ability to

bind its substrate efficiently.

Patients who do show a response to B_6 tend to have measurable residual enzymatic activity and the B_6 may act, in some of them, to enhance this activity, although not to near-normal levels. However, even this small enhancement may be metabolically important. We still do not know the minimum enzymatic activity required by the liver to adequately metabolize the methionine presented to the organ (62). Mudd has suggested that a small (1-3%) increase in cystathionine synthase activity, after pyridoxine treatment, could explain the improved metabolism of methionine to inorganic sulfate (113, 164). His group suggested that the clinical response to vitamin B_6 treatment correlated, not with the in vitro stimulation of residual synthase activity by addition of pyridoxal phosphate, but with the presence of traces of residual synthase activity (164). They found that less than 1% of the mean control value indicated a capacity to respond, and patients whose fibroblast extracts lack even this small activity do not respond to pyridoxine. They speculated that this pattern suggested that the B_6 response occurs by an effect on the synthase itself; this effect involving enhancement of residual synthase activity to a few (3-4) per cent of normal. Obviously heterozygotes, who produce less than half the amount of enzyme as do homozygous normal individuals, metabolize enough methionine to prevent the clinical problems seen in patients. That the normal person is producing much more cystathionine synthase than is needed daily has also been calculated (58).

It appears (62) that 15-30% of mean control cystathionine synthase activity is sufficient for maintainence of normal plasma amino acid concentrations, and sometimes for a near-normal ability to metabolize a methionine load.

If small stimulation of residual enzyme activity is physiologically significant, low methionine diets may prove harmful by reducing this activity in homocystinurics. However, at present it seems reasonable to maintain on vitamin therapy those patients who respond to B_6 and to recommend some dietary restrictions and/or supplementation to those who do not.

Tissue Culture and Inborn Errors of Amino Acid Metabolism

Advances in the past ten years in somatic cell genetics and tissue culture have made it possible to study an increasing number of biochemical disorders in vitro. Cell cultures derived from humans known to be homozygous or heterozygous for specific genetic lesions of a biochemical pathway have been employed for the in vitro analysis of a variety of mutant gene products. The initial application of tissue culture to biological investigations was the work of Harrison in 1907, who demonstrated that nerve cells would continue to proliferate and function in vitro (78). However, until recently the principal use of mammalian cell cultures was in the study of malignancy and virus-host interactions. The utilization of human cell culture techniques for the study of genetic diseases, especially the inborn errors of

metabolism, has taken place only since the early 1960's. Perhaps the major stimulus for this type of investigation was the demonstration by Tjio and Puck (163), and later by Hayflick and Moorhead (80), that cells derived from human tissues can be grown in culture without difficulty, and that these cells will maintain the chromosomal complement of their original tissue, as well as a number of enzymatic activities functional in vivo. It was also shown (79, 80, 158) that fibroblastic lines derived from human fetal and adult tissues have a predictable and limited lifespan in culture; the cells will double approximately fifty times during a period of thirty to fifty weeks.

Obviously, metabolic disorders can be studied in tissue culture only if the specific enzyme is detectable in normal cultured cells. Many biochemists believed that the limited metabolism of fibroblastic cells in vivo would restrict the biochemical variation that could be expressed in vitro. However, most of the enzyme defects associated with the inborn errors of metabolism are found in several tissues and are thus manifest in cells cultured from biopsies of skin or other sources. An important realization was that these cell lines continue to express the specific genotype of their tissue of origin. This "genetic stability" has been amply demonstrated by investigation of individuals affected with one of a number of biochemical genetic disorders. Fibroblast cultures from many of these patients have been shown to express the relevant biochemical defect. But, not all biochemical lesions are exhibited by cultured cells. Phenylketonuria,

which is caused by deficient activity of the enzyme phenylalanine 4-hydroxylase, cannot be studied in tissue culture because normal fibroblast cells lack this enzymatic activity. Similarly, cystinuria and Hartnup disease, both of which are characterized by defects in renal and intestinal amino acid transport, cannot be demonstrated in cultured cells (73).

Investigation of the skin fibroblast in tissue culture has proven quite productive. Fibroblast cultures offer a number of advantages not found with biopsies of solid tissue such as liver. Obviously, the ease and safety of obtaining the specimen is an important consideration. In addition, cultured cells provide a potentially large reservoir of material over an extended period of time, whereas a biopsy specimen furnishes a limited and often small amount of tissue. Cultured cells can also be used to analyze the secondary metabolic derangements resulting from specific biochemical defects, as well as the primary lesion itself. One can easily alter such environmental conditions as the constituents of the culture medium, or apply various stress factors to these living cells. Alternately, strictly controlled conditions can be maintained. Replicate experiments are easily performed, and drug and radiation therapy can be attempted without concern for the safety of the patient. For these and numerous other reasons, techniques employing human fibroblastic cells, grown and multiplied in tissue culture, have been repeatedly applied to the investigation of human biochemical genetics. These

studies have furthered the understanding of the mechanisms responsible for inherited metabolic diseases, substantiated possible genetic modes of transmission, and extended our awareness of the large degree of heterogeneity that is manifest. Furthermore, they have led to the development of new techniques for the diagnosis and treatment of homozygous affected individuals, and the identification of asymptomatic heterozygous carriers. Investigations of the cells cultured from patients have led to the detection of the specific enzymatic lesion responsible for a number of genetic disorders including hyperlysinemia (29) Refsum's disease (81, 82, 155) and the X-linked Lesch-Nyhan syndrome (140, 147). Disorders involving abnormal sulfur amino acid metabolism have recently begun to be elucidated through the use of tissue culture systems.

In "minimal" medium, and under ordinary conditions of cell culture, cystine is required for cellular survival and growth by a number of cultured human cells (37). However, Eagle et al. (38, 39) have found that many heteroploid human cell lines were capable of synthesizing cystine from methionine, via homocysteine and cystathionine, at high population densities. The normal cystine requirement results from the loss of the amino acid and its precursors from the cellular pool to the surrounding medium in amounts which exceed the biosynthetic capacity of the cell. Thus, at sufficiently high cell population this nutritional requirement disappeared, as the concentration of newly synthesized cystine and its precursors in the medium

and in the cellular pool could be raised to metabolically efficient levels before the cells succumbed to cystine deprivation. These observations provided evidence that the ability to synthesize cystine in vivo was a general biosynthetic capacity of human cells and not restricted to the liver.

In a later study (40), Eagle's group had the opportunity to investigate the synthesis of homocysteine, cystathionine and cystine by normal human diploid cell lines and, in addition, by lines derived from what appeared to be a familial case of cystathioninuria (47, 77). Cultured fibroblasts derived from the patient died in a cystine-free medium, even at the highest population density attained, and regardless of the precursors that were added, including cystathionine. However, normal human diploid cell lines appeared to behave in the same manner. None could survive in a cystine-free medium containing methionine or homocysteine, and most could not utilize cystathionine in lieu of cystine, however high the population density. Again the heteroploid lines were seen to carry out all of the reactions necessary for the synthesis of cystine from methionine: the synthesis of homocysteine from methionine, the synthesis of cystathionine from homocysteine, and the utilization of cystathionine for survival and growth in a cystine-free medium. The diploid lines were not blocked in the synthesis of homocysteine from methionine, although the average activity was less than that of the hetroploid cultures. Similarly, most of the diploid lines were

able to condense homocysteine and serine to form cystathionine. Yet all of the diploid cultures were blocked in the conversion of cystathionine to cystine, and thus were dependent upon the addition of cystine to the medium, due to lack of the cleaving enzyme cystathionase. Other evidence, including the report of Mudd (116), has supported the suggestion that weak or absent cystathionase activity may be an inherent property of many human tissues and not a reflection of in vitro culture factors. Thus, the non-essential role of cystine in vivo may be due to the biosynthetic capacity of only a few organs such as the liver, pancreas and kidney (116). Cystathionase activity has been detected in cultured long-term lymphocytes (132).

Cystathionine synthase activity has also been investigated in different mammalian tissues (116). Generally, enzymatic activity is present in liver, brain, pancreas and kidney, but absent from heart and skeletal muscle. In 1968 Uhlendorf and Mudd (165) demonstrated cystathionine synthase activity in tissue culture derived from human skin. They determined enzymatic activity of cystathionine synthase and of methionine-activating enzyme in cultured fibroblasts from normal controls and from patients affected with homocystinuria. There were no differences in culture characteristics between the two groups. Fibroblasts cultured from the skins of affected individuals demonstrated normal levels of methionine-activating enzyme when compared to control lines. However, fibroblasts from four of the

six patient lines showed no detectable cystathionine synthase activity and the other two exhibited enzymatic activity that was 2-3% of that seen in normals. This established that the metabolic lesion responsible for homocystinuria is demonstrable in tissue culture. Interestingly, neither methionine-activating enzyme nor cystathionine synthase could be detected in full-thickness human skin, dermis or epidermis. These investigators also assayed cystathionine synthase activity in cultured amniotic fluid cells, finding a much higher mean activity than that found in adult fibroblasts.

The utilization of cultured skin fibroblasts in the diagnosis of numerous cytogenetic, enzymatic and metabolic disorders in man has recently been followed by efforts at making specific prenatal diagnoses with the use of cultured amniotic fluid cells. Diagnostic amniocentesis has proven itself useful to the physician who is monitoring pregnancies at high risk for genetic disorders (126). This technique is also valuable for the study of the early stages of inborn errors of metabolism in man. Biochemical and/or structural abnormalities resulting from the genetic lesion have been found in the cells and organs of young fetuses affected with a number of different metabolic diseases including: Tay-Sachs disease (130, 145), Niemann-Pick disease (86), Pompe's disease (26, 124, 127, 141), Krabbe's disease (122), and Gaucher's disease (122).

Assuming that the cellular material found in amniotic fluid is derived from the fetus and amnion (166), the biochemical, cytological

and cytochemical analysis of cultured amniotic fluid cells would seem to be a reliable method for the prenatal detection of genetic disorders. More than thirty familial metabolic disorders are, at least potentially, diagnosable in cultured amniotic fluid cells, and indeed, half of these have already been detected in utero (123). However, the detection of inborn errors of metabolism by assay of enzymatic activity in cultured amniotic fluid cells rests upon two premises; that the enzyme detected in cultured cells derived from skin is the same isozyme deficient in the disorder under study, and that these data can be extended to amniotic fluid cells. Thus, the initial problem is to establish that cultured skin fibroblasts demonstrate the enzymatic aberration in question. The metabolic state of cells in tissue culture is obviously subject to numerous biologic, genetic, and in vitro culture variables. In addition, the extrapolation of this biochemical data from post-natal skin fibroblasts to cultured amniotic fluid cells must be preceded by the characterization of the enzyme during fetal life. Are the biochemical manifestations of cultured amniotic fluid cells representative of the metabolic state of the fetus? A comparison of the specific activity and the kinetics of the enzyme in cultured adult and fetal fibroblasts and amniotic fluid cells is essential. The range of activity in cultured skin fibroblasts from heterozygotes and homozygotes for the disease under study, as well as from an adequate number of normal controls must be determined. The ability to distinguish the heterozygous carrier from

the other two groups is critical. Finally, the range of activity in normal amniotic fluid cells from fetuses of appropriate gestational age must be established before any attempt is made to diagnose the defective state in utero. Throughout all of these studies strict control over culture conditions must be maintained so as to minimize biochemical variation.

As of 1972, 175 inborn errors of metabolism had been described in man, 35 of which are diagnosable by use of white blood cells (86). Thus, another system for the in vitro study of enzymes is provided by the utilization of cells derived from peripheral blood. Of enormous potential is the ability to establish virtually permanent cultures from peripheral blood and the fact that these continuously propagated lymphocytes are valuable for the study of human biochemical genetics. Long-term lymphocyte cell lines have been established in a number of laboratories from normal individuals and patients with various cytogenetic and biochemical abnormalities. A lymphocytoid line derived from the peripheral blood of an individual will retain its original genotype in continuous culture (83). These lines proliferate abundantly in easily maintained suspension cultures for an apparently indefinite period of time. In addition, many of the enzymes involved in the inborn errors of metabolism have been detected in lymphoid lines from normal donors. Thus, a biochemical defect involving one of these enzymes can be investigated in long-term lines derived from affected individuals. A large amount of material

can be produced, via rapid multiplication of these cells, all of identical cytogenetic and biochemical constitution. This allows for detailed study of the enzymatic lesion and the secondary metabolic aberrations resulting from it. These cultures provide a continuous system for the in vitro study of metabolic problems such as cofactor requirements and the effects of various metabolites on normal and deficient cells.

Thus, the tissue culture of human cells, derived from both skin biopsies and peripheral blood, has lent itself well to the in vitro investigation of numerous problems in human biochemical genetics, including the investigation of aberrant sulfur amino acid metabolism. Yet, there remain a number of unsolved problems in the study of homocystinuria due to cystathionine synthase deficiency. Clearly, the affected enzyme must be investigated using new techniques of tissue culture and microassay. Specific and sensitive assays must be developed in order to maximize the enzymatic activity found in cultured cells. The properties of the enzyme must be established and comparisons made between tissues of different origin and/or age. Are the characteristics of the synthase in cultured cells amenable to the hypothesis that we are studying the same enzyme in vitro as in vivo? Are the manifestations of genetic heterogeneity observable in cultured cells as they are in liver biopsy specimens? Can we determine, with relative ease and accuracy, the heterozygous state of the disease? And, are the techniques and knowledge at hand

sufficient to justify an attempt at the prenatal diagnosis of the disease? This dissertation is an attempt to answer some of these and additional questions concerning the nature of cystathionine synthase and its deficiency, by utilizing normal and deficient cultured human cells.

II. MATERIALS AND METHODS

Preparation of Cultured Cells

Skin fibroblasts. - Skin biopsies were obtained from the flexor surface of the forearm or from the foreskin of patients, obligate and potential heterozygotes, and normal controls. Biopsies were also taken from five second trimester fetuses that were aborted by hysterotomy. The fibroblast cultures were established using standard culture procedures. Each biopsy specimen was diced and the pieces allowed to attach to the floor of a plastic culture flask. After attachment, 5 ml of "complete" medium (McCoy's 5A modified medium containing 30% by volume of fetal calf serum, 100U penicillin and 100 μg streptomycin/100 ml, and 1% of 200 mM glutamine) were introduced into the flask. The cultures were maintained in a humid atmosphere of 10% CO_2 in air at 37°for approximately seven days. Following this, the culture medium was changed twice weekly. After several (3-5) weeks, during which time fibroblast cells migrated from the original explant and divided, the cultures were treated with 0.25% trypsin and established as monolayer cultures. These cultures were harvested for measurement of enzymatic activity between the third and eighth subculture.

Care was taken to assure that all the cultures were at a similar stage of early confluence before the final feeding program was initiated. This program consisted of feeding the cultures consecutively for three days and harvesting on the fourth day. On that day the cultures were treated with 0.25% trypsin for 1 minute at 37°, and with 0.02% Na EDTA for 15-20 minutes. The cells were then washed three times with 0.9% saline and lysed by freeze-thawing, using a slurry of acetone and dry ice.

Amniotic fluid cells. - Amniotic fluid, obtained from second trimester pregnancies by transabdominal amniocentesis, was divided into 5-ml aliquots and each aliquot was centrifuged for 10 minutes at 150 x G. The cell pellet from each tube was then resuspended in 5 ml of "complete" medium and the suspension placed in a plastic culture flask. The cultures were incubated for 4 days in a humid atmosphere of 10% CO_2 in air, at 37° . The culture medium was changed on the fifth day and new medium was introduced twice weekly thereafter. After several (3-4) weeks the cultures were trypsinized and established as monolayer cultures which were then handled in the same manner as the skin fibroblast cultures.

Long-term lymphoid cell lines. - Long-term lymphoid lines were established according to the method of Beratis and Hirschhorn (7). Blood (30-50 ml) was drawn from patients, obligate and potential heterozygotes and normal controls, using a plastic syringe with heparin as anticoagulant. The blood was allowed to sediment in the

syringe for approximately 1-2 hours and the supernatant plasma containing white blood cells was removed from the syringe through a bent needle. This plasma suspension was centrifuged at 150 x G for 15 minutes, the supernatant removed, and the cell pellet gently disrupted. The leukocytes were suspended in 10 ml of RPMI 1640 medium and the cell suspension was recentrifuged. The sedimented leukocytes were resuspended in "complete" medium (RPMI 1640 medium containing 30% by volume of fetal calf serum, 1% of 200 mM glutamine, 100U penicillin and 100 μg streptomycin per ml). Replicate cultures were prepared, each containing 1-2 x 10^7 lymphocytes in a volume of 6 ml of "complete" medium in a plastic culture flask. Purified phytohemagglutinin (PHA) was added at a concentration of 1 μg/ml, and the flasks were incubated at 37° in the presence of 10% CO_2 in air. Epstein-Barr virus (EBV) was added to some of the cultures as follows: After 48 hours the cultures were transferred to tubes and centrifuged at 150 x G for 15 minutes. The supernatant was removed, the pellet resuspended in 5 ml of "complete" RPMI medium, and the suspension transferred to the original flask. One ml of medium containing EBV at a concentration of 3.3 x 10^6 virus particles/ml was added to each flask. The cultures were then incubated at 37° in a 10% CO_2 atmosphere. The cells were fed every 4-5 days by centrifuging the culture flasks at 600 RPM for 10 minutes, removing 3 ml of the supernatant medium, gently shaking the flasks and replenishing with 3 ml of "complete" medium. For the estab-

lishment of lymphoid lines without EBV, the PHA was removed from the cultures at 48 hrs. and the cultures were fed with fresh "complete" medium.

Establishment of the lymphoid lines usually takes 4-6 weeks. Successful cultures exhibit a drop in the pH of the medium, an increase in cell number and formation of cell clusters. After establishment, the cultures were maintained by removing 4 ml of medium with suspended cells once a week, placing them in a new flask and feeding with 6 ml of RPMI 1640 medium containing 20% of fetal calf serum and glutamine, penicillin and streptomycin. Once the lines had been established, no gassing with CO_2 was necessary. Preparation of multiple initial flasks increased the chances of establishing a lymphoid line from a given donor, and it was not unusual for only one of several cultures from the same individual to become established. The cultures were fed with fresh medium 24 hrs. before cells were obtained for enzymatic analysis. The cells were then washed with normal saline and lysed by freeze-thawing.

Enzyme Assay

The assay procedure is based on the direct measurement, on an automatic amino acid analyzer, of cystathionine formed in the reaction. Conditions were established for the attainment of maximal enzymatic activity. The concentrations below were found to produce optimal results: In a total volume of 0.4 ml, the following, in micro-

moles, were incubated for 4 hours at 37°: Tris-HCl buffer (0.66 M, pH 8.4), 60.0; pyridoxal 5'-monophosphate (PLP) in tris buffer, 0.5 (0.015 umoles were used for substrate curves); EDTA in tris buffer, 1.0; L-serine in tris buffer, 10.0; and L-homocysteine (made from the thiolactone), 15.0. The homocysteine and PLP solutions were prepared fresh daily. To this reaction mixture, 250 microliters of cell lysate was added as the incubation was begun.

The remainder of the procedure has been described by Gaull et al. (60). The reaction was stopped by adding 0.4 ml of cold 10% trichloracetic acid, chilling in ice for 15 minutes, and centrifuging at 10,000g for 10 minutes. At this point, 0.5 ml of the supernatant was added to 20 ml of water and passed over a 0.9 x 2.9 cm. column of Dowex 50-X^4 (H^+) 200-400 mesh resin. After application of the sample, the column was washed with 18 ml of water, 35.5 ml of 0.4 N HCl, and 12 ml of water, all of which were discarded. Cystathionine was then eluted with 3.9 ml of 3.4 N NH_4OH. This fraction was collected in a round bottom flask and dried on a rotary evaporator. The sample was redissolved in 1 ml of 0.1 N HCl and appropriate amounts (50-500 microliters) were analyzed on a 6 cm. column of Beckman UR-30 acidic and neutral resin at 55° using pH 3.21 sodium citrate buffer for elution. The columns were regenerated between runs with 0.2 N NaOH followed by a wash with the eluting buffer. The retention time of cystathionine (approximately 32 minutes) was checked with each series of analyses by applying authentic L-cystathionine. The

product of the reaction was identified as cystathionine in two other ways (60): (a) it was demonstrated that when both the reaction product and authentic L-cystathionine were applied to a column, a single symmetrical peak was observed; (b) the peak disappeared after oxidation of the reaction product overnight at 4° in hydrogen peroxide, as expected for a sulfur-containing amino acid. Protein concentrations were determined according to the method of Lowry et al. (98). Enzymatic activity is expressed as nmoles cystathionine formed/mg protein/hour.

Tissue culture medium (McCoy's modified 5A medium and RPMI 1640 medium) and fetal calf serum (tissue culture select) were obtained from Baltimore Biological Laboratories (Bioquest). Penicillin-streptomycin solutions, L-glutamine, and trypsin were from Grand Island Biological Company. Plastic flasks and pipets were from Falcon Plastics (Bioquest). Purified phytohemagglutinin was received from Burroughs-Wellcome. L-serine and L-homocysteine thiolactone were obtained from Calbiochem, pyridoxal 5'-monophosphate from Sigma, EDTA from Fisher, and Tris from Nutritional Biochemicals. Dowex-50 resin was from Biorad. Standard reagents and chemicals were best grade available.

III. RESULTS

Detection of cystathionine synthase activity in cultured human fibroblasts: optimum assay conditions. - The presence of cystathionine synthase activity in cultured skin fibroblasts was first demonstrated by Uhlendorf and Mudd in 1968 (165). However, the assay system employed by this group utilized conditions that had been developed for use with crude tissue extracts from rat liver (116) and in addition, was rate-limited with respect to the concentration of one substrate, serine. Subsequent studies (68, 146) have also depended on only slight modifications of this system. The method developed in the present work involved several modifications of the conditions utilized in previous assays; these conditions were systematically established as optimum. After lysis of harvested fibroblasts and centrifugation to collect the supernatant fraction, the production of L-cystathionine was proportional to protein concentration up to 1.3 mg/vessel (Fig. 2) and to time of incubation up to 5 hours (Fig. 3). The enzymatic activity was greatest at pH 8.4 (Fig. 4). Cystathionine synthase activity was completely dependent upon the addition of homocysteine and the rate of reaction was maximal in the presence of 10-15 μmoles of L-homocysteine with the

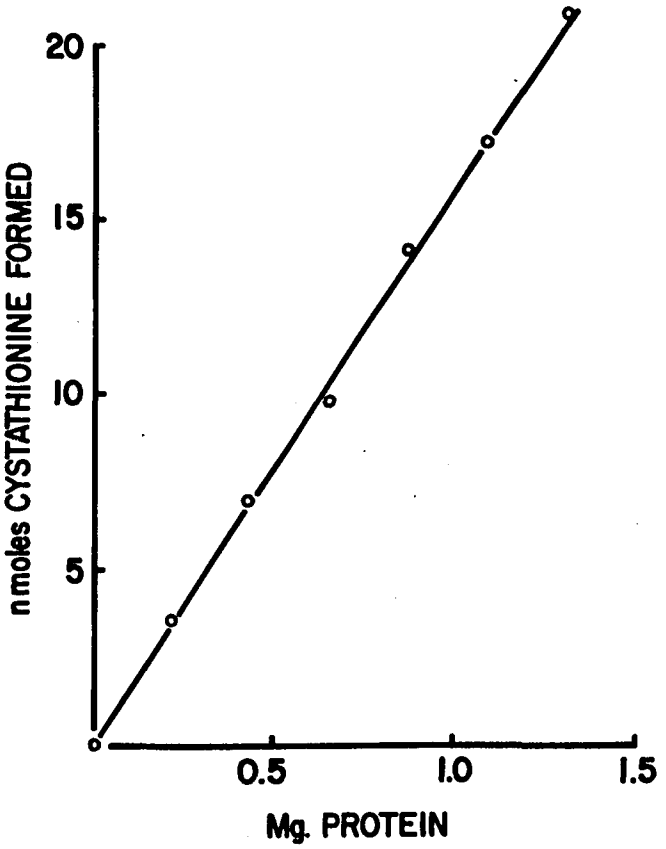


Figure 2. Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of protein concentration.

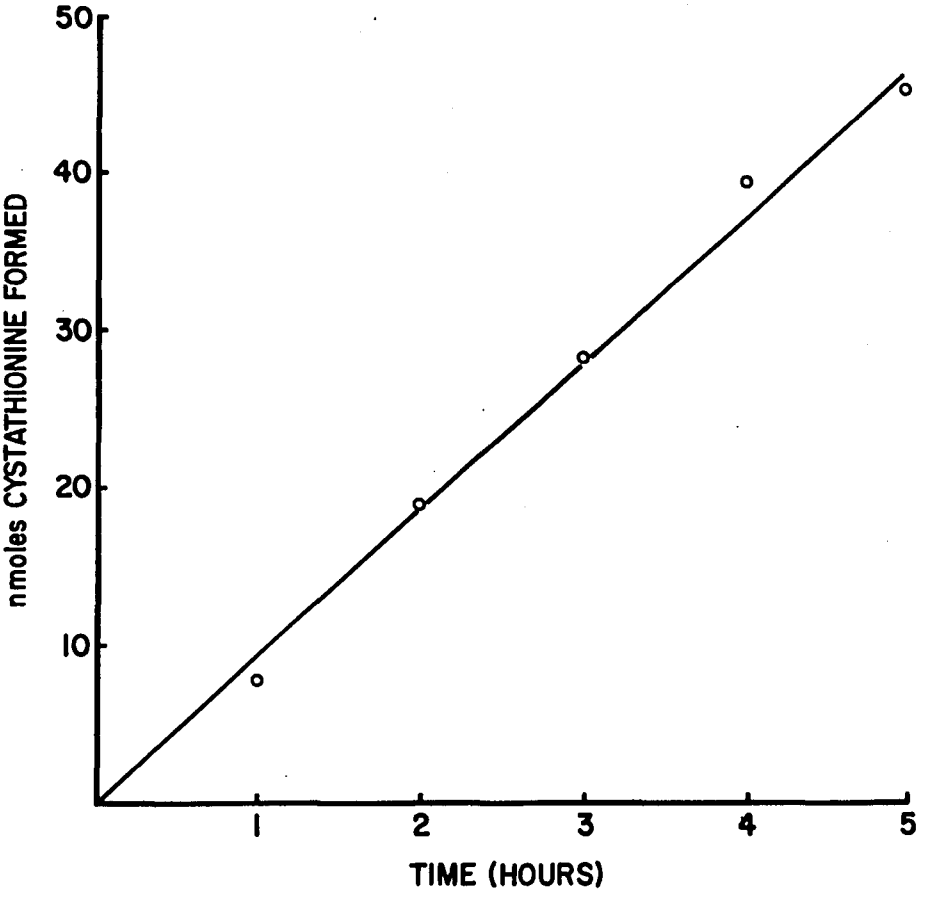


Figure 3. Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of time of incubation.

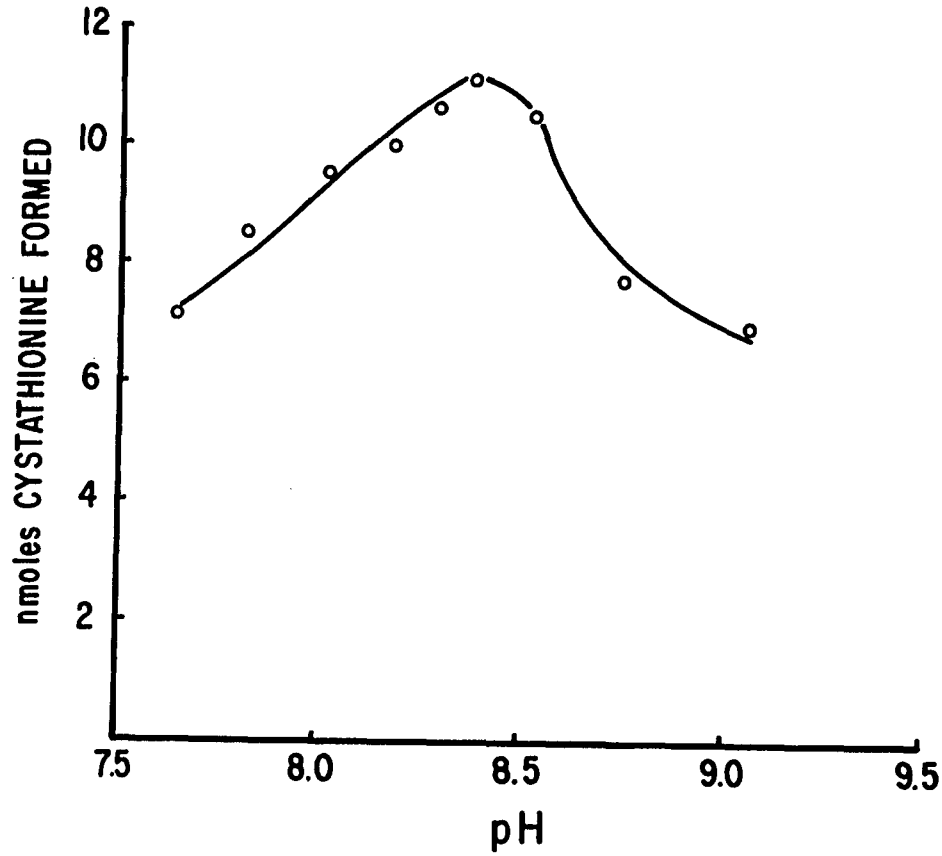


Figure 4. Effect of pH on cystathionine synthase from cultured human adult skin fibroblasts. Assay procedure as in the text, except that solutions and buffer were at the pH indicated.

highest activity consistently found at 15 μmoles (Fig. 5). A serine concentration of 8-13 μmoles produced the maximum reaction rate (Fig. 6), but it was noted that when no serine was added to the reaction mixture very small amounts of product were still detectable (<0.6 nmoles). These "blank" values, presumably the result of endogenous serine, were subtracted from the other points on the curve. The omission of pyridoxal phosphate from the assay mixture did not greatly alter the activity of the preparation from that obtained with the standard value of 0.015 μmoles. However, when the concentration of pyridoxal phosphate was increased to 0.5 μmoles, a significant increase in product formation was observed (Fig. 7). The omission of EDTA from the reaction mixture caused no reduction in L-cystathionine formation. Cystathionine synthase activity was completely destroyed when a boiled extract was used as a source of the enzyme.

Detection of cystathionine synthase activity in cultured fetal fibroblasts and amniotic fluid cells. - Cystathionine synthase activity was also determined in extracts of cells cultured from amniotic fluid obtained from women during the 14-20th week of pregnancy, and in extracts of cells cultured from the skin of human fetuses of the same gestational age, obtained by hysterotomy. Optimal substrate concentrations were established for both of these cell types and it was observed that a serine concentration of 8-15 μmoles (Fig. 8 and 9) and a homocysteine concentration of 10-15 μmoles (Fig. 10 and 11) produced maximal

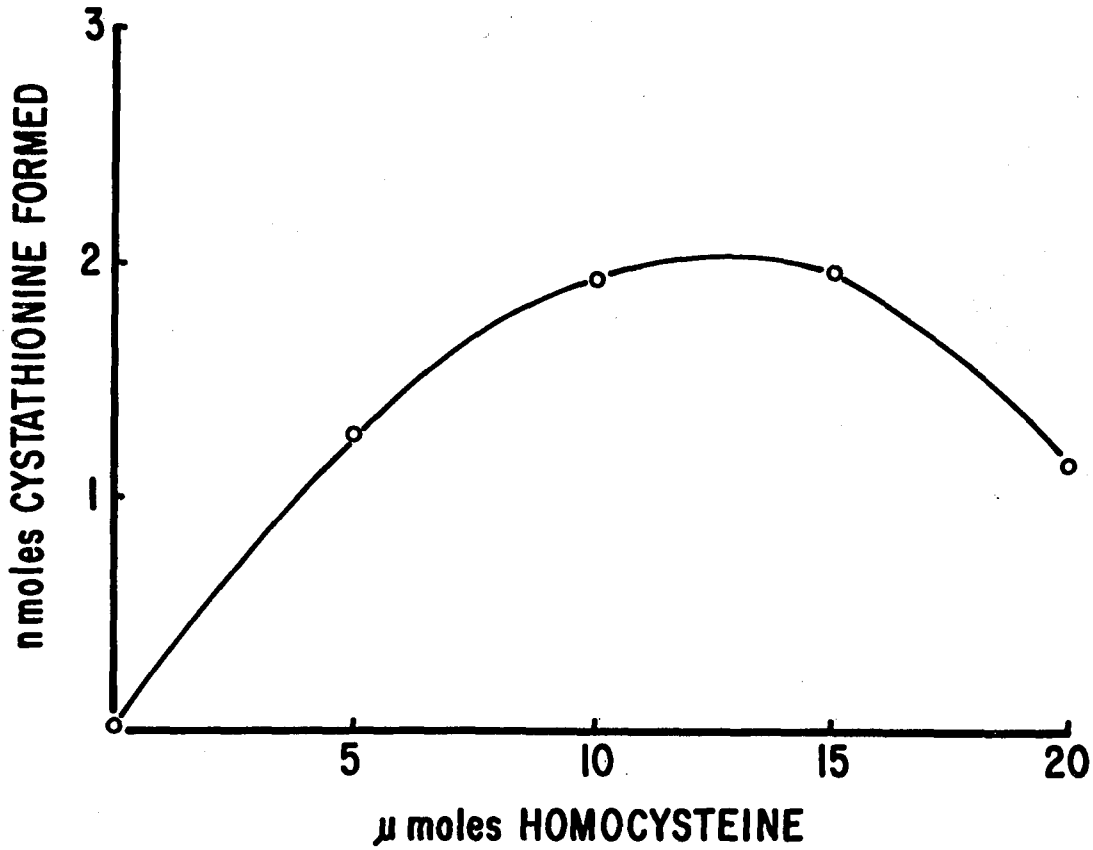


Figure 5. Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of L-homocysteine concentration.

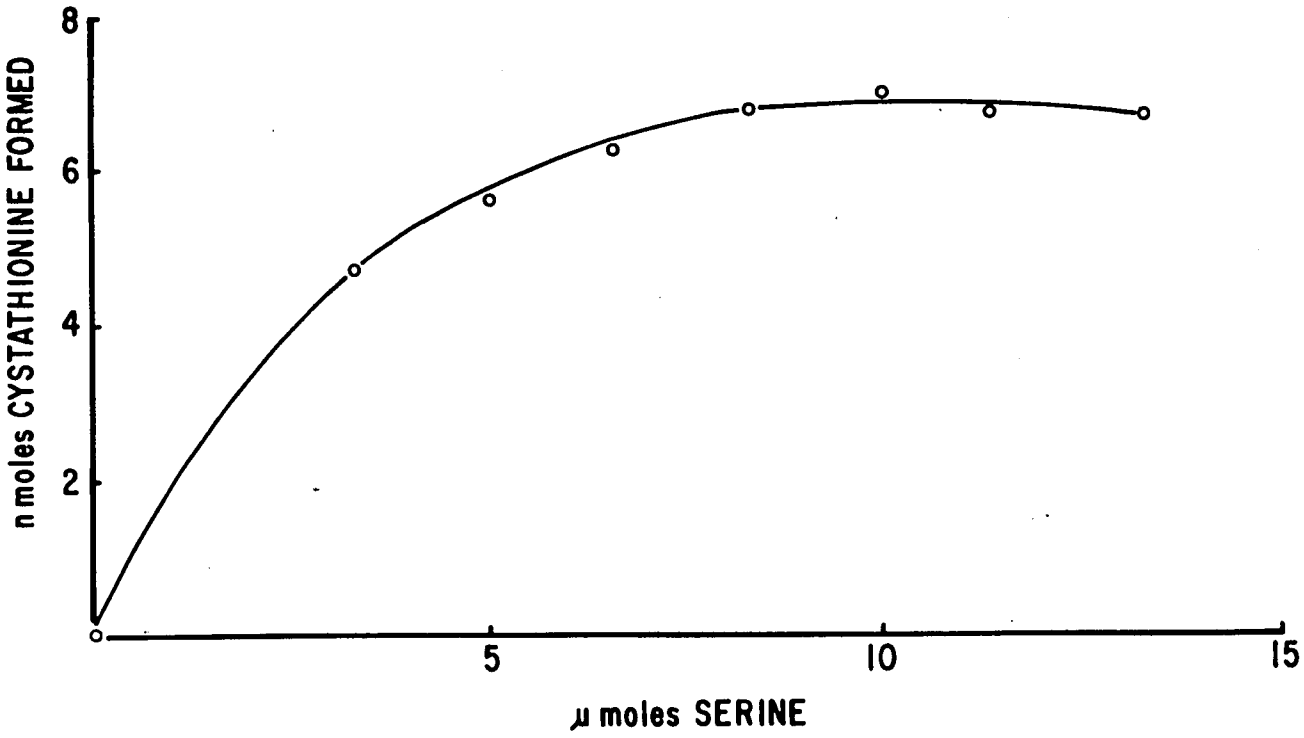


Figure 6. Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of L-serine concentration.

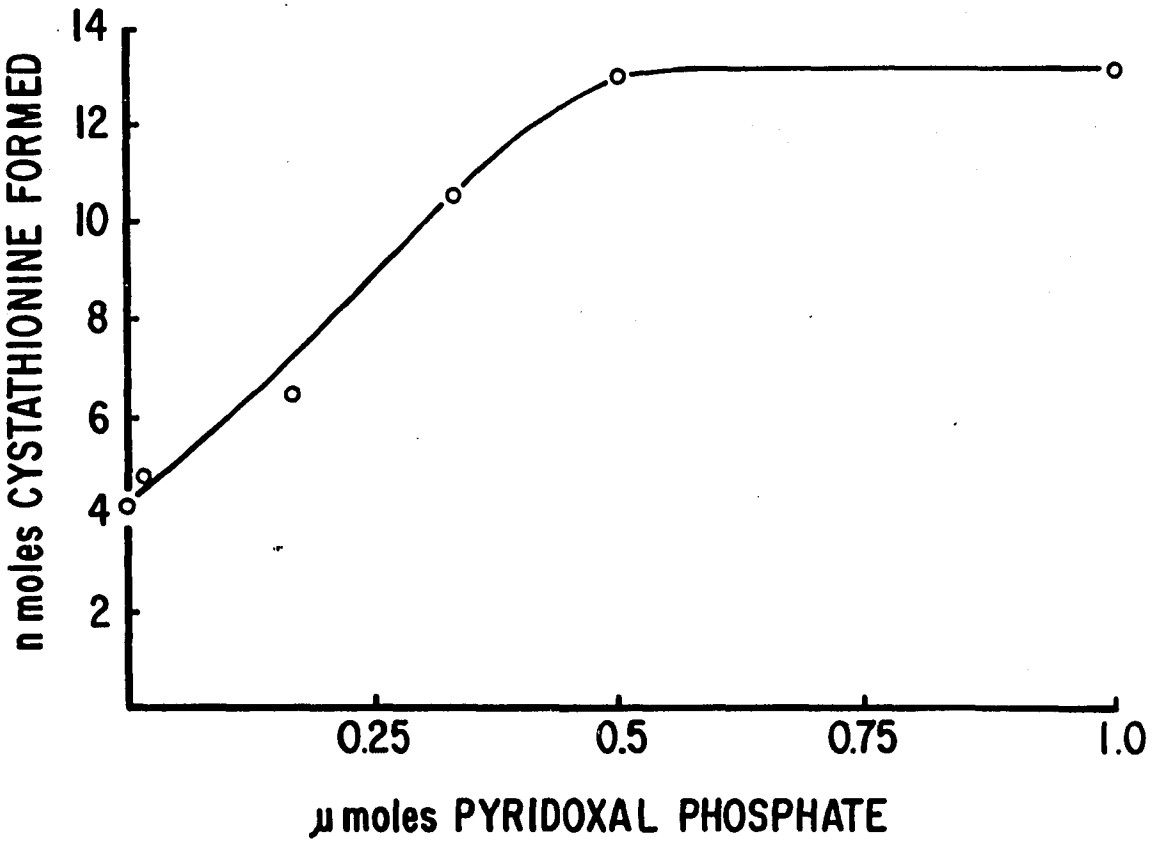


Figure 7. Rate of reaction of cystathionine synthase from cultured human adult skin fibroblasts as a function of pyridoxal phosphate concentration.

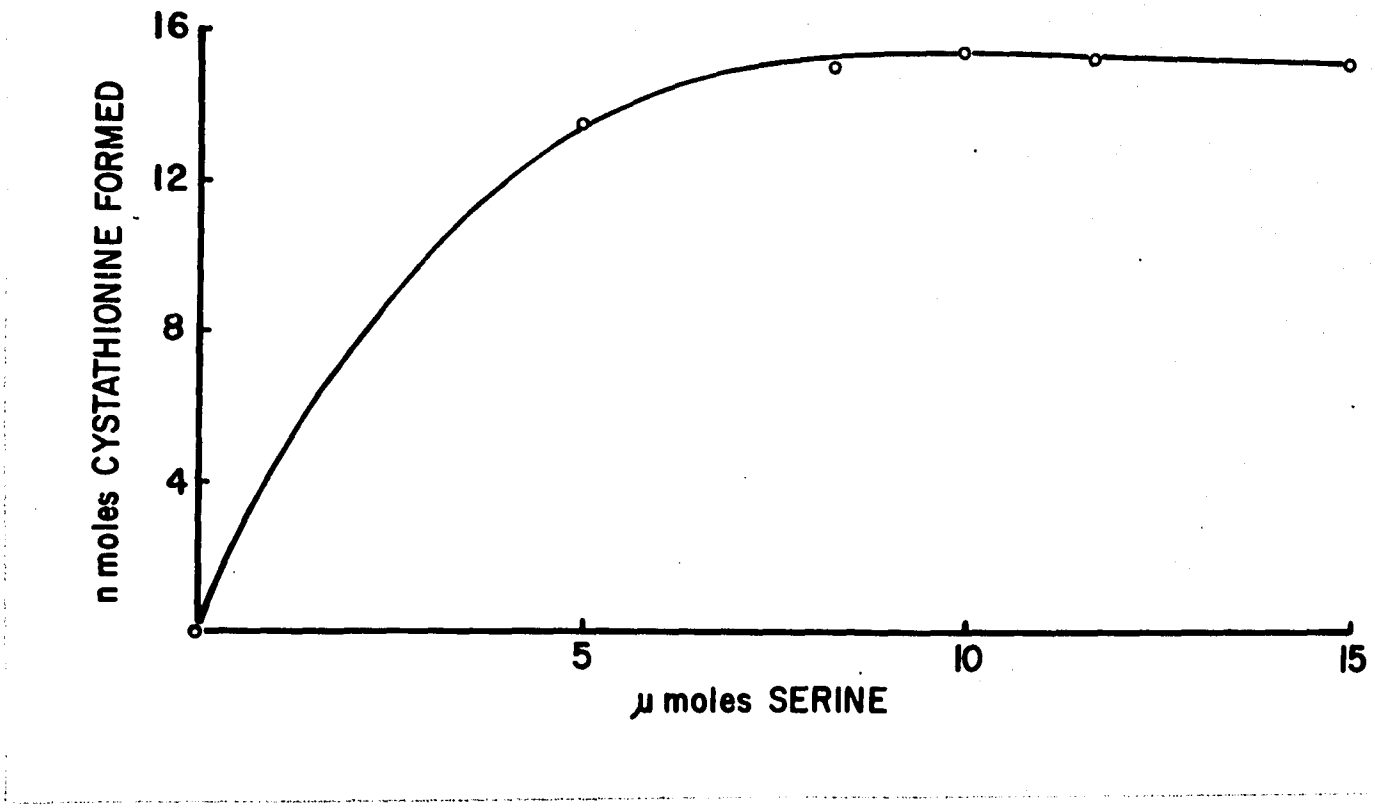


Figure 8. Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of L-serine concentration.

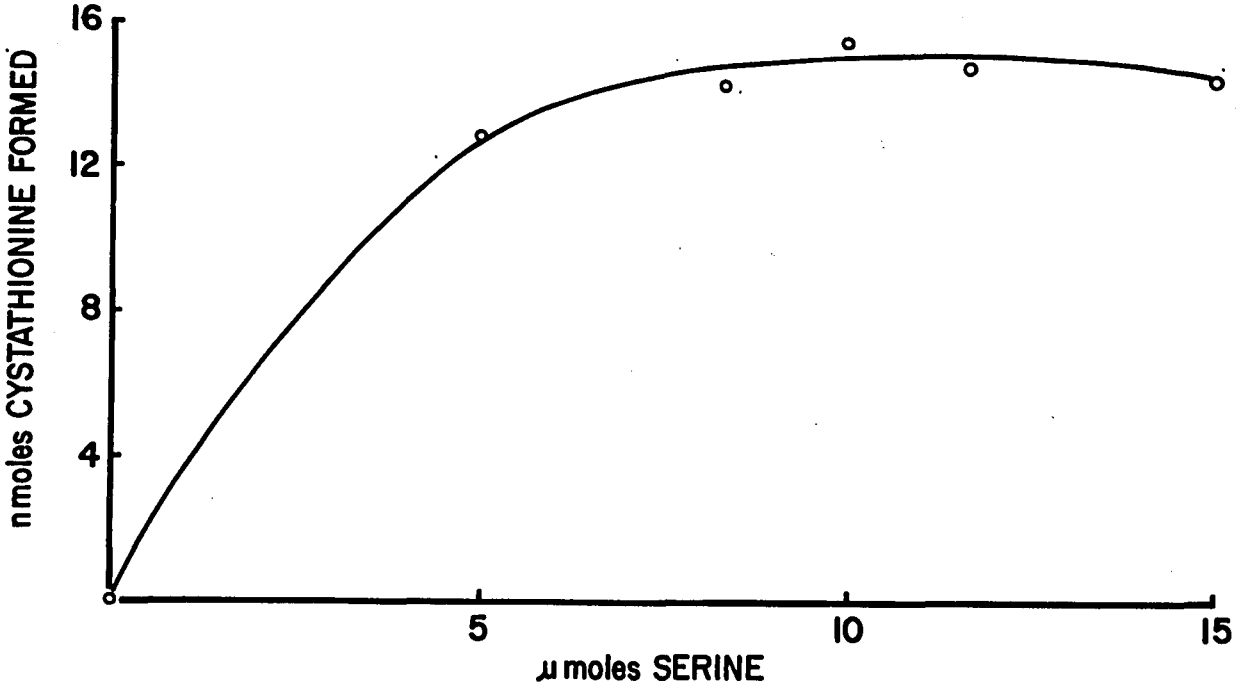


Figure 9. Rate of reaction of cystathionine synthase from cultured human fetal skin fibroblasts as a function of L-serine concentration.

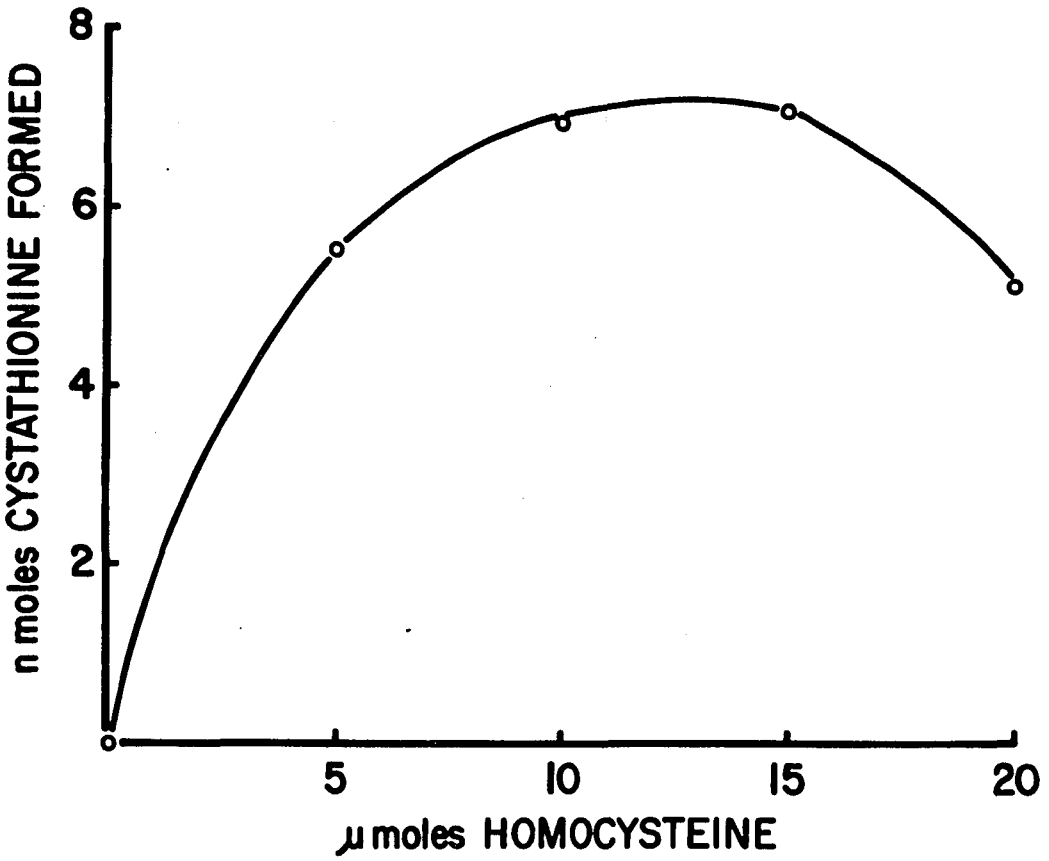


Figure 10. Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of L-homocysteine concentration.

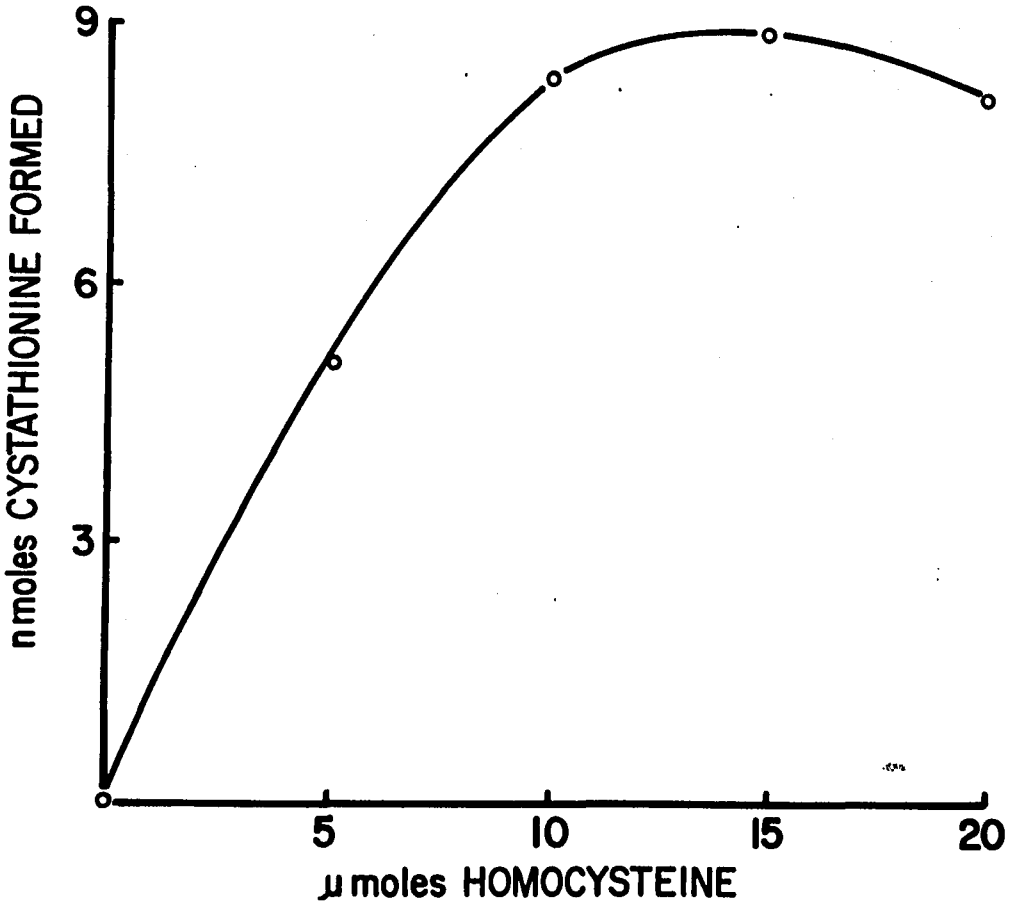


Figure 11. Rate of reaction of cystathionine synthase from cultured human fetal skin fibroblasts as a function of L-homocysteine concentration.

enzyme activity, as was found with the adult fibroblasts. Product formation was found to be maximal at pH 8.4 (Fig. 12). Production of L-cystathionine by the supernatant fraction of cultured amniotic fluid cells was linear with respect to amount of protein added up to 1.3 mg/tube (Fig. 13) and with respect to time of incubation up to 5 hours (Fig. 14). The increase in enzymatic activity with addition of larger concentrations of pyridoxal phosphate (up to 1 μmole) was much less than that observed with adult fibroblasts (Fig. 15).

Detection of cystathionine synthase activity in cultured long-term lymphoid cell lines. - Utilizing optimum assay conditions, cystathionine synthase activity was detected in extracts from cultured long-term lymphoid cells. The conditions were identical, in most respects, to those determined for cultured skin fibroblasts. A serine concentration of 10-15 μmoles (Fig. 16) and a homocysteine concentration of 15 μmoles (Fig. 17) were optimal. The pH optimum was found to be 8.4 (Fig. 18) and the production of cystathionine was linear with respect to time of incubation up to 5 hours (Fig. 19). Interestingly, the change in enzymatic activity in response to an increased concentration of pyridoxal phosphate was small (Fig. 20), resembling amniotic fluid cells rather than adult fibroblasts in this respect. Enzymatic activity could not be detected when a boiled extract was used as a source of the enzyme.

Establishment of mean control values of cystathionine synthase activity in cultured skin fibroblasts and in vitro detection of homozy-

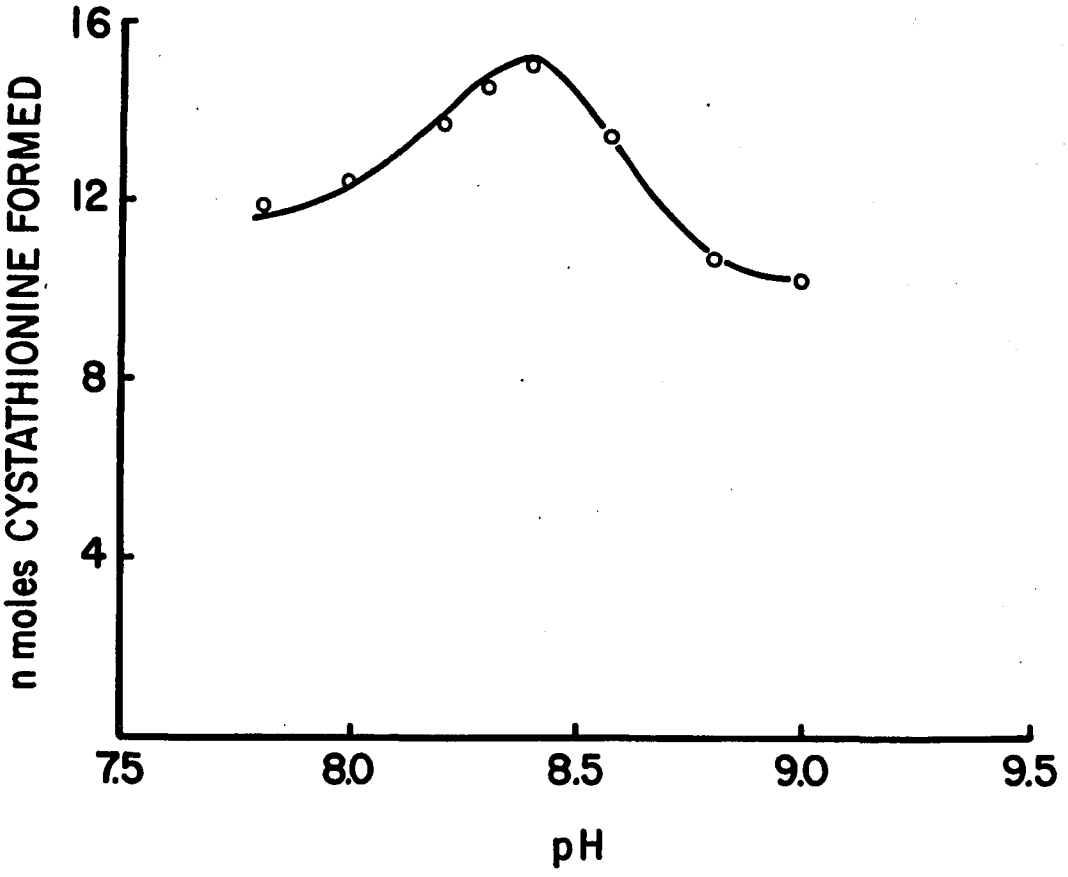


Figure 12. Effect of pH on cystathionine synthase from cultured human amniotic fluid cells. Assay procedure as in the text, except that solutions and buffer were at the pH indicated.

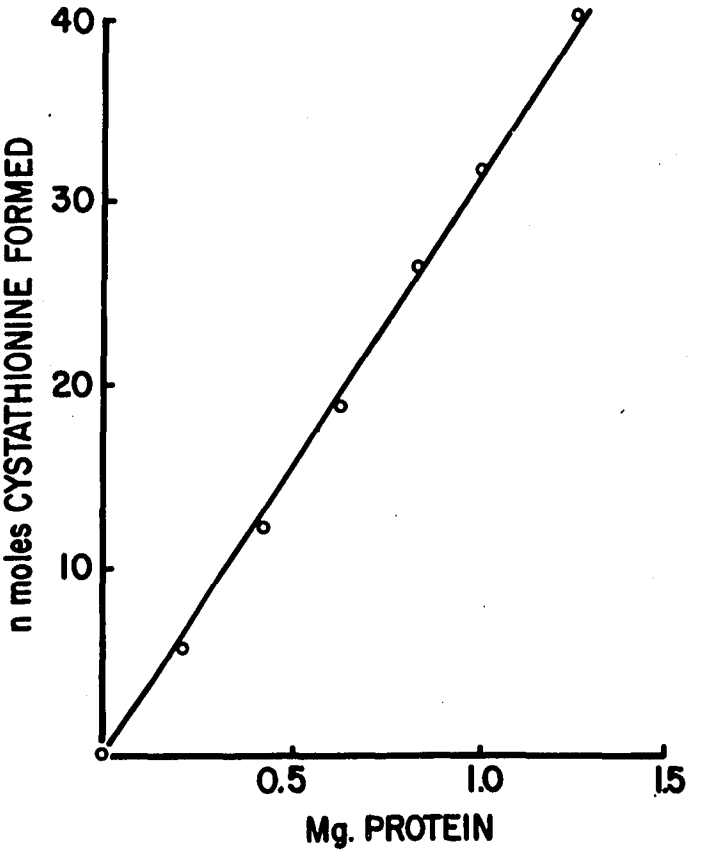


Figure 13. Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of protein concentration.

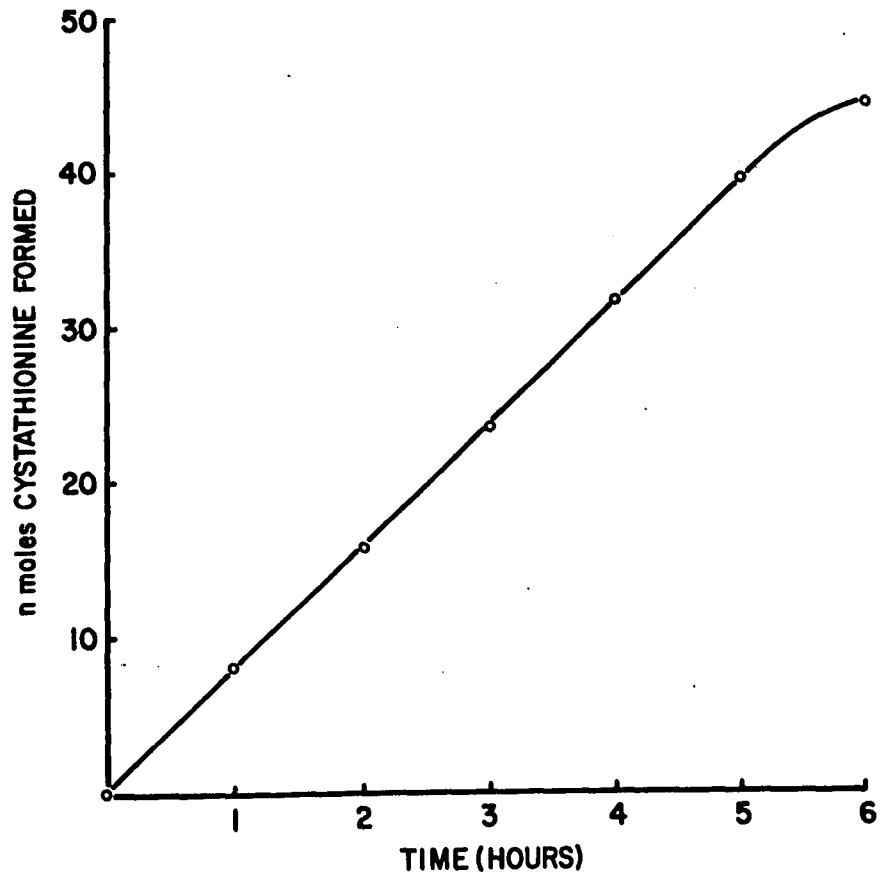


Figure 14. Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of time of incubation.

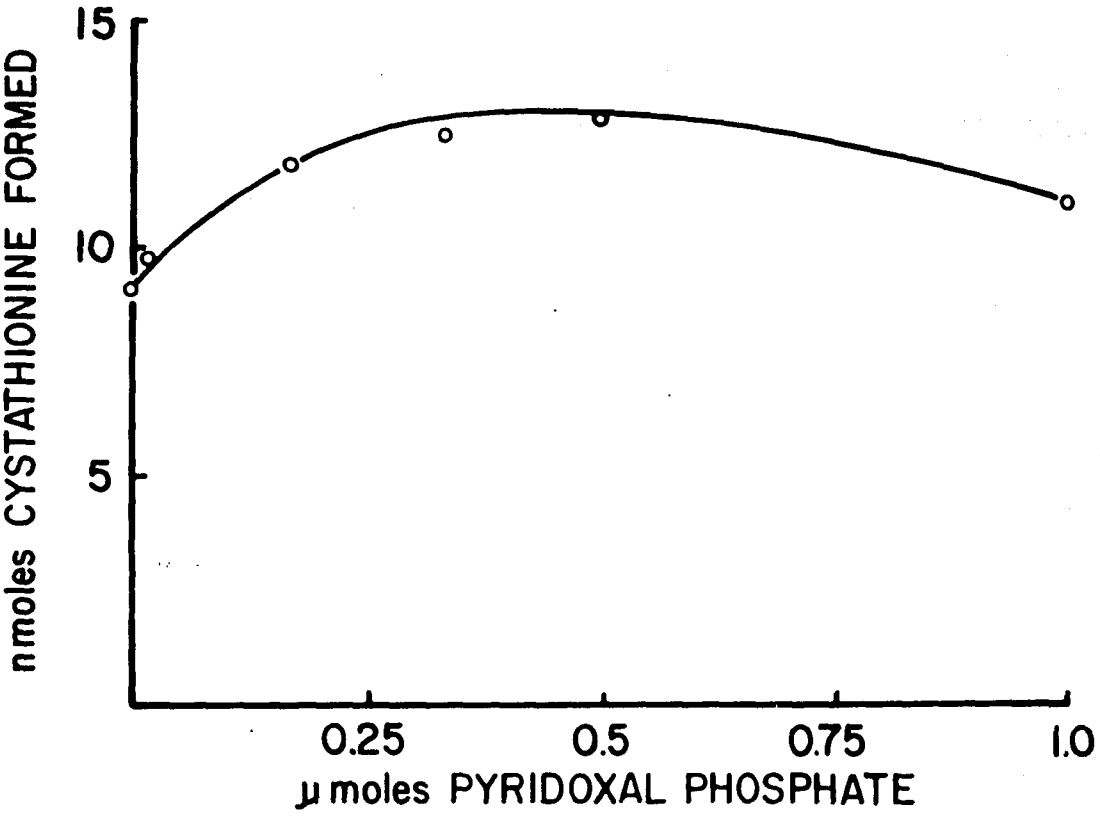


Figure 15. Rate of reaction of cystathionine synthase from cultured human amniotic fluid cells as a function of pyridoxal phosphate concentration.

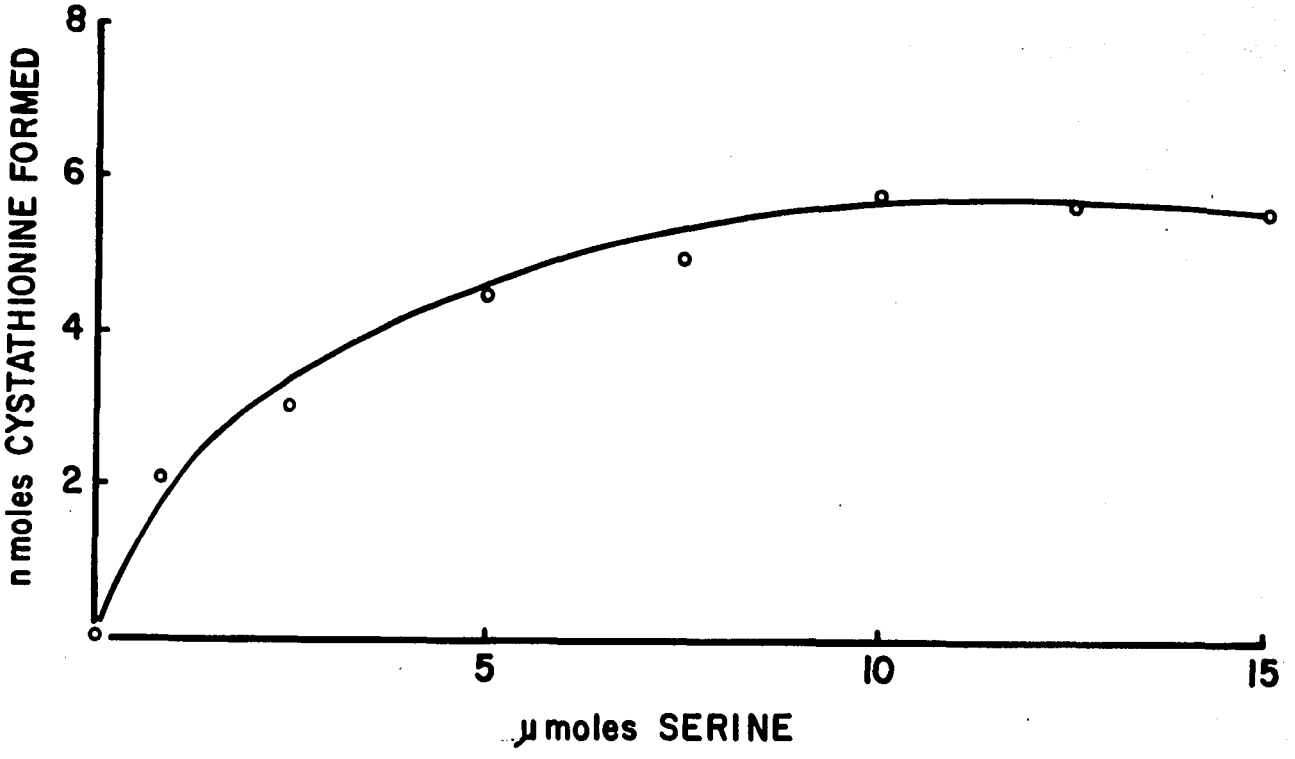


Figure 16. Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of L-serine concentration.

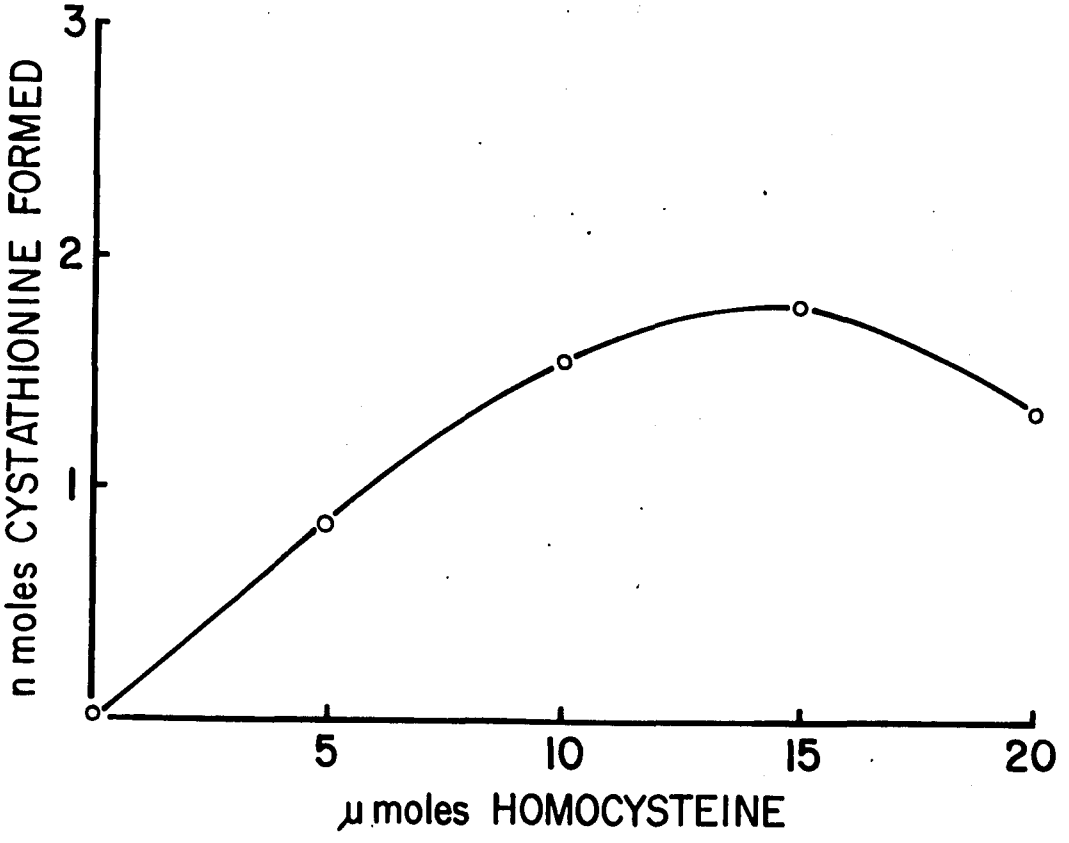


Figure 17. Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of L-homocysteine concentration.

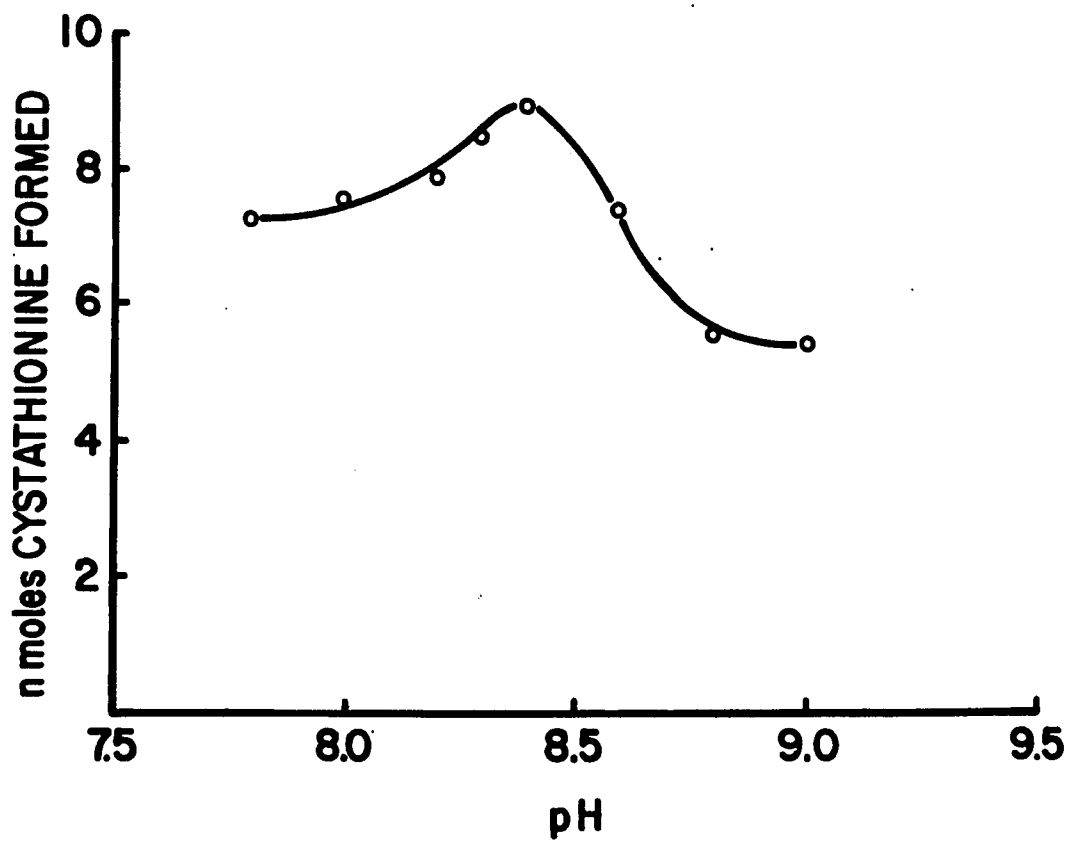


Figure 18. Effect of pH on cystathionine synthase from human long-term lymphoid cell lines. Assay procedure as in the text, except that solutions and buffer were at the pH indicated.

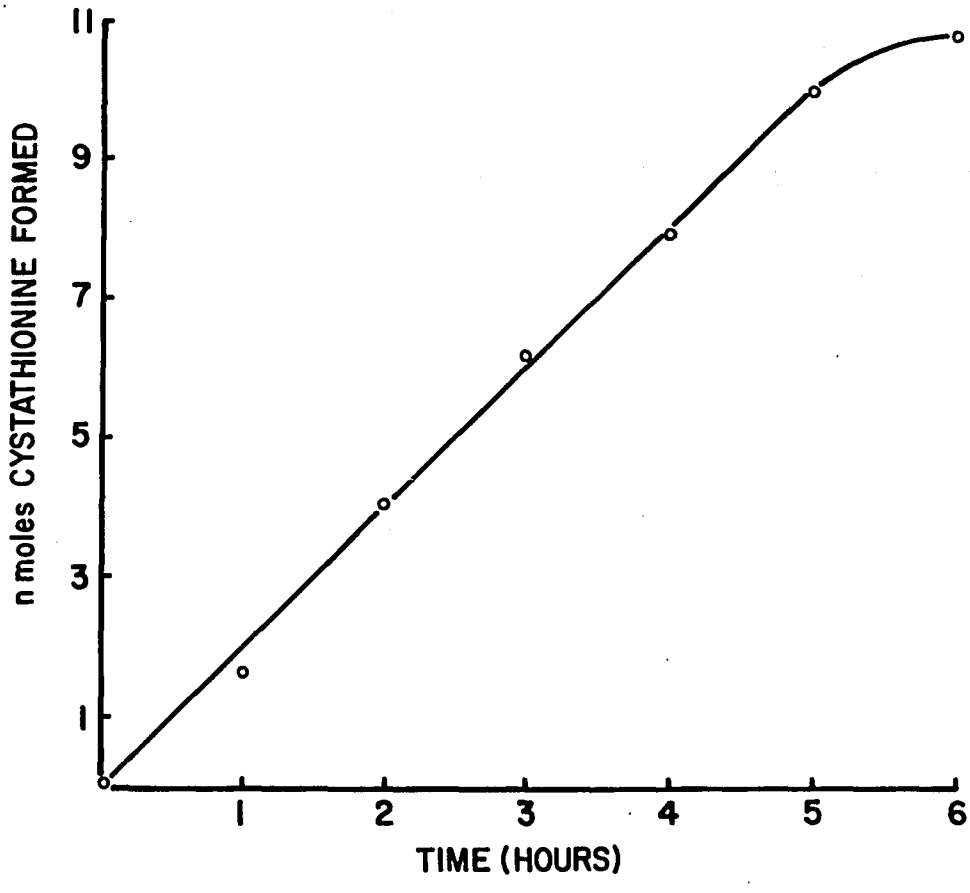


Figure 19. Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of time of incubation.

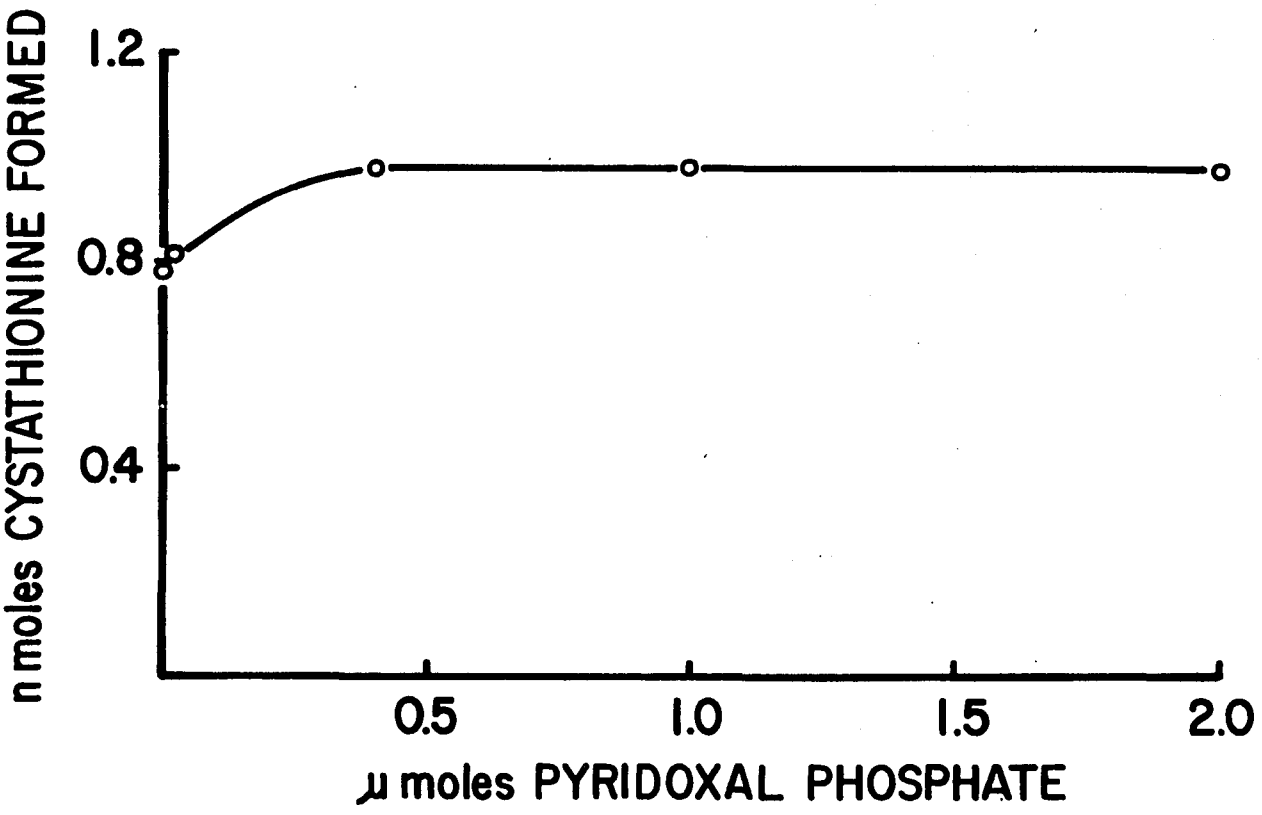


Figure 20. Rate of reaction of cystathionine synthase from human long-term lymphoid cell lines as a function of pyridoxal phosphate concentration.

gotes and heterozygotes for homocystinuria. - Values for cystathionine synthase activity in cultured skin fibroblasts derived from normal donors, homozygotes, and heterozygotes for homocystinuria are presented in Tables 1 and 2, and in Figure 21. Enzymatic activity was highest in the control group, which demonstrated mean activity of 20.97 nmoles/mg protein/hour with a standard error of 1.81. Obligate heterozygotes demonstrated intermediate enzymatic activity with a mean value of 4.40 and a standard error of 0.92. Of the five homozygotes studied, cystathionine synthase activity was detectable in three, two of whom demonstrated values of 0.74 and 0.83. The third patient demonstrating enzymatic activity had a value of 2.29, the highest observed in the homozygote group. However, this patient has been documented as being both clinically and biochemically atypical, and his hepatic cystathionine synthase activity was also unusually high for a homocystinuric (62). The differences between the means of the three groups were highly significant, and the only overlapping value was that of the atypical patient. On the basis of the above data, cultured skin fibroblasts from two potential heterozygotes were investigated to determine their genetic status with respect to homocystinuria. The first demonstrated a value of 21.60 and was thus classified as normal, while the second showed activity of 6.76, suggesting a heterozygous condition.

Mean control values of cystathionine synthase activity in cultured fetal fibroblasts and amniotic fluid cells. - Extracts of cultured skin

TABLE 1

CYSTATHIONINE SYNTHASE ACTIVITY IN CULTURED ADULT SKIN FIBROBLASTS FROM CONTROLS, AND FROM HETEROZYGOTES AND HOMOZYGOTES FOR SYNTHASE DEFICIENCY

Cell Line	Cystathionine formed*	Mean ± SEM
Controls:		
GM 41	12.04	
GM 38	12.50	
1909	13.29	
GM 76	14.38	
1993	16.89	
1897	17.20	
1928	17.71	
1736	18.15	
1922	18.37	
HS	19.22	20.97 ± 1.81
GM 75	20.81	
FS 1	23.98	
GM 43	24.85	
1927	27.86	
FS 3	28.57	
MN	31.11	
FS 2	39.54	
Obligate Heterozygotes:		
Mr. C	2.15	
Mrs. M	2.84	
Mr. M	3.25	4.40 ± 0.92
Mrs. G	4.10	
Mr. H	5.84	
Mrs. N	8.19	

TABLE 1 (Cont'd)

Cell Line	Cystathionine formed*	Mean ± SEM
Homozygotes:		
AG	0.00	
JG	0.00	
RN	0.74	0.77 ± 0.42
LM	0.83	
CH	2.29	
Potential Heterozygotes:		
BN	6.76	
SN	21.60	

*nmoles/mg protein/hour

TABLE 2

COMPARISON OF CYSTATHIONINE SYNTHASE ACTIVITY (MEAN ± SEM) IN CULTURED ADULT SKIN FIBROBLASTS FROM CONTROLS, AND FROM HETEROZYGOTES AND HOMOZYGOTES FOR SYNTHASE DEFICIENCY

Phenotype (no. tested)	Cystathionine formed*	
Controls (17)	20.97 ± 1.81	
		$p < .001$
Obligate Heterozygotes (6)	4.40 ± 0.92	
		$.005 < p < .01$
Homozygotes (5)	0.77 ± 0.42	
Potential Heterozygotes: BN	6.76	
SN	21.60	

*nmoles/mg protein/hour

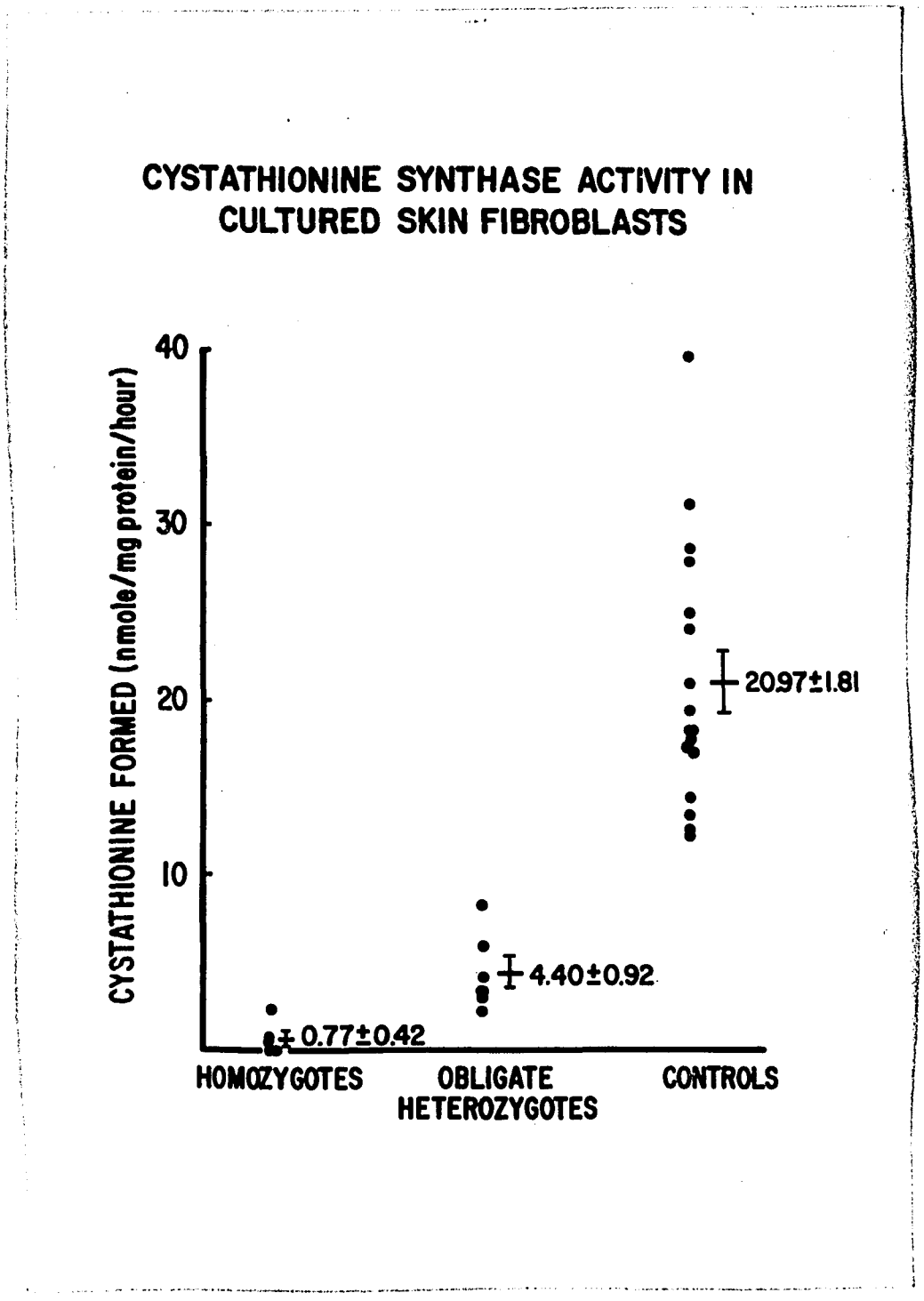


Figure 21. Distribution of cystathionine synthase activities from cultured human adult skin fibroblasts derived from control subjects and from homozygotes and heterozygotes for cystathionine synthase deficiency. The value for each group gives the mean ± SEM.

fibroblasts from five second trimester fetuses were assayed for cystathionine synthase activity. A mean value of 32.89 nmoles/mg protein/hour was determined, with a standard error of 5.06 (Table 3). The mean enzymatic activity of cultured amniotic fluid cells was found to be 40.70 with a standard error of 4.58. There was no significant difference between the means of these two groups, but both differed significantly from the mean found in cultured adult fibroblasts ($p < .001$) (Table 4). Amniotic fluid cells obtained during the sixteenth week of pregnancy, from the obligate heterozygote mother of two children with homocystinuria, were cultured and their extracts assayed to attempt a prenatal diagnosis. Cystathionine synthase activity of 73.79 nmoles/mg protein/hour was found, and the suggestion was made that she was carrying a normal fetus.

Cystathionine synthase activity in cultured long-term lymphoid cell lines from controls, heterozygotes, and homozygotes for homocystinuria. - Data on cystathionine synthase activity in cultured lymphoid cells is presented in Table 5. Mean control enzymatic activity was found to be 9.49 nmoles/mg protein/hour with a standard error of 0.98. Mean enzymatic activity in the heterozygote group was 3.21 ± 0.37. The homozygous affected individual had cystathionine synthase activity of 0.88.

TABLE 3

CYSTATHIONINE SYNTHASE ACTIVITY IN CULTURED FETAL FIBROBLASTS AND AMNIOTIC FLUID CELLS

Cell lines	Cystathionine formed*	Mean ± SEM
Control Fetal Fibroblasts:		
113	19.99	
117	25.32	
GM 10	30.01	32.89 ± 5.06
115	42.73	
GM 11	46.42	
Control Amniotic Fluid Cells:		
128	27.90	
127	30.82	
1920	31.34	
1975	31.78	40.70 ± 4.58
1947	35.35	
129	54.78	
1963	56.01	
1938	57.60	
Amniotic Fluid Cells From Obligate Heterogygote Mother:	73.79	

*nmoles/mg protein/hour

TABLE 4

COMPARISON OF MEAN CYSTATHIONINE SYNTHASE ACTIVITY IN CULTURED CONTROL CELLS

Type of Cell Line	Mean ± SEM Cystathionine formed*
Long-term lymphocytes	9.49 ± 0.98
Adult skin fibroblasts	20.97 ± 1.81
Fetal fibroblasts	32.89 ± 5.06
Amniotic fluid cells	40.70 ± 4.58

*nmoles/mg protein/hour

TABLE 5

CYSTATHIONINE SYNTHASE ACTIVITY IN LONG-TERM LYMPHOID CELL LINES FROM CONTROLS AND FROM HETEROZYGOTES AND HOMOZYGOTES FOR SYNTHASE DEFICIENCY

Cell Lines	Cystathionine formed*	Mean ± SEM
Controls:		
EBV 4B	5.17	
EBV 4A	5.27	
EBV 4C	6.30	
EBV 1	7.32	
NB 16	8.18	
NB 14	8.73	
EBV 13	9.63	9.49 ± 0.98
NB 13	10.27	
EBV 7B	11.03	
EBV 3	11.92	
EBV 8	13.73	
EBV 7A	16.33	
Heterozygotes:		
Mrs. M	2.51	
Mr. M	3.38	3.21 ± 0.37
Mrs. N	3.75	
Homozygote:		
PM	0.88	

*nmoles/mg protein/hour

IV. DISCUSSION

In 1964 Mudd et al. (114) suggested that deficient activity of cystathionine synthase was the basic metabolic defect resulting in the clinical syndrome of homocystinuria. To test this hypothesis they developed assays (114, 116) for the synthase and for two of the other enzymes involved in the mammalian transsulfuration pathway, methionine adenosyltransferase and cystathionase. The micro-methods they utilized were based on the differential adsorption of labelled product and the system was sufficiently sensitive to make use of the tissue obtained from a single needle biopsy of liver. By application of these assays, they were able to demonstrate cystathionine synthase activity in both fresh and postmortem human liver. They were also able to show that patients with homocystinuria were deficient in hepatic synthase activity, although their hepatic methionine adenosyltransferase and cystathionase were comparable in activity to controls. However, their assay system depended for its sensitivity on the presence of serine at a concentration well below the K_m for the enzyme, as they noted. Obviously, with both enzyme and substrate rate-limiting in an assay, conclusions about differences between enzymes in the same tissue are difficult. Thus, in 1969,

Gaull et al. (60) developed new assays for these three enzymes and established control values for their activities in fresh tissues. Like the previously existing methods, the new assays were sensitive enough to assay all three enzymatic activities from a single needle biopsy of liver, but unlike the previous methods, they did not depend for sensitivity on rate-limiting substrates. When Gaull et al. (60) used the assay of Mudd's group (116) they obtained average values of 310 nmoles/mg protein/135 minutes for partially purified rat liver preparations and 94 nmoles/mg protein/135 minutes for crude extracts of rat liver. However, when the preparations were assayed utilizing their new method, they obtained average values of 988 nmoles/mg protein/135 minutes for partially purified preparations and 346 nmoles/mg protein/135 minutes for crude extracts. Thus, the utilization of saturating concentrations of both substrates tripled the amount of activity detected in these rat liver preparations.

In 1971 Tallan et al. (161) were investigating homolanthionine synthesis by human liver cystathionase. It became obvious to them that since the assays of the transsulfuration enzymes were necessarily performed under conditions established as optimal for the enzymes from rat liver, studies of the human enzymes were needed. Until that time, little had been reported concerning the human enzymes, and this group found that the enzymes of rat and human origin differed in certain physical, enzymatic and immunochemical properties. They modified the assay for cystathionine synthase

which had been developed for rat liver, and determined optimal conditions for the enzymatic assay in human liver. Utilizing this new system, they obtained a mean value for the synthase of human liver of 466 nmoles/mg protein/135 minutes as compared to 252 nmoles/mg protein/135 minutes, which was the mean value for human liver synthase obtained by Mudd using his "rat" assay. Tallan et al. also found that the same human liver preparation demonstrated twice as much activity, when assayed by their method, than it did with Mudd's system (159).

The presence of cystathionine synthase in cultured skin fibroblasts was first demonstrated in 1968 by Uhlendorf and Mudd (165). They determined synthase activity in fibroblasts grown in tissue culture, derived from small skin biopsies of patients with homocystinuria and controls. They found synthase activity in the patients' fibroblasts absent or severely deficient, although methionine adenosyltransferase activity was normal. Their mean control value for cystathionine synthase in cultured fibroblasts was 14.4 nmoles/mg protein/hour. In later studies, Mudd (112, 164) reported that the specific activity of cystathionine synthase in extracts of fibroblasts from obligate heterozygotes fell at the low end of, or below, the control range, but higher than the activities of the cells derived from patients. He concluded that heterozygotes for synthase deficiency could not be detected with certainty by utilizing a tissue culture system, although their cells did demon-

strate intermediate levels of enzyme activity. Again however, the assay system they utilized was based on conditions developed for use with crude tissue extracts from rat liver.

Rather than depend on rate-limiting substrate in a radioactive assay (114,116), I attempted to obtain the requisite sensitivity by an examination of each of the assay conditions and determination of optimal activity. The "fibroblast" synthase assay presented here is based upon the measurement of cystathionine formed under optimal conditions for enzymatic activity in cultured cells and quantified by the use of short columns on an automatic amino acid analyzer. The product cystathionine is separated from the substrates, serine and homocysteine, by the use of Dowex-50 ion exchange resin. Appropriate experiments demonstrated that under these newly established conditions enzymatic activity was linear with time and proportional to protein concentration. The optimal pH was established, as were the substrate concentrations producing the maximal rate of reaction. As expected, the enzymatic activity was dependent upon the addition of substrate and no activity was observed when a boiled extract was used as a source of the enzyme. Particular attention was paid to the elution time of cystathionine on the amino acid analyzer. Care was taken to avoid confusion with pyridoxal phosphate adducts known to simulate cystathionine in some chromatographic systems (60).

Care was also taken with the culturing and preassay condition of the cells. Obviously, the quantity of available material from cultured fibroblasts is small. No attempt was made to purify or dialyze the enzyme. However, culture conditions were established so as to provide the cells at their most metabolically efficient state, to achieve optimum lysis of the cells, and to maintain the enzymatic activity at a maximum until the time of assay. There appeared to be no correlation between enzymatic activities and the site from which the skin biopsy was taken, the sex or the age of the donor (except fetal tissue, to be discussed later), the presence or absence of EBV, the number of subcultures (2-7), or the length of time the cells had been stored after harvest. The attempt was made to harvest all the cultures at the same stage of confluence in order to minimize differences resulting from varying stages of the cell growth cycle.

Utilizing this improved system, synthase activity in cultured adult human fibroblasts was quantified. A mean of 20.97 nmoles/mg protein/hour was determined for these cells, as compared to 14.4 found by Uhlendorf and Mudd (165), 3.29 found by Seashore et al. (146) using Mudd's system and 8 by the latter group, using an amino acid analyzer and a serine concentration tenfold higher. Thus, the new assay system seems to increase the measurable activity in these cells.

During the course of these experiments an interesting effect of pyridoxal phosphate (PLP) concentration was noticed. Mudd et al.

(116) had noted that with their system, an increase in PLP concentration consistently stimulated synthase activity. They found no decrease when PLP was omitted from the reaction mixture as compared to the value obtained with their standard concentration of 0.015 micromoles. However, as they increased the concentration of PLP to 0.4 micromoles, they found a 10% increase in cystathionine formation. Seashore et al. (146) also studied the effects of varying concentrations of PLP in the assay system of both normal and homocystinuric fibroblasts and the effects of increasing the PLP concentration in the culture medium. When synthase assays were performed on cells grown in culture medium that had been supplemented one hundred fold with pyridoxine, no enhancement of activity was noted, either in control or homocystinuric cells even after three generations in culture. However, the addition of increasing amounts of PLP to the incubation mixture did stimulate synthase activity in control cells. When small amounts of PLP (0.016 micromoles) were added to the in vitro assay system, no stimulation of synthase activity was noted in control cell extracts, relative to that found with no additional PLP. However, PLP concentrations of 0.4 micromoles and 2 micromoles did stimulate activity (twofold at a 2 micromole concentration). They also found a 2.5-4-fold increase in synthase activity in extracts from fibroblasts of one of their "pyridoxine-responsive" patients when the concentration of PLP in the assay system was

increased to 0.4 micromoles. Lysates from fibroblasts of another patient who showed an *in vivo* response to vitamin B_6 showed no response to PLP added *in vitro* at that time. However, the same group has since reported (91) that they examined partially purified extracts from the second patient and demonstrated a direct stimulation. Recently, Uhlendorf *et al.* (164) studied the effect of varying the concentration of PLP in the assay for cystathionine synthase activity in extracts of cultured fibroblasts from a large group of synthase-deficient patients. They reported that in 24 out of 25 clinically responsive patients, cystathionine synthase activity could be detected in fibroblast extracts that were assayed without the addition of PLP. The synthase activity of these patients was stimulated to the same degree as was control synthase activity by additional PLP (30-50% stimulation was obtained with 0.4 micromoles of PLP). The only enzymatic activity that demonstrated notably greater stimulation with increasing concentrations of PLP was that of a non-responsive patient, the only non-responder to show any detectable synthase activity.

In the present study, utilizing the new conditions established for cultured cells, it was found that the omission of PLP from the *in vitro* reaction mixture did not greatly alter the activity of the preparation from that obtained with a low concentration of 0.015 micromoles. However, an increase in PLP concentration to 0.5 and 1.0 micromoles did result in a three-fold enhancement of enzy-

matic activity. Thus, the cofactor requirement of cystathionine synthase for PLP would seem to be higher than the 0.015 micromoles employed in the assay system developed by Mudd (116) and used in several subsequent studies by other workers (9,68,69) (Uhlendorf et al. (164) have recently changed the concentration of PLP in their synthase assay from 0.015 to 0.4 micromoles). A concentration of 0.5 micromoles appears to produce maximal enzymatic activity.

The data presented in this study confirm the fact that homozygotes for homocystinuria are severely deficient in cystathionine synthase activity (MEAN $\pm$ SEM = 0.77 $\pm$ 0.42) as compared to a random group of normal controls (20.97 $\pm$ 1.81). However, a closer look at the actual values for the production of cystathionine suggests an intriguing hypothesis. It can be seen that two of the patients (sibs) demonstrated a complete absence of synthase activity, and two other unrelated patients showed activity of less than 1 nmoles/mg protein/hour. Nevertheless, the fifth patient produces cystathionine at the rate of 2.29 nmoles/mg protein/hour, a value that places him at the low end of the heterozygote range. This patient has been documented as being both clinically and biochemically atypical (62), and his hepatic synthase activity is also unusually high for a patient with homocystinuria (15% of mean control hepatic synthase activity). His classification as an atypical, minimally affected homozygote, rather than a severely affected hetero-

zygote is uncertain. Both of his parents are clearly heterozygous for synthase deficiency, as his mother's hepatic synthase activity is 29% of the mean control hepatic value and his father demonstrates enzymatic activity in lysates of his skin fibroblasts that is 27% of the normal value.

Another problem in the classification of a case of synthase deficiency was noted by Finkelstein et al. (43). They investigated an asymptomatic cousin (MAG) of one of their symptomatic patients with homocystinuria who was also found to excrete homocystine in her urine. Her hepatic synthase activity was significantly below the mean activity of the control group, and indeed, well below the value for the obligate heterozygotes, parents of the original patient. The patient himself had no detectable cystathionine synthase activity. Thus, the genotype of MAG remained in question. Her synthase activity was intermediate between that of a homozygote and two obligate heterozygotes for homocystinuria, and she did excrete homozystine into her urine. Yet no other clinical signs of the disease were apparent.

A third case of questionable genetic status was presented by Gaull et al. (62). They reported the investigation of a sib of one of their patients who showed hepatic synthase activity that was about 60% of their mean control value. Thus, it was unclear whether to classify him as a normal at the low end of the normal range or a heterozygote at the high end of the heterozygous range.

However, his hepatocytes showed the same changes under the electron microscope that this group had observed in both patients and obligate heterozygotes for synthase deficiency, and he was thus classified as a probable carrier of the disease.

The ambiguity described above, as well as in numerous other cases documented in the literature, is best explained in the light of the concept of genetic heterogeneity; that is, the occurence of a phenotype which actually represents more than one genotype. Often a particular syndrome, originally believed to be a homogeneous entity determined by a particular mutant gene, has been ascertained to delineate an assemblage of specific disorders, each with its own underlying molecular defect, its own characteristic pathological changes, and its own treatment. It has been observed, especially among the rare inborn errors of metabolism, that although a specific enzyme may be deficient in a particular disease, the nature of the defect and the degree of metabolic disarrangement it causes often appear to differ among patients. However, several very different mutant genes can theoretically result in a series of closely related, possibly clinically indistinguishable disorders. Variation is the foundation upon which selection acts to create the multiplicity of phenotypes obvious to even the most casual observer. The variation, both normal and aberrant, detectable in any population may result from factors effective at numerous levels.

Heterogeneity may result from the loss of function of one or another of a sequence of enzymes, which are all involved in a particular metabolic pathway or physiologically associated with one another. Thus, several quite unrelated genes, possibly located on different chromosomes, and each affecting a distinct enzyme, may all result in analogous clinical manifestations. In addition, genetic diversity may be the consequence of mutations at one of several distinct gene loci, each producing its unique polypeptide chain, all of which are involved in the formation of a single enzyme or protein. Another locus, regulating the rate of synthesis of the protein, can add to this pool of variation. Furthermore, any particular locus may be occupied by one of a variety of different alleles. Mutations occurring during DNA replication result in changes in the sequence of the amino acids in a polypeptide chain or in alterations of the rate of synthesis. These mutations are, of course, the actual source of all genetic heterogeneity. They result in the production of numerous alleles, any one of which can occupy the same locus and each of which will code for a distinct peptide chain. Thus, at any gene locus determining a specific enzyme, a variety of heterogeneous mutations may take place, each resulting in a unique enzyme defect with characteristic clinical abnormalities. In fact, even the term "allele" may be redefined in terms of the actual difference produced by a mutation. Two different alleles may result from mutations at different base triplets within the locus, at the same triplet but at different nucleo-

tides, or at the same nucleotide. Thus, alleles may result in polypeptides differing in several amino acids, in a single amino acid, or actually identical in amino acid sequence.

The investigation of human genetics involves the identification of the multitude of loci demonstrating variation, the discovery of the numerous alleles at each locus and the correlation of each allele with the phenotypic characteristic that it directs. Although a large number of the inherited inborn errors of metabolism, resulting from defects in specific enzymes, are known to demonstrate varying clinical manifestations, often the extent of genetic and phenotypic heterogeneity remains unclear. Though our knowledge in this area is rapidly expanding, it is still impossible to interpret most genetic variation in terms of differences in the amino acid sequences of the relevant proteins, not to mention the nucleotide sequence of the gene itself. One notable example, in which the correlation between phenotypic diversity and biochemical structure has been achieved, involves the locus determining the β chain of human hemoglobin. Amino acid sequencing has revealed the molecular nature of the various alleles capable of occupying this locus, and resulting in numerous and widely disparate phenotypes. Generally, our most valuable clues are derived from the observable activity or function of the enzyme or protein. If a specific enzyme demonstrates reduced activity, this can be due to one of several causes: the enzyme protein may be altered in structure, its synthesis may be reduced, or its

activity may be inhibited in some way. And, each of these causes may be secondary to one of several alternate reasons. A mutant gene may be responsible for the synthesis of a structurally abnormal enzyme protein with defective catalytic properties, or one whose molecular stability is severely reduced so that it breaks down very quickly; either way leading to deficient enzymatic activity.

In man, the test for genetic heterogeneity is presently confined to a comparison of quantitative measurements of enzymatic activity, and to a contrast of clinical manifestations, among members of sibships and families on the one hand, and between unrelated persons on the other hand. There are numerous examples in the literature of enzymatic deficiencies with phenotypic variations suggesting more than one genotype. It is assumed that the genes involved are alleles because the same enzyme system is affected in each case. Each of these deficiencies has a common type and in addition, some less common variant forms. For example, twenty-two variant forms of glucose-6-phosphate dehydrogenase deficiency have been noted (8, 152, 162). Examination of the enzymatic characteristics of all the variants, which range in activity from zero to near normal, reveals molecular differences in each. But it must be kept in mind that similar phenotypes can also result from unrelated genes. Hemolytic anemia, for example, may result from at least ten different enzyme deficiencies (24). Although the patho-

logical findings in each of the diseases are quite similar, requiring enzymatic assay for precise diagnosis, a different protein system is involved in each variant form, and thus different genetic mutations are likely.

When an enzymatic deficiency has been documented in a particular disease state, the possibility of allelism, as opposed to the possibility of genes at different loci, can be best supported by demonstrating different degrees of deficiency of the same protein (76). However, this is not positive proof of allelism, as many steps may be involved before an enzyme reaches its final catalytic state, and each of these steps may be under separate genetic control. Also, the enzyme may be composed of two or more structurally different subunits, each the product of a separate genetic locus. This phenomena of different subunits in enzymes appears to be rather frequent (76) and diseases involving a deficiency of one of these enzymes, and exhibiting phenotypic diversity, may have a non-allelic basis for the observed variation. Although the most conclusive proof of allelism would be a demonstration that the disorders in question result from different amino acid substitutions in the same polypeptide chain, failure of complementation (e. g. physiological, molecular, or by cell hybridization) is supportive evidence. Thus if the values obtained by enzymatic assay are similar in related persons, but significantly different between unrelated persons, genetic heterogeneity is a sound guess, but alle-

lism, although a reasonable hypothesis, is not a certainty. Indeed, when no activity or very low levels are detected, without evidence of a structural defect, the possibility always exists that the lesion involves not the structural gene locus specifying the enzyme peptide chains, but a "regulator" gene controlling the quantity or rate of enzyme protein synthesized. It is also necessary to distinguish between variation due to mutant alleles at different loci and variation due to the existence of several "normal" alleles at the same locus. It has been shown that allelic genes exist, their effects qualifying quantitatively as normal, and that these distinct alleles have different effects on function without actually resulting in pathological changes. These "isoalleles" (157), when present in combination with an allele specifying an abnormally deficient enzymatic activity, may account for the wide range of enzymatic activity demonstrated by heterozygotes. This type of situation could also influence the expression of the clinical picture, and will result in high intrafamily correlations. In addition, affected sibs will resemble one another more closely in the characteristics of a particular disease than will affected parents and their children. And finally, mention must be made of the fact that variation in the expression of an inherited disease in different individuals may be due to differences in the "genetic background" of the individuals in whom the mutant gene occurs. Certain combinations of genes at various unrelated

loci may indirectly act to minimize the pathological consequences of a particular disease, whereas others may accentuate it, both situations adding to the observable heterogeneity.

Thus, it is conceivable that the three patients mentioned above are examples of the genetic heterogeneity manifest in cystathionine synthase deficiency. This variation has been cited recently (62) and is demonstrable in both the response of patients to megavitamin therapy with pyridoxine and the effect of the vitamin on the synthase itself. Genetic heterogeneity is suggested by the fact that the affected members of a given sibship are either all responsive or all non-responsive to vitamin B_6. Uhlendorf et al. (164) demonstrated that the differences in synthase activity between affected sibships are greater than the differences within the sibships. Since affected sibs have probably inherited the same two mutant genes determining synthase activity, the variation encountered within any sibship gives an indication of the scatter, produced by experimental conditions, between individuals in whom the genetic factors are constant. The greater variation found between sibships suggests that the sibships differ from one another in their genetic determinants of cystathionine synthase. Thus, heterogeneity appears to exist between patients who are responsive to vitamin B_6 treatment and those who are not responsive and, in addition, within each of these two groups. Patients unresponsive (or only partially responsive) to massive doses of B_6 in vivo evidence no stimulation of the

synthase by the vitamin either *in vivo* or *in vitro* and generally show the least residual hepatic synthase activity. On the other hand, clinically-typical patients exhibiting a complete biochemical response to the vitamin therapy appear to have more residual synthase activity. Some of these "responsive" patients display stimulated hepatic synthase activity in response to the vitamin *in vivo* (174), while others do not (62, 113). Seashore *et al.* (146) and Uhlendorf *et al.* (164), also noted this heterogeneity, in the response to the addition of pyridoxal phosphate to the *in vitro* assay of the synthase activity in lysates of cultured skin fibroblasts, as mentioned above.

The fifth patient investigated in the present paper demonstrated the highest synthase activity of the homocystinuric group, both in lysates of skin fibroblasts (11% of mean control value) and in liver biopsy material (15% of mean control value). This level of enzymatic activity places him at the low end of the heterozygote range and poses the question of his actual genetic status with respect to cystathionine synthase deficiency. One obvious possibility is that he is homozygous for a recessive gene which does not cause as severe a deficiency of the synthase as that manifest in the four other patients. Thus, both his enzymatic activity and his clinical situation are closer to normal. Some doubt is cast on this hypothesis however, by the fact that both of his parents, who would be obligate heterozygotes for this "milder" mutant gene, show enzyme levels intermediate in the heterozygote group, as cited above. Another feasible theory would

be that this individual is actually heterozygous for a mutant synthase gene and also for an "isoallele" which determines less synthase activity than the "common" normal allele. As mentioned above, the combination of one of several mutant alleles with one of several "normal" alleles could account for a wide variation in enzymatic activity of heterozygotes. This variation might result in synthase activity in the heterozygous group which overlaps at one extreme with that of homozygous recessive individuals and at the other extreme with that of homozygous normals. Of course, genes at other loci may be playing a role in the synthase activity detectable in this patient.

Another potential explanation would suggest that this particular case may represent neither a true homozygote for synthase deficiency nor an actual heterozygote for the disease. Most of the genetically-determined deficiencies in man are assumed to be due to the presence of two identical mutant genes and their carriers to be homozygous for the mutant gene in question. If it is possible to detect consanguinity in the family background this idea is further strengthened, and indeed, if the parents of a patient are related, there is a strong possibility that the two recessive genes present in their offspring are identical. However, if no consanguinity can be demonstrated, and especially if the disease is rare, the greatest likelihood is that the patient is actually a "genetic compound" (107), possessing two rare mutant alleles at a particular locus, both of which are responsible for

reduced enzymatic activity, but which are not identical. Genetic studies on microorganisms, as well as biochemical investigations, suggest that the average gene specifying a single polypeptide chain of a protein may range in size from 300 to 4500 or more nucleotide pairs, thus coding for polypeptide chains of 100 to 1800 amino acid residues (the limits in polypeptide chain length in most proteins). Since the smallest mutable site on a gene is a single nucleotide pair and inasmuch as all parts of a gene appear to be equally vulnerable to mutation, which in itself is a rare event, it is unlikely that the various mutant genes randomly distributed in a population are identical. The heterozygotes for two rare mutant genes should be much more frequent than homozygotes for either allele. Thus, many of the inborn errors of metabolism probably occur in persons heterozygous for two rare genes, rather than homozygous for one; e.g. individuals heterozygous for the common gene responsible for galactosemia and for the Duarte variant (24). Each of the mutant genes expresses itself with a distinctive decrease in enzymatic activity and/or molecular variation. The genes demonstrate segregation patterns expected of alleles. There also may be chemical indications that these genes are allelic. If the genes occupied different loci the effect of each mutant gene should be balanced by its normal allele and the maximum total reduction would be that of the gene effecting the greater loss. However, if the genes are allelic the reduction in activity should be additive, and this appears to be the

case in most examples investigated thus far. Another possibility is the existence of different and partially complementary mutant alleles resulting in the individual possessing the "genetic compound" demonstrating greater enzymatic activity than either of the homozygous types. Nadler and his group (125) claim to have demonstrated this "interallelic complementation" in hybrid human diploid cells deficient in galactose-1-phosphate uridyl transferase activity, although nonallelic complementation could also explain their findings (107). Complementary mutant alleles may underlie the unusually high enzymatic activity detected in the fifth patient of the present study. Obviously, these "genetic compounds" will add to the observable heterogeneity of a disease. Several mutant genes occuring in combination with one another, or with one of several "normal" genes, can exhibit immense variability of both the homozygous and heterozygous states. This will be demonstrable as variations in enzymatic insufficiency, as well as in clinical manifestations, and would explain the occurrence of mildly and severely affected patients and the heterogeneity observable both among sibs and between unrelated individuals.

The data presented here demonstrate for the first time, the clear differentiation of the heterozygous state of homocystinuria due to cystathionine synthase deficiency from both the homozygous state of the disease, and from normal controls, utilizing easily obtainable cells grown in tissue culture (45). Obligate heterozygotes demon-

strated enzymatic activity (Mean $\pm$ SEM = 4.40 $\pm$ 0.92) intermediate between that of the homozygous affected group (0.77 $\pm$ 0.42) and the normal controls (20.97 $\pm$ 1.81). In addition, cultured skin fibroblasts from two potential heterozygotes were investigated to determine their genetic status. The first demonstrated a value of 21.6 nmoles/mg protein/hour and was thus classified as normal, while the second showed activity of 6.76 nmoles/mg protein/hour, suggesting a heterozygous condition.

Heterozygote detection has been attempted with cultured skin fibroblasts (9,164), but overlap between the three genotypes was found, making identification uncertain. Heterozygote detection on the basis of loading tests has not proved feasible, although Fowler et al. (46) reported that the plasma concentration of cysteine-homocysteine disulfide after methionine load provided a reliable biochemical parameter for the differentiation between heterozygotes and normal subjects. Detection of heterozygous individuals has been performed more routinely by assay of synthase activity in liver biopsy material. Interestingly, in the obligate heterozygotes who have been investigated, hepatic synthase activity averaged considerably less than 50% of the mean control value. Finkelstein et al. (43) reported the mean synthase activity of two obligate heterozygotes to be 42% of their control group, although when another unusually high control value was included the heterozygote level fell to 31% of the controls. Gaull et al. (62) demonstrated

synthase activity of obligate heterozygotes ranging from 21-46% of the mean control value. It should be noted that the 46% value was obtained after an oral methionine load and may represent a "stimulated" value, similar to that seen in this individual's homocystinuric son following such a load. In addition, this heterozygous father demonstrates activity of 27% of the mean control value in extracts of his cultured skin fibroblasts. Very recently, Goldstein et al. (69) reported the detection of heterozygotes for cystathionine synthase deficiency using phytohemagglutinin (PHA)-stimulated lymphocytes. Although their mean enzymatic activity for the obligate heterozygotes was significantly below that of the control group, the values for three of the heterozygotes overlapped with the control range. This group also found that the activities of the obligate heterozygotes was lower than expected, with a mean value 17-20% that of the controls. The present results confirm and extend the previous observations, and demonstrate mean synthase activity in lysates of cultured skin fibroblasts derived from obligate heterozygotes that is 21% of that seen in the control group. It is intriguing that these results do not follow the "normal" pattern demonstrable in most inborn errors of metabolism, with heterozygotes showing about 50% of the enzymatic activity found in controls, and consistent with a simple gene-dose relationship.

The vast majority of inborn errors of metabolism are inherited as "recessive" conditions. Persons affected with the characteristic

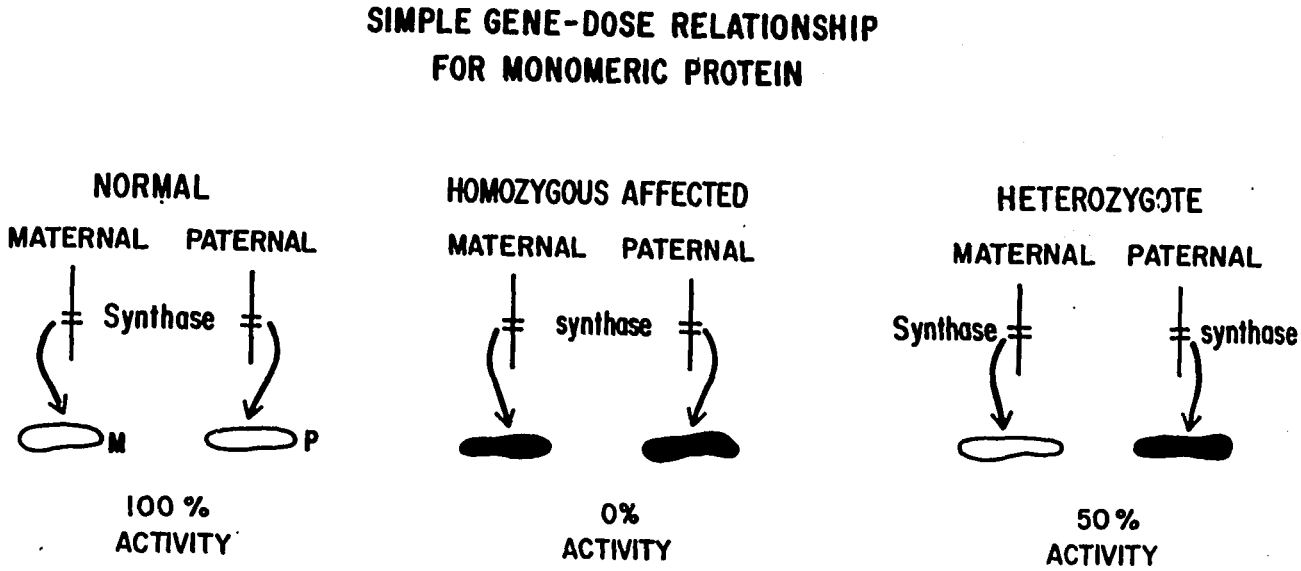
clinical and metabolic abnormalities of the disease are generally found to be homozygous for a rare mutant gene (or, as noted above, heterozygous for two different mutant alleles). The corresponding heterozygous carriers of the disease possess one normal allele and one mutant allele at the locus in question and are usually clinically normal. There is a characteristic familial distribution and segregation data conform to expectation. The asymptomatic heterozygotes generally exhibit a partial deficiency of the enzyme that is severely deficient in the homozygous state, and often also a minor metabolic disarrangement paralleling qualitatively that observed in patients, but much milder in degree.

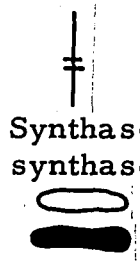
The value of heterozygote detection in the investigation of human biochemical genetics includes the ability to more accurately diagnose a genetic trait, the more precise estimation of gene frequencies, a better understanding of heritable biochemical variability, and the obvious significance in genetic counseling and in intrauterine metabolic studies and diagnosis. Numerous methods have been developed for the biochemical detection of heterozygous carriers of hereditary metabolic diseases. These include the use of loading tests, the use of cloning techniques with cells from females heterozygous for sex-linked disorders, and the direct measurement of the enzyme or metabolite in the carrier. Recently, the identification of carriers through studies of PHA-stimulated lymphocytes and of leukocytes and fibroblasts grown in tissue culture has taken on added

importance. Enzyme assay in easily accessible tissues such as erythrocytes, leukocytes, and cultured skin fibroblasts has proven extremely useful in the investigation of the heterozygous state for mutant genes determining a number of the inborn errors of metabolism. It is of interest to note that the metabolic changes observed in skin fibroblasts often tend to parallel closely those seen in white blood cells, as can be clearly demonstrated for cystinosis (144). In addition, a better measurement of tissue enzyme activity can often be obtained by employing white blood cells and fibroblasts, and frequently a biochemical defect can be demonstrated which is not detectable in erythrocytes. In Type II glycogen storage disease (Pompe's disease) a deficiency of α-1,4-glucosidase was demonstrated in liver and muscle, but could not be found in erythrocytes. Later studies revealed the enzymatic deficiency in both leukocytes and fibroblasts of homozygous patients, and in obligate heterozygotes approximately half of the normal enzyme activity was detected (129). Interestingly, the heterozygous individual could not be distinguished in a reliable manner with unstimulated leukocytes (84). After stimulation of lymphocytes with phytohemagglutinin the levels of α-1,4-glucosidase were suggestive, but not uniformly diagnostic, of the heterozygous state. However, when the phytohemagglutinin-induced changes in the affected enzyme were compared to a "control" enzyme, identification of the heterozygote could be made with certainty. These results are due to the fact that the response of a particular

"affected" enzyme in the stimulated lymphocytes of the heterozygote is less than that of another "control" enzyme, whereas the response is similar in normal individuals.

At the present time, a large number of different inherited enzymatic deficiencies have been investigated (76). Generally the average enzymatic activity demonstrated by the heterozygotes is intermediate between the very low levels of the abnormal homozygotes, and the levels found in normal controls. It should be remembered however, that there is always some variation about the mean. The distributions may overlap so that it is not always feasible to detect the heterozygous state by means of enzyme assay. Nonetheless, if the homozygous affected individual exhibits a complete (or nearly complete) absence of an enzymatic activity, the heterozygote will usually demonstrate "approximately 50%" of the mean control value. Thus, there would appear to be a simple gene-dose relationship (Figure 22); a double dose of the normal allele in the healthy homozygote leading to the production of twice as much enzymatic activity as a single dose in the heterozygote. However, there are a number of examples in which the data would suggest other than 50% of normal enzymatic activity in heterozygotes. The mean activity of heterozygotes appears to be more than 50% of the control value in the Swiss type of acatalasia (1) and less than 50% in orotic aciduria (42) and metachromatic leukodystrophy (6,48). It now seems that cystathionine synthase deficiency is also one of these "unusual" inborn





Legend

= chromosomal locus for cystathionine synthase

Synthase = locus for normal enzyme

synthase = locus for mutant enzyme

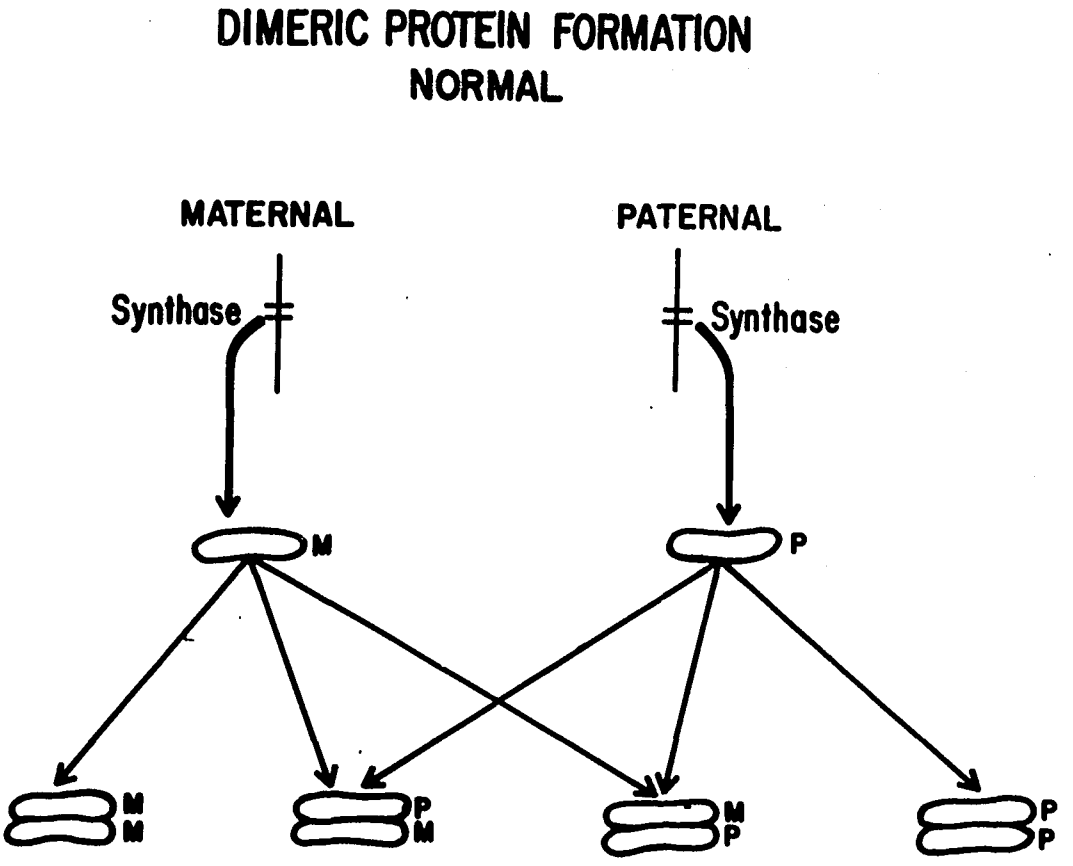
= normal polypeptide

= mutant polypeptide

Figure 22. Polypeptide formation and enzymatic activity in normal individuals and in individuals homozygous and heterozygous for cystathionine synthase deficiency. The enzyme is conceived of as being functional as a monomeric protein and activities are as would be expected on the basis of a simple gene-dose relationship.

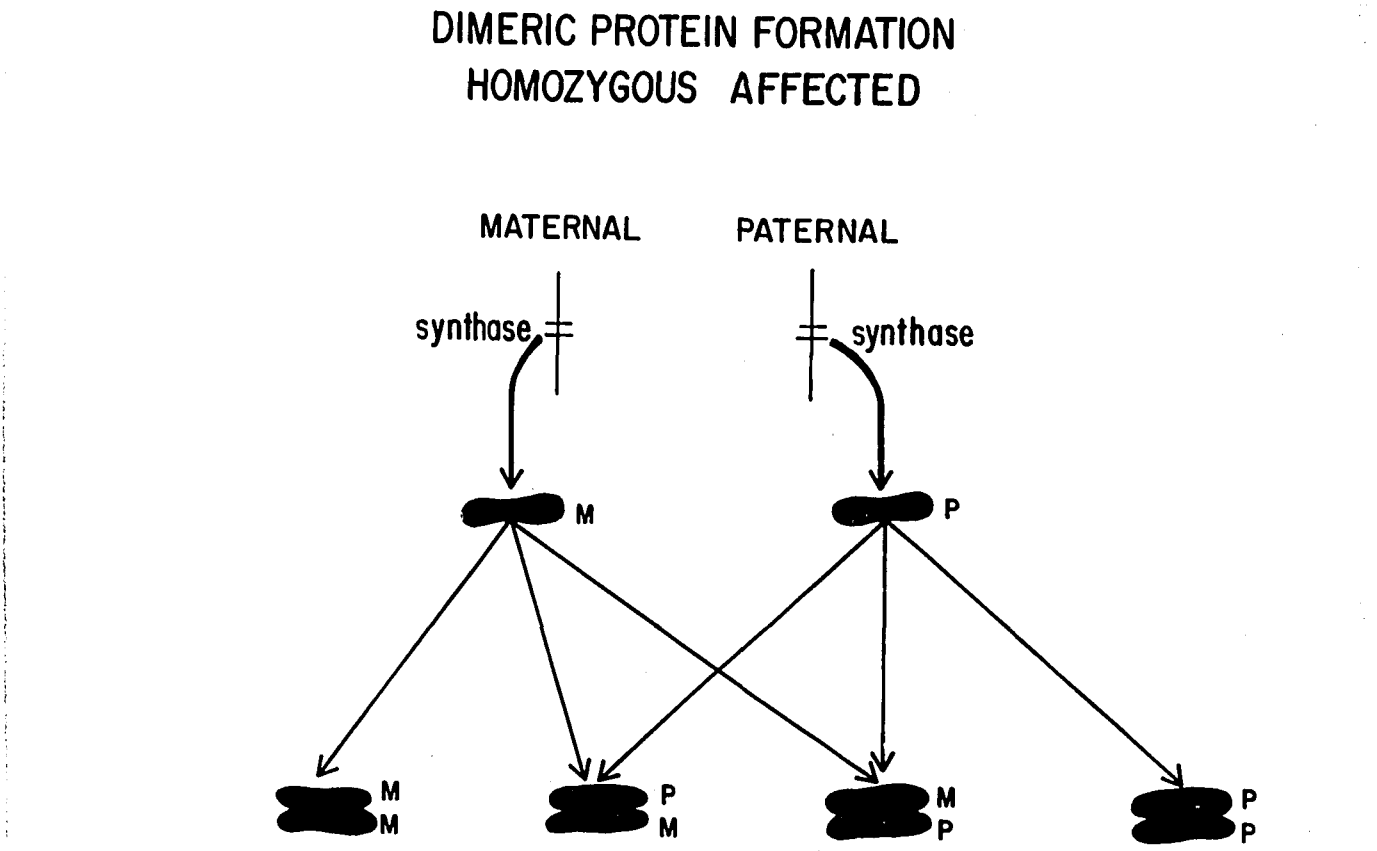
metabolic errors, demonstrating enzyme activity in heterozygotes that is significantly below the anticipated 50% of control values.

A conceivable hypothesis to account for the low enzymatic activity of the heterozygous group would conceive of the synthase as a protein consisting of more than one polypeptide chain. It could exist as a dimer, a tetramer or a larger polymer. If, for example, the enzyme protein were a dimer consisting of two polypeptide subunits, and if there were a series of various possible alleles at a single locus, each determining a molecularly different polypeptide chain, in a homozygous individual only one kind of polypeptide chain would be synthesized and thus one form of the dimeric enzyme protein would result. In the homozygous normal individual this protein would be 100% functional and this person would demonstrate 100% of the normal enzymatic activity (Figure 23). Similarly, the homozygous affected individual would possess two identical alleles coding for identical polypeptide subunits (Figure 24). However, as both of these alleles are mutant, and if we assume that the dimer formed by the combination of two of them possesses no enzymatic activity, these individuals would demonstrate a complete lack of functional enzyme. In the heterozygote two different alleles are present, and two kinds of polypeptide subunits would be synthesized, one normal and one aberrant (Figure 25). If the mutation did not affect the rate of synthesis (or the stability) of the corresponding polypeptide, the subunits would be formed in equal amount. Subsequently, these two



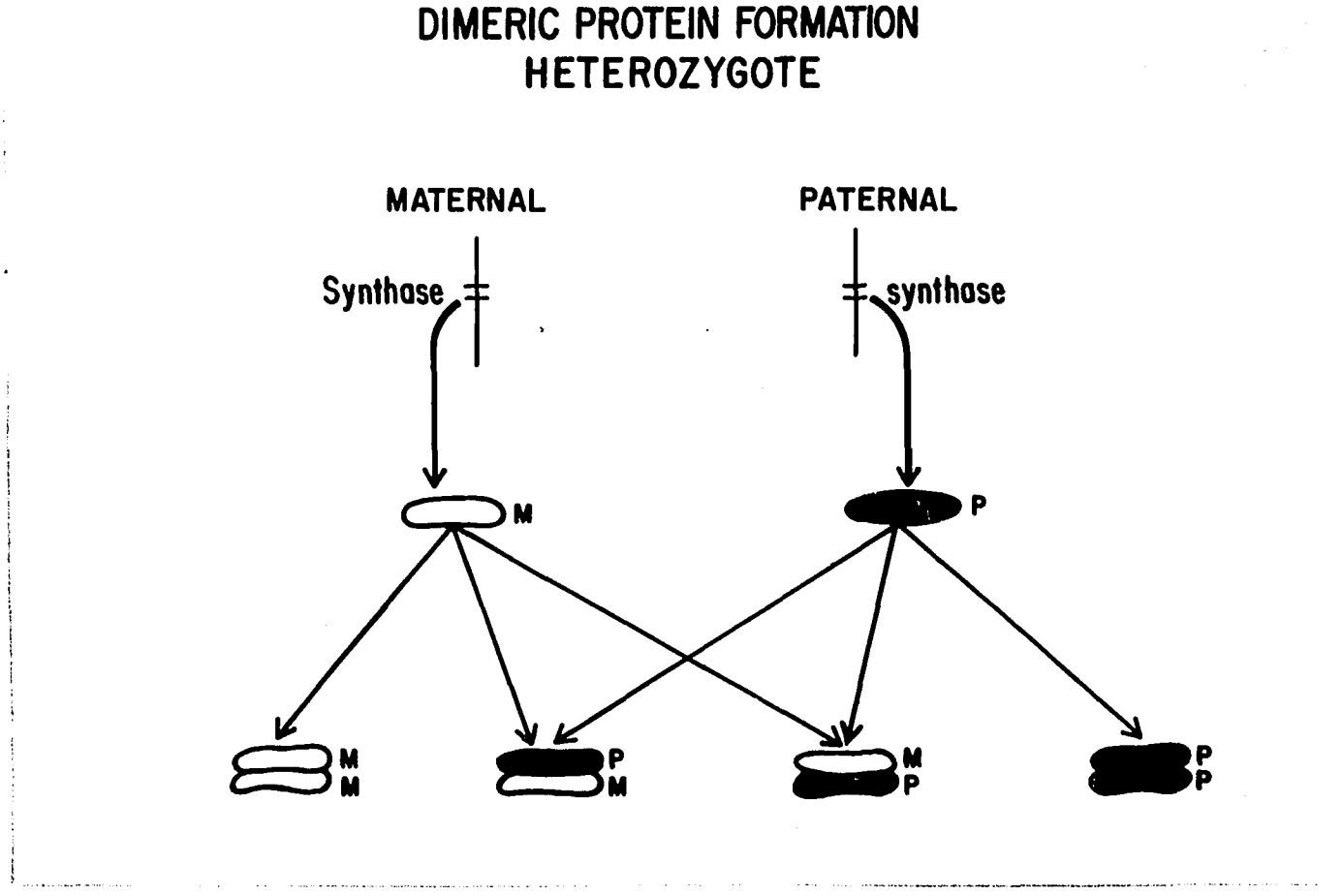
See legend for Figure 22

Figure 23. Formation of cystathionine synthase, conceived of as a dimeric protein, in a normal individual. These enzyme molecules will demonstrate 100% (normal) enzymatic activity.



See legend for Figure 22

Figure 24. Formation of cystathionine synthase, conceived of as a dimeric protein, in an individual homozygous for synthase deficiency. These enzyme molecules will demonstrate 0% of normal enzymatic activity.



See legend for Figure 22

Figure 25. Formation of cystathionine synthase, conceived of as a dimeric protein, in an individual heterozygous for synthase deficiency. The enzymatic activity of each type of dimeric molecule can be expressed as follows:

	Normal Dimers	Hybrid Dimers	Mutant Dimers	Totals
Enzymatic activity/molecule	100	0-100	0	
% of total enzyme protein in heterozygote.	25	50	25	100
Contribution to total enzymatic activity (%) in heterozygote.	33-100	0-67	0	100
Contribution to enzymatic activity (%) in heterozygote relative to normal	25	0-50	0	25-75

heterogeneous subunits could combine to form a dimer by associating in three different ways: normal-normal, normal-mutant (and mutant-normal), and mutant-mutant. This would result in the formation of three types of proteins, differing in molecular structure. Two of them would be analogous to the single forms seen in each of the corresponding homozygotes, and the third would be a "hybrid" molecule consisting of both kinds of polypeptide chains. If combination occurred at random, these three types of dimers would be produced in a 1:2:1 ratio. Thus, the normal-normal dimer would account for 25% of the total enzyme protein in this individual, the normal-mutant for 50%, and the mutant-mutant for 25%. The normal-normal dimer should again be totally functional, as in the homozygous normal individual, and the mutant-mutant dimer completely inactive. The nature of the defect of the mutant polypeptide chain, and the degree of metabolic disturbance it produces in combination with a normal polypeptide, would determine the functional state of the hybrid (normal-mutant) dimer. If the mutant polypeptide formed a completely active dimer in combination with the normal polypeptide, these hybrid dimers would contribute their portion of the enzyme activity (50%) which, when added to the 25% contributed by the normal-normal dimer, would endow this particular heterozygote with 75% of the total normal enzymatic activity. If on the other hand, the mutant-normal dimer were functionless, the individual heterozygous for this allele would demonstrate only 25% of the normal level of activity. Clearly, the contribu-

tion of the hybrid molecule could vary from zero to 50% depending upon the effect of the mutant polypeptide in the heterodimer, and thus, the total activity observable in a heterozygous individual could vary from 25-75% of normal. Perhaps the 50% value so often reported in populations of heterozygous carriers of enzymatic deficiencies actually represents a mean of individuals with enzymatic activities ranging from 25-75%. Of course, more complex patterns of hybrid molecule formation in heterozygotes could occur if the enzyme protein is comprised of more than two subunits. If, for example, it is a tetramer consisting of four identical polypeptide units in the homozygote, then the heterozygote could possess five molecular forms of the protein, three of which would be "hybrid". Lactate dehydrogenase has been shown (3, 100, 101) to be a protein comprised of four polypeptide subunits which are the products of two different loci. This results in five characteristic "isozymes", two of which contain identical subunits and three of which are hybrid molecules. Mutation at either of the two loci would result in 15 different tetramers and lead to even greater possible variation in enzymatic activity. Although the subunits in the case of LDH are coded by nonallelic genes, the principle is analogous if the tetramer is composed of four subunits which are all products of the same locus. A heterozygous individual would possess five different tetrameric molecules and, depending upon the activity of the hybrid molecules, the enzymatic activity demonstrated in the heterozygote could

show quite a wide variability. Kashiwamata et al. (89) have reported that native rat liver cystathionine synthase consists of four subunits; two identical components which migrate relatively rapidly during acrylamide gel electrophoresis, and two identical components which migrate relatively slowly. On the basis of this finding, Uhlendorf et al. (164) have postulated that human synthase also has an "$\alpha_2\beta_2$" composition. This would suggest that man possesses at least two pairs of structural genes for cystathionine synthase, each pair determining a polypeptide subunit. Each of these loci could be occupied by one of several different alleles, and one would expect to find individuals who are homozygous for the same mutant allele (although these would be very rare), individuals who are genetic compounds possessing two different mutant alleles at the same locus, and individuals who are double heterozygotes, carrying single mutant alleles at each of the two loci. Thus, the opportunity for genetic heterogeneity would be immense.

Hybrid protein formation in heterozygotes, resulting in the production of a single protein molecule containing each of the structurally different polypeptide chains coded by two of a variety of alleles, seems to occur with some frequency and many examples involving a variety of proteins and enzymes have been reported. Harris (76) has listed a series of human enzymes, classified according to whether or not heterozygous individuals demonstrate an electrophoretic pattern suggestive of hybrid protein formation.

Usually, in electrophoresis, the hybrid molecules can be seen to migrate at a rate intermediate between that of the "pure" proteins, and often the intensity of the stain of the middle (hybrid) band is greater than that of the other two, suggesting the existence of more of the hybrid than of either of the others. In almost half of the cases categorized by Harris, hybrid enzyme formation appears to occur.

It is intriguing to note that "hybrid" proteins, by definition represent unique molecules, limited to the heterozygous state. Perhaps selection may favor certain heterozygotes because of the metabolic advantage associated with possessing three enzyme forms rather than just one. It is known for example, that the multiple molecular forms of LDH differ in their metabolic capacities (30). These differences could prove adaptive under certain conditions.

This hypothesis of a dimeric (or multimeric) synthase protein could conceivably explain the notably low enzymatic activity demonstrated by heterozygotes for cystathionine synthase deficiency. It is also a plausible explanation for the situation presented by Gaull et al. (62) and cited above. A sib of one of their patients demonstrated synthase activity of approximately 60% of the mean control value, thus higher than the "expected" 50% and considerably higher than the values demonstrated by obligate heterozygotes for synthase deficiency. His classification as a presumed heterozygote was based on the observation that his hepatocytes, when viewed under the electron microscope, demonstrated the same aberrations as those of patients

and obligate heterozygotes. It must be kept in mind however, that the unusually high enzymatic activity demonstrated by this potential heterozygote may alternately result from the combination of a mutant allele and an isoallele specifying greater than normal activity.

The occurrence of these ultrastructural changes in the hepatocytes of obligate heterozygotes as well as in patients (62) raises interesting questions. The pathogenesis of these alterations is unknown. There appears to be no direct relationship between plasma concentrations of methionine, homocystine, or cystine and the changes in hepatocyte morphology, as these abnormalities remain in the B_6-responsive patients even after their plasma amino acid concentrations are brought to normal levels, and are also exhibited by heterozygotes who demonstrate no significant plasma amino acid aberrations. However, it is conceivable that these ultrastructural changes could be a function of abnormal tissue concentrations of the amino acids in the liver of patients, both treated and untreated, and of heterozygotes.

In any case, the fact that these changes occur in the "asymptomatic" heterozygote emphasizes the concept that the terms "dominant" and "recessive" have meanings limited to a specific characteristic or phenotype. A good example of the possible ambiguity of these terms is afforded by the pattern of inheritance of sickle-cell anemia (76). This disease is known to be inherited in a recessive manner, i.e. the disease itself is observed only in homo-

zygotes for the abnormal gene. However, the sickling phenomenon is actually inherited as a dominant characteristic, since both heterozygotes and homozygotes for the mutant allele possess cells which sickle when subjected to sufficiently reduced oxygen tension. It would now appear that in synthase deficiency too, although extremely deficient enzymatic activity is expressed only in the homozygote and thus appears to show a recessive pattern of transmission, the ultrastructural changes in the hepatocytes are also demonstrable in the heterozygote and would therefore seem to be inherited in a dominant manner.

In enzyme deficiency diseases, the distinction between dominant and recessive modes of inheritance may depend on the enzymatic activity demonstrable in homozygous normal individuals, and on the minimum activity actually necessary to maintain sound function. Heterozygotes for such diseases usually possess enzymatic activity intermediate between that of homozygous affected and normal individuals. Since they are generally quite healthy, the implication would be that the normal individual possesses much more than the minimum activity required for normal metabolism, and thus has somewhat of a functional reserve. Only the abnormal homozygotes will demonstrate clinical aberrations, because of their extreme deficiency of enzymatic activity. It has been estimated (58) that considerably more synthase activity is normally present than is needed for the liver to metabolize its free methionine. From the data on hepatic synthase it would appear that enzymatic activity of 15-30% of mean control value ade-

quately maintains normal plasma amino acid concentrations. However, if the morphological changes seen in the hepatocytes of heterozygotes for synthase deficiency do result from the aberrant intracellular metabolism of methionine, more than 60% of normal enzymatic activity is necessary to prevent their occurrence. Perhaps the actual functional reserve of cystathionine synthase is less than had been anticipated.

The present data demonstrate that cystathionine synthase activity can be detected in cultured long-term lymphoid cell lines, and strongly suggest that the in vitro detection of homozygotes and heterozygotes for synthase deficiency can be accomplished using this system. The enzymatic activity detected in these cells demonstrated properties almost identical to those of cultured fibroblasts. Activity was totally dependent upon the addition of homocysteine to the reaction mixture and was completely destroyed when a boiled blank was used as a source of the enzyme. The one interesting difference was in the response of the enzyme to the addition of higher concentrations of pyridoxal phosphate to the in vitro assay system. Only a very small increase in enzyme activity could be detected with higher levels of the coenzyme, as opposed to the much greater stimulation exhibited by cultured skin fibroblasts. In this respect, the cultured lymphocytes show a stronger resemblance to fetal than to adult cells. The suggestion has been made that established lymphoid cells represent "an early type of stem cell

with multiple potentials"(83), thus less differentiated and more like a fetal cell. The present observation would support this idea.

Lymphocytoid lines offer a multitude of potentialities for the investigation of human biochemical genetics. Studies of the metabolic situation in hereditary enzyme deficiencies are often limited by the relatively small quantity of cellular material available from any single patient. Detailed enzyme studies, including characterization of the enzyme protein, are especially hampered by this. There are a number of characteristics of lymphoid cell lines which are of particular significance for this type of investigation. A long-term lymphoid line derived from peripheral blood will retain the unique genotype of the donor in continuous culture, perhaps indefinitely. In addition, the increasing success rate of establishing these cultures makes them feasible tools for use in many laboratories. Many of the enzymes involved in the inborn errors of metabolism have been detected in lymphoid lines derived from normal donors. Thus, a biochemical defect of one of these enzymes could be investigated in long-term lines from affected individuals. The lines proliferate abundantly in easily maintained suspension cultures and thus, once established, can be multiplied rapidly into large quantities. In this way a sufficient amount of cellular material, all of identical genotype, can be produced for biochemical studies, including much more detailed investigation of the enzyme protein lesion. These cells can be stored and transported in a frozen state

and reestablished at a future time, thus providing a degree of "immortality" for the cultures and facilitating comparisons of enzyme defects in patients from different laboratories, which is of special significance because of the rarity of most of these disorders. The establishment of long-term lymphoid cell "banks" is feasible. In addition, lymphocytoid lines allow for systematic investigation of the secondary metabolic aberrations resulting from the enzymatic lesion. The culture conditions can be altered at will, and a variety of stress factors can be applied. Alternately, strictly controlled conditions can be maintained. These studies will be valuable in the investigation of numerous metabolic problems such as cofactor and substrate requirements, and the effects of various metabolites on both normal and deficient, continually proliferating human cells.

It is interesting to note that cystathionine synthase, an enzyme present in high activity only in liver and brain (116), and not detected in human skin, erythrocytes, or leukocytes (114,165), is demonstrable in cultured fibroblasts and amniotic fluid cells (165), PHA-stimulated lymphocytes (68), and long-term lymphoid cell lines (44). An obvious possibility is that the presence of the synthase in cultured cells, but not in skin or blood cells, is a result of differences in cellular response to <u>in vitro</u> and <u>in vivo</u> conditions (165). It has been observed that mammalian cells in culture tend to retain or lose specialized functions of their tissue of origin, including some enzymatic activities (94). Very few mammalian cells have been seen to realize

new properties in vitro. Yet, the above results would indicate that lymphocytes in short and long-term culture, and also cultured fibroblasts, acquire cystathionine synthase activity in vitro. This possibility raises interesting questions concerning the regulation of synthase activity and the effect of culture conditions upon this regulation. In addition, it suggests that the number of enzymes that can be investigated in vitro may be greater than that suggested by their occurrence in leukocytes and their general distribution in vivo.

In the present study, cystathionine synthase activity of cultured fetal fibroblasts and amniotic fluid cells from second trimester pregnancies was also investigated. The skin biopsies were obtained from fetuses aborted by hysterotomy, and the amniotic fluid by transabdominal amniocentesis. It was observed that fetal fibroblast cells have an enhanced capability for multiplication in vitro, in contrast to cells of non-fetal origin. Optimum assay conditions were established for both of these cell types and compared to those determined for adult (non-fetal) cells. Most conditions were found to be identical. The only discrepancy was the observation that enzymatic activity was stimulated to a much smaller degree by addition of large concentrations of pyridoxal phosphate (up to 1 micromole) to the in vitro reaction mixture of amniotic fluid cell extracts than had been demonstrated with adult fibroblasts.

The mean ($\pm$ SEM) enzymatic activity determined for cultured fetal fibroblasts was 32.89 ± 5.06 nmoles/mg protein/hour, and that

for cultured amniotic fluid cells was 40.70 $\pm$ 4.58 nmoles/mg protein/hour. There was no significant difference between the means of these two groups, as would be expected if the suggestion is true that amniotic fluid cells actually are derived from sloughed fetal cells, but both differed significantly from the mean found in cultured adult skin fibroblasts of 20.97 $\pm$ 1.81 ($p<.001$). These results confirm and extend those of Uhlendorf and Mudd (165), who assayed synthase activity in four amniotic fluid cell lines. They also found the activity in these cells to be significantly higher than in adult fibroblast cells. The relative activities of several other enzymes in cultured adult, fetal, and amniotic fluid cells have been investigated. β-galactosidase, like cystathionine synthase, demonstrates greater enzymatic activity in amniotic fluid than in maternal (adult) cells (87). However, unlike the synthase which, as shown here, also exhibits high activity in fetal fibroblasts, β-galactosidase shows the lowest relative activity in this cell type. Conversely, arylsulfatase A (87), argininosuccinase (150) and ornithine keto acid transaminase (151) display lower specific activity in cultured amniotic fluid cells than in adult skin fibroblasts. Arylsulfatase A also demonstrates its least relative activity in cultured fetal cells (87). Investigation of β-D-N-acetylglucosaminidase showed that enzymatic activity of cultured amniotic fluid cells and maternal cells was not significantly different, although both showed higher specific activity than did fetal cells (87). Thus, although highly significant differences were found in the mean speci-

fic activities of all of these disease-related enzymes, when compared in adult and fetal fibroblasts and cultured amniotic fluid cells, the pattern of variation in the three cell types was different for each enzyme. Clearly, the relative specific activities of enzymes in normal cells of these three groups are particularly germane to the task of intrauterine diagnosis of metabolic disease. It cannot be assumed, on the basis of morphological and biochemical similarities between cultured fibroblasts and amniotic fluid cells, that all biochemical parameters will vary in parallel. Every distinct enzyme must be individually appraised and the appropriate comparisons made for each.

During multiple passages in culture, fibroblastic cell lines retain their original chromosome complement and, in addition, a number of enzymatic activities functional in vivo. Indeed, many of the enzyme defects involved in the inborn errors of metabolism are found in several tissues and are also manifested by cells cultured from biopsies of skin or other sources. However, the extrapolation of this biochemical data from adult skin fibroblasts to cultured amniotic fluid cells and fetal fibroblasts, and on to the metabolic state of the fetus, is precarious. In an investigation of glycolytic differences between fetal and non-fetal human fibroblast cells, Condon et al. (25) demonstrated that cells in long-term culture, derived from human fetal skin, could be distinguished metabolically from non-fetal cells, whereas cells derived from children and adults

were alike with respect to glucose metabolism. They suggested that fibroblasts retain the metabolic capabilities present in their original state of development and thus, that fetal cells in culture could be utilized to investigate the metabolism of the fetus at various stages of development. However, not all enzymatic activities of cultured fetal cells are representative of the situation in the developing fetus. Cystathionine synthase activity is seen from the present study to have a mean value approximately twice as high in cultured cells of fetal origin as in adult fibroblasts. However, Gaull et al. (61), during an investigation of the development of mammalian sulfur metabolism, demonstrated that the synthase is present in the liver and brain of human fetuses from six weeks of gestation, but at 25-35% of the activity exhibited by similar preparations from mature humans. After establishing the developmental pattern of methyltransferase activities of human fetal tissues, in addition to the absence of cystathionase, they postulated (63) that in fetal liver and brain the transsulfuration pathway for the further metabolism of homocysteine is reduced in activity in favor of the N-5-methyl-tetrahydrofolate-B_{12} remethylation pathway, which appears to be more active in these organs of the fetus than in the mature human. Thus, synthase activity in cultured skin fibroblasts does not appear to vary with development of the individual in the same manner as does the enzymatic activity in liver and brain.

Nevertheless, the metabolic lesion associated with cystathionine synthase deficiency is demonstrable in cultured fibroblasts. Enzymatic activity is exhibited by cell lines derived from normal donors, and severely deficient or absent from lines derived from documented cases of synthase deficiency. Heterozygotes for the disease display intermediate synthase activity. Amniotic fluid cells and fetal fibroblasts in culture, obtained during the second trimester of normal pregnancies, demonstrate extremely high enzymatic activity. This latter observation is of utmost consequence for the proper diagnosis of cystathionine synthase deficiency, and its carrier state, in utero. As mentioned above, it is crucial to determine if the specific activity of an enzyme in cultured amniotic fluid cells is the same, greater, or lower than in adult skin fibroblasts. If the specific activity of the disease-related enzyme is higher in control amniotic fluid cell cultures than in adult fibroblasts, and if obligate heterozygotes for the deficiency possess intermediate activity, an affected fetus could be misdiagnosed as a normal or a heterozygote. Conversely, if the enzymatic activity is lower in cultured amniotic fluid cells, detection of the heterozygous state and differentiation of carriers from affected fetuses might be difficult. In addition, a normal fetus could be interpreted as having deficient enzymatic activity and aborted in error. Thus, the comparison of relative specific activities and enzyme kinetics between normal cultured amniotic fluid cells and control skin fibroblasts is essential to any attempt at prenatal diagno-

sis. In addition, the range of quantitative variation in second trimester amniotic fluid cell cultures, as well as in adult fibroblasts, must be established. This should ideally be determined in cells that have been maintained for approximately four to six weeks in culture; the time during which the diagnosis would have to be completed. Genetic heterogeneity has been sufficiently discussed above for it to be apparent that this inherent variability could be hazardous if unanticipated in this type of situation.

Furthermore, it is crucial that the asymptomatic heterozygous carrier be distinguishable from the homozygous affected individual, and from the normal, by means of enzymatic assay in cultured skin fibroblasts. Ideally, heterozygous amniotic fluid and fetal material should be available for comparison, but as the cases of any particular enzyme deficiency are few and far between, this is unlikely. Another vital prerequisite is the establishment of the range of enzymatic activity in normal cultured fibroblasts derived from second trimester fetuses and in addition, the normal range in any other fetal material that might be used for corroboration of the diagnosis. Obviously, if material obtained via abortion is to be the basis of comparison, it would be preferable that control and "at risk" fetal material be obtained by the same abortion procedure.

It is generally accepted that early midtrimester amniocentesis and analysis of cultured amniotic fluid cells can be unique tools in the management of pregnancies at "high risk" for cytogenetic and sex-

linked disorders in the fetus. However, if quantitative enzymatic data from amniotic fluid cell cultures is to be used as the foundation of the prenatal diagnosis of heritable enzyme deficiencies, the above prerequisite investigations must be carried out for each specific enzymatic problem, in order to establish baseline values. Having determined these necessary control data, intrauterine diagnosis of inborn errors of metabolism becomes a potential reality.

The data exhibited in the present investigation demonstrate that the prenatal diagnosis of homocystinuria due to cystathionine synthase deficiency is feasible. After these studies were completed it was discovered that one of the obligate heterozygotes for this disease, the mother of two young children with cystathionine synthase deficiency, was three months pregnant. Cells from amniotic fluid, drawn during the sixteenth week of pregnancy, were cultured for approximately five and one half weeks, at which time lysates from the cell cultures were analyzed for synthase activity. Enzymatic activity was demonstrated to be extremely high (actually above the range of control amniotic fluid cells) and the diagnosis was made and later confirmed, that the woman carried a normal fetus.

LITERATURE CITED

1. Aebi, H., Bossi, E., Cantz, M., Matsubara, S., and Suter, H. in E. Beutler (Ed.), (1968), Hereditary Disorders of Erythrocyte Metabolism, Grune and Stratton, N.Y.

2. Allen, J.D., Cusworth, D.C., Dent, C.E., and Wilson, V.K. (1958), Lancet 1, 182.

3. Apella, E., and Markert, C.L. (1961), Biochem. Biophys. Res. Commun. 6, 171.

4. Barber, G.W., and Spaeth, G.L. (1967), Lancet 1, 337.

5. Barber, G.W., and Spaeth, G.L. (1969), J. Pediat. 75, 463.

6. Beratis, N.G. personal communication.

7. Beratis, N., and Hirschhorn, K. (1973), Birth Defects IX (1), 247.

8. Beutler, E., Mathai, C.K., and Smith, J.E. (1968), Blood 31, 131.

9. Bittles, A.H., and Carson, N.A.J. (1973), J. Med. Genet. 10, 120.

10. Brenton, D.P., and Cusworth, D.C. (1966), Clin. Sci. 31, 197.

11. Brenton, D.P., Cusworth, D.C., Dent, C.E., and Jones, E.E. (1966), Quart. J. Med. 35, 325.

12. Brenton, D.P., Cusworth, D.C., and Gaull, G.E. in Proc. Internat. Congr. on Scient. Study of Mental Retard. (1964), Copenhagen, p. 79.

13. Brenton, D.P., Cusworth, D.C., and Gaull, G.E. (1965), J. Pediat. 67, 58.

14. Brenton, D.P., Cusworth, D.C., and Gaull, G.E. (1965), Pediatrics 35, 50.

15. Brett, E.M. (1966), Proc. Roy. Soc. Med. 59, 484.

16. Brown, C., and Mallady, S. (1966), Fed. Proc. 25, 341.

17. Brown, F.C., Mallady, S., and Roszell, J.A. (1966), J. Biol. Chem. 241, 5220.

18. Carbon, J., and David, H. (1968), Biochemistry 7, 3851.

19. Carey, M.C., Donovan, D.E., Fitzgerald, O., and McAuley, F.D. (1968), Amer. J. Med. 45, 7.

20. Carson, N.A.J., and Carre, I.J. (1969), Arch. Dis. Childhood 44, 387.

21. Carson, N.A.J., Cusworth, D.C., Dent, C.E., Field, C.M.B., Neill, D.W., and Westall, R.G. (1963), Arch. Dis. Childhood 38, 425.

22. Carson, N.A.J., Dent, C.E., Field, C.M.B., and Gaull, G. E. (1965), J. Pediat. 66, 565.

23. Carson, N.A.J., and Neill, D.W. (1962), Arch. Dis. Childhood 37, 505.

24. Childs, B., and DerKaloustian, V.M. (1968), N. Engl. J. Med. 279, 1267.

25. Condon, M.A.A., Oski, F.A., DiMauro, S., and Mellman, W. J. (1971), Nature (New Biol.) 229, 214.

26. Cox, R.P., Douglas, G., Hutzler, J., Lynfield, J., and Dancis, J. (1970), Lancet 1, 893.

27. Cusworth, D.C., and Dent, C.E. (1969), Brit. Med. Bull. 25, 42.

28. Cusworth, D.C., Dent, C.E., and Flynn, F.V. (1955), Arch. Dis. Childhood 30, 150.

29. Dancis, J., Jansen, V., Hutzler, J., and Levitz, M. (1963), Biochim. Biophys. Acta 77, 523.

30. Dawson, D.M., Goodfriend, T.L., and Kaplan, N.O. (1964), Science 143, 929.

31. Dent, C.E. (1946), Lancet 2, 637.

32. Dent, C.E. (1949), Symp. Biochem. Soc. 3, 34.

33. Donahue, S., Sturman, J.A., and Gaull, G.E., unpublished observations.

34. Dunn, H.G., Perry, T.L., and Dolman, C.L. (1966), Neurology 16, 407.

35. duVigneaud, V. (1952). A Trail of Research in Sulfur Chemistry and Metabolism, and Related Fields. Ithaca, New York: Cornell University Press.

36. Eagle, H. (1955), J. Exp. Med. 102, 37.

37. Eagle, H. (1955), Science 122, 501.

38. Eagle, H., and Piez, K. (1962), J. Exp. Med. 116, 29.

39. Eagle, H., Piez, K.A., and Oyama, V.I. (1961), J. Biol. Chem. 236, 1425.

40. Eagle, H., Washington, C., and Friedman, S.M. (1966), Proc. Nat. Acad. Sci. 56, 156.

41. Efron, M.L. (1965), N. Engl. J. Med. 272, 1058, 1107.

42. Fallon, H.J., Smith, L.H., Graham, J.B., and Burnett, C. H. (1964), N. Engl. J. Med. 270, 878.

43. Finkelstein, J.D., Mudd, S.H., Irrevere, F., and Laster, L. (1964), Science 146, 785.

44. Fleisher, L.F., Beratis, N.G., Hirschhorn, K., and Gaull, G.E. (1972), Lancet 2, 482.

45. Fleisher, L.D., Tallan, H.H., Beratis, N.G., Hirschhorn, K., and Gaull, G.E. (1973), Biochem. Biophys. Res. Commun. 55, 38.

46. Fowler, B., Sardharwalla, I.B., and Robins, A.J., (1973), Proc. Biochem. Soc. p. 23p.

47. Frimpter, F.W., Haymovitz, A., and Horwith, M. (1963), N. Engl. J. Med. 268, 333.

48. Gabreels, F., Lamers, K., Kok, J., Loonen, M., and Lommen, E. (1971), Neuropadiatrie 2, 461.

49. Gaitonde, M.K., and Richter, D. (1959), in Metabolism of the Nervous System, Pergamon Press, N.Y.

50. Garrod, A.E. (1908), Lancet 2, 1, 73, 142, 214.

51. Gaudier, B., Francois, P., Biserte, G., Dautrevaux, M., Nuyts, J.P., and Bombart, E. (1968), Arch. Fr. Pediat. 25, 541.

52. Gaull, G.E. (1967), Amer. J. Dis. Child. 113, 103.

53. Gaull, G.E. (1967), in Advances in Tetralogy, III, Academic Press, N.Y., p. 101.

54. Gaull, G.E. (1968), in Brain Damage in Inborn Errors of Metabolism, E. van Praag, (Ed.), Bohn Publ., Haarlem, p. 67.

55. Gaull, G.E. in Cellular and Molecular Basis of Neurological Disease, M. Shy, E. Goldensohn, S. Appel, (Eds.), in press.

56. Gaull, G.E., Carson, N.A.J., Dent, C.E., and Field, C.M.B. (1964), Proc. Inter. Congr. on Scient. Study of Ment. Retard., Copenhagen, p. 91.

57. Gaull, G.E., and Gaitonde, M.K. (1966), J. Med. Genet. 3, 194.

58. Gaull, G.E., Rassin, D.K., Solomon, G.E., Harris, R.C., and Sturman, J.A. (1970), Pediat. Res. 4, 337.

59. Gaull, G.E., Rassin, D.K., and Sturman, J.A. (1968), Lancet 2, 1302.

60. Gaull, G.E., Rassin, D.K., and Sturman, J.A. (1969), Neuropadiatrie 1, 199.

61. Gaull, G.E., Sturman, J.A., and Raiha, N.C.R. (1972), Pediat. Res. 6, 538.

62. Gaull, G.E., Sturman, J.A., and Schaffner, F. (1973), J. Pediat., in press.

63. Gaull, G.E., VonBerg, W., Raiha, N.C.R., and Sturman, J.A. (1973), Pediat. Res. 7, 527.

64. Gerritsen, T., Vaughn, J.G., and Waisman, H.A. (1962), Biochem. Biophys. Res. Commun. 9, 493.

65. Gerritsen, T., and Waisman, H.A. (1964), Pediatrics 33, 413.

66. Gerritsen, T., and Waisman, H.A. (1964), Science 145, 588.

67. Gibson, J.B., Carson, N.A.J., and Neill, D.W. (1964), J. Clin. Pathol. 17, 427.

68. Goldstein, J.L., Campbell, B.K., and Gartler, S.M. (1972) J. Clin. Invest. 51, 1034.

69. Goldstein, J.L., Campbell, B.K., and Gartler, S.M. (1973), J. Clin. Invest. 52, 218.

70. Goodman, S.I., Moe, P.G., Hammond, K.B., Mudd, S.H., and Uhlendorf, B.W. (1970), Biochem. Med. 4, 500.

71. Gordon, A.H., Martin, A.J.P., and Synge, R.L.M. (1943), Biochem. J. 37, xiii.

72. Greengard, O. (1967), Advan. Enzyme Regul. 5, 397.

73. Groth, U., and Rosenberg, L.E. (1972), J. Clin. Invest. 51, 2130.

74. Hagborg, B., and Hambraeus, L. (1968), Develop. Med. Child. Neurol. 10, 479.

75. Hambraeus, L., Wranne, L., and Lorrentsson, R. (1968), Clin. Sci. 35, 457.

76. Harris, H., The Principles of Human Biochem. Genetics. Amer. Elsevier, N.Y., 1970.

77. Harris, H., Penrose, L.S., and Thomas, D.H.H. (1959), Ann. Hum. Genet. 23, 442.

78. Harrison, R.G. (1967), Proc. Soc. Exp. Biol. Med. 4, 140.

79. Hayflick, L. (1965), Exp. Cell Res. 37, 614.

80. Hayflick, L., and Moorhead, P.S. (1961), Exp. Cell Res. 25, 585.

81. Herndon, J.H., Steinberg, D., and Uhlendorf, B.W. (1969), J. Clin. Invest. 48, 1017.

82. Herndon, J.H., Steinberg, D., and Uhlendorf, B.W. (1969), N. Engl. J. Med. 281, 1034.

83. Hirschhorn, K. (1973), Birth Defects 9 (1), 9.

84. Hirschhorn, K., Nadler, H.L., Waithe, W.I., Brown, B.I., and Hirschhorn, R. (1969), Science 166, 1632.

85. Hooft, C., Carton, D., and Samyn, W. (1967), Lancet 1, 1384.

86. Hsia, D.D.Y. (1972), Enzyme 13, 161.

87. Kaback, M.M., Leonard, C.O., and Parmley, T.H. (1971), Pediat. Res. 5, 366.

88. Kashiwamata, S., and Greenberg, D.M. (1969), Fed. Proc. 28, 668.

89. Kashiwamata, S., Kotake, Y., and Greenberg, D.M. (1970), Biochim. Biophys. Acta 212, 501.

90. Kennedy, C., Shih, V.E., and Rowland, L.P. (1965), Pediatrics 36, 736.

91. Kim, V.J., and Rosenberg, L.E. (1973), Pediat. Res. 7, 291, (abstract).

92. Klavins, J.V. (1963), Brit. J. Exp. Pathol. 44, 507.

93. Komrower, G.M., Lambert, A.M., Cusworth, D.C., and Westall, R.G. (1966), Arch. Dis. Childhood 41, 666

94. Krooth, R.S., Darlington, G.A., and Velasquez, A.A. (1968), Ann. Rev. Genet. 2, 141.

95. Laster, L., Mudd, S.H., Finkelstein, J.D., and Irreverre, F. (1965), J. Clin. Invest. 44, 1708.

96. Laster, L., Spaeth, G.L., Mudd, S.H., and Finkelstein, J.D. (1965), Ann. Intern. Med. 63, 117.

97. Levy, H.L., Mudd, S.H., Schulman, J.D., Dreyfus, P.M., and Abeles, R.H. (1970), Amer. J. Med. 48, 390.

98. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951), J. Biol. Chem. 193, 265.

99. Mahoney, M.J., Rosenberg, L.E., Mudd, S.H., and Uhlendorf, B.W. (1971), Biochem. Biophys. Res. Commun. 44, 375.

100. Markert, C.L. (1963), Science 140, 1329.

101. Markert, C.L. (1968), Ann. N.Y. Acad. Sci. 151, 14.

102. McCully, K.S. (1969), Amer. J. Pathol. 56, 111.

103. McCully, K.S. (1970), Amer. J. Pathol. 59, 181.

104. McCully, K.S. (1972), Amer. J. Pathol. 66, 83.

105. McCully, K.S., and Ragsdale, B.D. (1970), Amer. J. Pathol. 61, 1.

106. McKusick, V. (1966), in Heritable Diseases of Connective Tissue, 3rd ed. C.V. Mosby Co., N.Y. Ch. 4.

107. McKusick, V.A. (1973), Amer. J. Hum. Genet. 25, 446.

108. Michl, H. (1952), Monatsh, Chem. 83, 737.

109. Moore, S., Spackman, D.H., and Stein, W.H. (1958), Anal. Chem. 30, 1185.

110. Moore, S., and Stein, W.H. (1951), J. Biol. Chem. 192, 663.

111. Mudd, S.H. (1971), Fed. Proc. 30, 970.

112. Mudd, S.H. (1971), in Inherited Disorders of Sulfur Metabolism, Carson, N.A.J. and Raine, D.N. (Eds.), Churchill Livingtone, London.

113. Mudd, S.H., Edwards, W.A., Loeb, P.M., Brown, M.S., and Laster, L. (1970), J. Clin. Invest. 49, 1962.

114. Mudd, S.H., Finkelstein, J.D., Irreverre, F., and Laster, L. (1964), Science 143, 1443.

115. Mudd, S.H., Finkelstein, J.D., Irreverre, F., and Laster L. (1965), Biochem. Biophys. Res. Commun. 19, 565.

116. Mudd, S.H., Finkelstein, J.D., Irreverre, F., and Laster L. (1965), J. Biol. Chem. 240, 4382.

117. Mudd, S.H., Laster, L., Finkelstein, J.D., and Irreverre, F. (1965), in Symp. on Amino Metab. in Schizophrenia. Atlantic City.

118. Mudd, S.H., Levy, H.L., and Abeles, R.H. (1969), Biochem. Biophys. Res. Commun. 35, 121.

119. Mudd, S.H., Levy, H.L., and Morrow, G. III. (1970), Biochem. Med. 4, 193.

120. Mudd, S.H., Uhlendorf, B.W., Frieman, J.M., Finkelstein, J.D., and Shih, V.E. (1972), Biochem. Biophys. Res. Commun. 46, 905.

121. Mudd, S.H., Uhlendorf, B.W., and Hinds, K.R. (1970), Biochem. Med. 4, 215.

122. Nadler, H.L. (1972), Pediatrics 49, 329.

123. Nadler, H.L. in Harris, H., and Hirschhorn, K., (Eds.), (1972), Advances in Human Genetics, Vol.III, Plenum Publishers, N.Y., p. 35.

124. Nadler, H.L., Bigley, R.H., and Hug, G. (1970), Lancet 2, 369.

125. Nadler, H.L., Chacko, C.M., and Rachmeler, M. (1970), Proc. Nat. Acad. Sci. 67, 976.

126. Nadler, H.L., and Gerbie, A.B. (1970), N. Engl. J. Med. 282, 596.

127. Nadler, H.L., and Messina, A.M. (1969), Lancet 2, 1277.

128. Nakagawa, H., and Kimura, H. (1968), Biochem. Biophys. Res. Commun. 32, 208.

129. Nitowsky, H.M., and Grunfeld, A. (1967), J. Lab. Clin. Med. 69, 472.

130. O'Brien, J.S., Okada, S., Fillerup, D.L., Veath, M.L., Adornato, B., Brenner, P.H., and Leroy, J.G. (1971), Science 172, 61.

131. Osborne, T.B., and Mendel, L.B. (1915), J. Biol. Chem. 20, 351.

132. Pascal, T., Beratis, N., and Gaull, G., personal communication.

133. Perry, T.L., Dunn, H.G., Hansen, S., MacDougall, L., and Warrington, P.D. (1966), Pediatrics 37, 502.

134. Perry, T.L., Love, D.L., Hansen, S., Crawford, L.E., and Tischler, B. (1968), Lancet 2, 474.

135. Rama Rao, P.B., Norton, H.W., and Johnson, B.C. (1961), J. Nutr. 73, 38.

136. Rose, W.C., Coon, M.J., Lockhart, H.B., and Lambert, G.F. (1955), J. Biol. Chem. 215, 101.

137. Rose, W.C., and Rice, E.E. (1939), J. Biol. Chem. 130, 305.

138. Rose, W.C., and Wixon, R.L. (1955), J. Biol. Chem. 216, 763.

139. Rosenberg, L.E. (1969), N. Engl. J. Med. 281, 145.

140. Rosenbloom, F.M., Henderson, J.F., and Caldwell, I.C. (1968), J. Biol. Chem. 243, 1166.

141. Salafsky, I., and Nadler, H.L. (1971), J. Pediat. 79, 794.

142. Sardharwalla, I.B., Jackson, S.H., Hawke, H.D., and Sass-Kortsak, A. (1968), Can. Med. Ass. J. 99, 731.

143. Schimke, R.N., McKusick, V.A., Huang, T., and Pollack, A.D. (1965), J. Amer. Med. Ass. 193, 87.

144. Schneider, J.A., Rosenbloom, F.M., Bradley, K.H., and Seegmiller, J.E. (1967), Biochem. Biophys. Res. Commun. 29, 527.

145. Schneck, L., Valenti, C., Amsterdam, D., Friedland, J., Adachi, M., and Volk, B.W. (1970), Lancet 1, 582.

146. Seashore, M.R., Durant, J.L., and Rosenberg, L.E. (1972), Pediat. Res. 6, 187.

147. Seegmiller, J.E., Rosenbloom, F.M., and Kelley, W.N. (1967), Science 155, 1682.

148. Selim, A.S.S.M., and Greenberg, D.M. (1960), Biochim. Biophys. Acta. 42, 211.

149. Shih, V.E., and Efron, M.L. (1970), N. Engl. J. Med. 283, 1206.

150. Shih, V.E., and Littlefield, J.W. (1970), Lancet 2, 45.

151. Shih, V.E., and Schulman, J.D. (1970), Clin. Chim. Acta. 27, 73.

152. Shows, T.B. (1967), Biochem. Genet. 1, 171.

153. Spaeth, G.L., and Barber, G.W. (1965), Amer. Acad. Opthal. and Otolaryng. 69, 912.

154. Spiro, H.R., Schimke, R.N., and Welch, J.P. (1965), J. Nerv. Ment. Dis. 141, 285.

155. Steinberg, D., Herndon, J.H., and Uhlendorf, B.W., (1967), Science 156, 1740.

156. Stekol, J.A., and Szaron, J. (1962), J. Nutr. 77, 81.

157. Stern, C., and Schaeffer, E,W. (1943), Proc. Nat. Acad. Sci. 29, 361.

158. Swim, H.E., and Parker, R.F. (1957), Amer. J. Hyg. 66, 235.

159. Tallan, H.H. personal communication.

160. Tallan, H.H., Moore, S., and Stein, W.H. (1958), J. Biol. Chem. 230, 707.

161. Tallan, H.H., Pascal, T.A., Schneidman, K., Gillam, B.M., and Gaull, G.E. (1971), Biochem. Biophys. Res. Commun. 43, 303.

162. Tashian, R.E., and Shaw, M.W. (1962), Amer. J. Hum. Genet. 14, 295.

163. Tjio, J.H., and Puck, T.T. (1958), J. Exp. Med. 108, 259.

164. Uhlendorf, B.W., Conerly, E.B., and Mudd, S.H. (1973), Pediat. Res. 7, 645.

165. Uhlendorf, B.W., and Mudd, S.H. (1968), Science 160, 1007.

166. Van Leeuwen, L., Jacoby, H., and Charles, D. (1965), Acta Cytol. 9, 442.

167. Werder, E.A., Curtius, H., Anders, P.W., and Prader, A. (1966), Helv. Paediat. Acta 21, 1.

168. Werman, R., Davidoff, R.A., and Aprison, M.H. (1966), Life Sci. 5, 1431.

169. White, H.H., Rowland, L.P., Araki, S., Thompson, H.L., and Cowen, D. (1965), Arch. Neurol. 13, 455.

170. Womack, M., Kremmerer, K.S., and Rose, W.C. (1937), J. Biol. Chem. 121, 403.

171. Womack, M., and Rose, W.C. (1941), J. Biol. Chem. 141, 375.

172. Wollaston, W.H. (1810), Phil. Trans. 100, 223.

173. Yeh, H.L., Frankl, W., Dunn, M.S., Parker, P., Hughes, B., and Gyorgy, P. (1947), Amer. J. Med. Sci. 214, 507.

174. Yoshida, T., Tada, K., Yokoyama, Y., and Arakawa, T. (1968), Tohoku, J. Exp. 96, 235.