Cysteine Activation in Cultured Cystinotic Cells

THE SPECIFIC ACTIVITY OF CYSTEINYL-tRNA SYNTHETASE AND $tRNA_{Cys}$ AND THE DETERMINATION OF THE MICHAELIS-MENTEN CONSTANTS FOR CYSTEINYL-tRNA SYNTHETASE

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ABSTRACT This study explored the possibility whether an altered cysteinyl-tRNA synthetase might lead to the faulty regulation of cyst(e)ine levels in cystinotic cells. This hypotheses is attractive, since amino acid activation is important in the regulation of amino acid metabolism in microorganisms. By using cultured fibroblasts from patients with cystinosis, those cell components responsible for cysteine activation were examined: cyst(e)ine, the cysteinyl-tRNA levels, cysteinyl-tRNA synthetase activity, and the K_m of cysteine, ATP, and $tRNA_{Cys}$ for cysteinyl-tRNA synthetase. Fibroblasts from two patients with the infantile form of cystinosis were labeled for three days with [^{35}S]-cystine. In comparison with normal cells, these cells contained high levels of free cysteine and cystine. Labeled fibroblasts from a patient with the adolescent form of the disease contained elevated levels of cystine, although elevated cysteine levels were not detected. The ratio of acceptor activity of $tRNA_{Cys}$ to $tRNA_{Leu}$ in cystinotic cells was 0.46 in cystinotic cells and 0.54 in normal cells. The specific activity of cysteinyl-tRNA synthetase measured in fibroblasts of two infantile and one adolescent form was: 6.1, 2.2, and 2.1 pmol of [^{14}C]aminoacyl-tRNA formed/μg protein/10 min, respectively. In addition, the cysteine K_m's for the same cells, respectively, were: 3.1 μM, 1.5 μM, and 1.2 μM. The corresponding data for specific activities of two normal cell lines were 2.0 and 5.1 pmol [^{14}C]aminoacyl tRNA formed/μg protein/10 min, with K_m's of 3.0 μM and 1.7 μM. These data indicate that cystinotic cells contain levels of $tRNA_{Cys}$ and Cys-tRNA synthetase comparable to normal cells. In addition, within the cystinotic cells, the relative level of the Cys-tRNA synthetase and $tRNA_{Cys}$ to those of leucine and alanine are comparable to normal cells. Finally, the K_m of Cys-tRNA synthetase for ATP and tRNA is similar in normal and cystinotic cells.

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INTRODUCTION

Cystinosis is an autosomal recessive disease characterized by renal tubular dysfunction and growth retardation, which leads to death within the first decade of life (1). It can be diagnosed by the detection of cystine crystals in bone marrow and the cornea. Although the serum levels of cystine are normal, there is a marked increase of free cystine in all cells. The cause of the increased cystine levels has remained enigmatic. The disease does not appear to be a storage disease in the usual sense, since ^{35}S-labeled cystine is depleted from cystinotic cells at the same rate as from normal cells and is available for cellular metabolism (2, 3). In addition, no deficiency of enzymes in cysteine and cystine metabolism or cysteine-cystine interconversion has been identified (4, 5). It has been shown that cystine accumulates in lysosomes, yet it is not clear whether this is a cause or effect of the disease (6, 7).

Autosomal recessive traits are often the consequence of the deficiency of a single enzyme activity. Since cyst(e)ine accumulates in lysosomes and perhaps in other organelles, it is reasonable to suspect that an enzyme of cyst(e)ine metabolism is involved. We wish to report on an aspect of cysteine metabolism which has not previously been examined—that of cysteine aminoacyl synthetase. We have chosen to examine this particular aspect of cyst(e)ine metabolism since it has been shown in microorganisms that aminoacyl-tRNA synthetase and aminoacyl tRNA are important factors in the

regulation of intracellular levels of amino acids and their biosynthetic enzymes (8, for review see ref. 9). Since a total deficiency of this system would be incompatible with life, we have examined both its activity and its kinetics. The K_m's for the substrates, cysteine, ATP, and tRNA were determined. Controls were obtained by comparing cysteine activation activities to that of other amino acids in cystinotic cells and controls.

METHODS

Culture of cells. The fibroblast cells were grown from skin biopsies. Two of the cystinotic cell types were from individuals with the infantile form of the disease (nos. 1 and 2), and one had the adolescent onset form of the disease (10). The "normal" fibroblasts contained no detectable metabolic defect. The cells were all less than 20 generations from the first transfer from the skin biopsy, and no detectable viral or pleuropneumonia-like organism infection was found.

The cells were grown in Eagle's minimal essential medium (MEM) [1] with 10% fetal calf serum in Blake flasks and roller bottles. At confluency, the cells were washed with wash solution (0.83 g/liter Tris-base and 2.8 g/liter Tris HCl, 0.4 g KCl, 8.0 g/liter NaCl, and 0.201 g/liter EDTA). After washing, the cells were trypsinized by using 2.5 g/liter trypsin (Difco Laboratories, Detroit, Mich.) in wash solution. The trypsinized cells were resuspended in approximately 100 ml of ice cold MEM/ml of cells. The cells were pelleted by centrifugation at 400 *g* and were resuspended in a buffer containing 0.3 M sucrose, 0.01 M Tris-HCl (pH 7.5 at 25°C), and 0.005 M $MgAc_2$ (TM buffer), and repelleted. The second pellet was resuspended in TM buffer, 1 ml/g of cells, and stored at −90°C until used.

Labeling for measurement of free cyst(e)ine. Fibroblast cells were labeled in a manner described by Schulman, Fujimoto, Bradley, and Seegmiller (11). They were cultured in MEM containing 0.1 mCi of [^{35}S]cystine (Amersham/Searle Corp., Arlington Heights, Ill.). At the end of the 3 days of labeling, the cells were harvested by trypsinization and washed in 5 ml of MEM, and the number of cells was counted in a hemacytometer chamber. The suspension was then centrifuged, and the pellet was resuspended in 1 ml of Hanks' Salt Solution. The cells were lysed by the addition of 0.5 ml of 10% sodium lauryl sulfate solution in the presence of 7 mM *N*-ethylmaleimide (NEM) at pH 7.5. At this point, 1.5 ml of saturated picric acid solution was added. This solution was filtered by suction with Millipore filters and added to another tube with Dowex 2X10 in the chloride form (Dow Chemical Co., Midland, Mich.). This was added in small amounts, until all trace of yellow color was removed from the liquid. This solution was taken to dryness of 40°C *in vacuo* and redissolved in 2 ml of pH 2.2 amino acid analyzer starting buffer.

Amino acid analysis. Analyses of the cell extracts were carried out on the Beckman 120C Automatic Amino Acid Analyzer (Beckman Instruments, Inc., Fullerton, Calif.). A 0.9 × 60-cm column of UR30 resin was used. The tubing leading from the flow meter was replaced with a length of AWG-22 Teflon tubing which delivered the effluent to a fraction collector. Effluent fractions were collected at 1-min intervals. Samples collected as described above were suspended in 18 ml of toluene-based scintillant containing 45% vol/vol Triton X (Rohmand Haas Co., Philadelphia, Pa.). Counting was carried out in a liquid scintillation spectrometer. Quench corrections were made by using the automatic external standard (Packard Instrument Co., Inc., Downers Grove, Ill.).

Preparation of aminoacyl-tRNA synthetase. The procedure used to prepare aminoacyl-tRNA synthetase was adapted from Yang and Novelli (12). All operations were carried out at 0–4°C. Frozen cells prepared as described above were thawed in 2 ml of TM buffer/g frozen cells in the presence of 0.001 M dithiothreitol (DTT). The cells were homogenized in a Dounce homogenizer with 30 strokes, or until the nuclei were essentially free of cytoplasm, as judged by phase microscopy. The nuclei and cellular debris were removed by centrifugation at 20,000 *g* for 30 min. The resulting supernate was centrifuged in 2 ml nitrocellulose tubes at 40,000 rpm (100,000 *g*) for 90 min. The supernate (100S) was removed from the top of the pellet (1.5 ml/tube) and chromatographed on a 30-ml sephadex G100 column equilibrated in buffer A (15% glycerol, 0.01 M KPO_4 at pH 7.5, 0.005 M KCl, 0.001 M $MgAc_2$, 0.001 M DTT). The large molecular weight fractions (≥ 25,000 mol wt) were pooled and adsorbed to a 1 ml DEAE cellulose column equilibrated with buffer A. The column was washed with 5 vol of buffer A. The synthetases were eluted as a single peak with a buffer containing 15% glycerol, 0.01 M KPO_4 (pH 6.5), 0.25 M KCl, 0.005 M $MgAc_2$, and 0.001 M DTT. The active fractions were pooled and stored at −90°C.

Preparation of tRNA. The tRNA's were prepared after the procedure of Delihas and Staehelin (13). Human placental tRNA was prepared from a fresh human placenta. The placenta was immediately minced, washed in 0.9% NaCl, and frozen by placing the tissue in acetone-dry ice. From 300 g of this material, 320 A_{260} units of tRNA were prepared and used for all determinations of aminoacyl-tRNA synthetase except where stated. Fibroblast tRNA was prepared from approximately 1.4 g of fibroblasts grown from a patient with the infantile type of cystinosis, and 1.5 g of fibroblasts from a normal person. From the infantile type cells, 4.9 A_{260} units of tRNA were obtained, and from the normal cells 2.7 A_{260} units were obtained.

Determination of aminoacyl-tRNA synthetase activity. The assay used to determine both the amounts of aminoacyl-tRNA synthetase and tRNA was adapted from Yang and Novelli (12). Each reaction mixture contained per milliliter the following components which are referred to as the "common components" in the figure and table legends: 50 μmol Tris-HCl (pH 7.4); 50 μmol KCl; 10 μmol magnesium acetate in the alanyl-tRNA synthetase and cysteinyl-tRNA synthetase reactions, or 4 μmol in the leycyl-tRNA synthetase reactions; 2 μmol DTT; and 4 μmol ATP. The amounts per assay of [^{14}C]amino acid, tRNA, and aminoacyl-tRNA synthetases are given in the legends to the figure and tables. The ionic concentrations of K^+ and Mg^{++} have been shown by concentration dependence studies to be the concentration to give maximum enzyme activity for Cys-tRNA synthetase found in normal fibroblasts. Cysteine was added to the assays as [^{14}C]-cystine. Before starting the charging reaction by the addition of the synthetase preparation, the components were incubated at 30°C for 5 min to reduce cystine to cysteine

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; MEM, Eagle's minimal essential medium; NEM, *N*-ethylmaleimide; PPLO, pleuropneumonia-like organism; TCA, trichloroacetic acid; TM buffer, buffer containing 0.3 M sucrose, 0.01 M Tris-HCl (pH 7.5 at 25°C), and 0.005 M $MgAc_2$.

TABLE I

Amino Acid Analysis of the Nonprecipitable Radioactivity in Cells Grown in Media Containing [^{35}S]cystine

Cell	Substance in cpm/300,000 cells			Cystine/ glutathione	Total cystine*	Cystine sp act‡
	Glutathione	Cysteine-NEM	Cystine			
Normal	21,400	0	0	—	0	—
Infantile-1	29,500	930	13,700	0.47	5.25	2,600
Infantile-2	20,200	880	8,800	0.46	1.98	4,400
Adolescent	20,200	0	2,200	0.11	1.5	1,500
Mixture§	25,000	570	4,700	0.22	1.62	2,900

* In nmol of free cystine in 300,000 cells.

‡ cpm /nmol cystine.

§ Cells of infantile-1 grown with equal number of normal fibroblasts.

by DTT. After the addition of aminoacyl-tRNA synthetase, the mixtures were incubated at 30°C for 10 min. The reaction was terminated by the addition of ice-cold 5% trichloroacetic acid (TCA). The protein and tRNA (aminoacyl-tRNA plus tRNA) which precipitated were collected on Millipore filters and washed with ice-cold 5% TCA. The filters were dried and counted in 10 ml of a toluene-based scintillation fluid. Protein determinations were measured by the technique of Lowry, Rosebrough, Farr, and Randall (14).

RESULTS

Free cellular cystine. The free cellular cyst(e)ine was determined in the various cell lines. Approximately 300,000 cells were labeled with [^{35}S]cystine in a 25 cm^2-plastic culture flask as described in Methods. The cells were treated as described in Methods, and the deproteinized filtrate was chromatographed in the amino acid analyzer. The results for a 3-day label are shown in Table I. Approximately 6% of the soluble counts were eluted before aspartic acid and were not further characterized. Both cysteine and cystine were elevated in the infantile-type cells, although only cystine was detected in the adolescent-type cells.

When equal numbers of normal and cystinotic cells were grown in the same flask after mixing, [^{35}S]cyst(e)ine incorporation was equal to that of the cells grown separately. This is in contrast to the mucopolysaccharide storage diseases in which the defective cells are "corrected" when grown together (15).

The results shown in Table II compare the acceptor activity of tRNA isolated from three different cell types. The lowest activity was seen in the infantile-1 cells. The level of tRNA acceptor activity of a cell would reflect the level of protein synthesis of a cell at the time of harvest. In an attempt to discover whether $tRNA_{Cys}$ was increased or decreased in relation to other species, we have compared the ratio of $tRNA_{Cys}$ to $tRNA_{Ala}$ and $tRNA_{Leu}$ in the three cell types. In all of the cells examined, there was a close correlation between acceptor activity for one amino acid compared to another.

Table III shows the specific activity of Cys-tRNA synthetase in each cystinotic cell line tested. The values in cystinotic cells ranged from 2.1 to 6.1 U. The values in normal cells ranged from 2.0 to 5.8 U. As seen in Table III, no cell line was deficient in Cys-tRNA syn-

TABLE II

Specific Acceptor Activities of tRNA's which were Isolated from Normal Placenta and from Cystinotic Cells

tRNA preparation	sp act*			Ratio	
	$tRNA_{Cys}$	$rRNA_{Leu}$	$tRNA_{Ala}$	$tRNA_{Cys/Leu}$	$tRNA_{Cys/Ala}$
Human placenta	41 (39)	111	94	0.36	0.44
Infantile-1	19 (22)	41	45	0.46	0.42
Normal fibroblasts	69	109	127	0.54	0.64

* Specific activity of the tRNA's was determined by allowing the charging reaction to run to completion. In addition to the common components given in Methods, the 0.15-ml reaction mixtures contained 3 μmol of [^{14}C]amino acid; 1.0 A_{260} units of the indicated tRNA and no tRNA for the control; and 3.3 μg of normal cell synthetase. The results of the addition of 4.5 μg of the infantile-1 synthetase is shown in parentheses. Samples of 0.025 ml were taken at 3, 6, 12, 24, and 36 min. The reaction was complete by 24 min. These results are net values, i.e. sample minus control. The specific activity of the tRNA's is expressed as pmol of amino acid/A_{260} units.

thetase. To further demonstrate that Cys-tRNA synthetase was not abnormally low in cystinosis, its activity was compared with Ala-tRNA synthetase and Leu-tRNA synthetase in infantile-1 and normal cells. The specific activity of these synthetases were all found to be comparable. The ratios of one synthetase activity to another was similar for normal and abnormal cells.

To determine whether the Cys-tRNA synthetase had an abnormally low affinity for cysteine in cystinotic cells, as compared to the affinity in normals, its K_m's for cysteine were measured. Those K_m's for cysteine were: 3.1 μM for infantile-1, 1.5 μM for infantile-2, 1.2 μM for the adolescent form, and from 1.7 to 3.0 μM for the normal cells. Examples of some of the K_m determinations are given in Fig. 1.

Furthermore, to determine whether Cys-tRNA synthetase had an abnormally low affinity for either ATP or $tRNA_{Cys}$ in cystinotic cells as compared to normals, these K_m's were also measured. The K_m for ATP was

TABLE III

Determination of Specific Activities of Aminoacyl-tRNA Synthetase Activities

Enzyme source	Units of sp act for each amino acid			Ratio	
	Cys	Leu	Ala	Cys/Leu	Cys/Ala
Normal-1	5.8	5.2	4.5	1.1	1.3
Normal-2	2.7	2.0	3.1	1.4	0.9
Infantile-1	6.1	4.2	4.7	1.4	1.3
Infantile-2	2.2	—	—		
Adolescent	2.1	—	—		

* Specific activities were determined for each enzyme as described in Methods. In addition to the common components detailed in Methods, each 0.05-ml-reaction mixture contained 1 μ[^{14}C]amino acid; 0.5 A_{260} units of human placental tRNA; and varying amounts of synthetases. 1 U of enzyme activity is defined as pmol [^{14}C]aspartic acid-tRNA formed/μg protein/10 min.

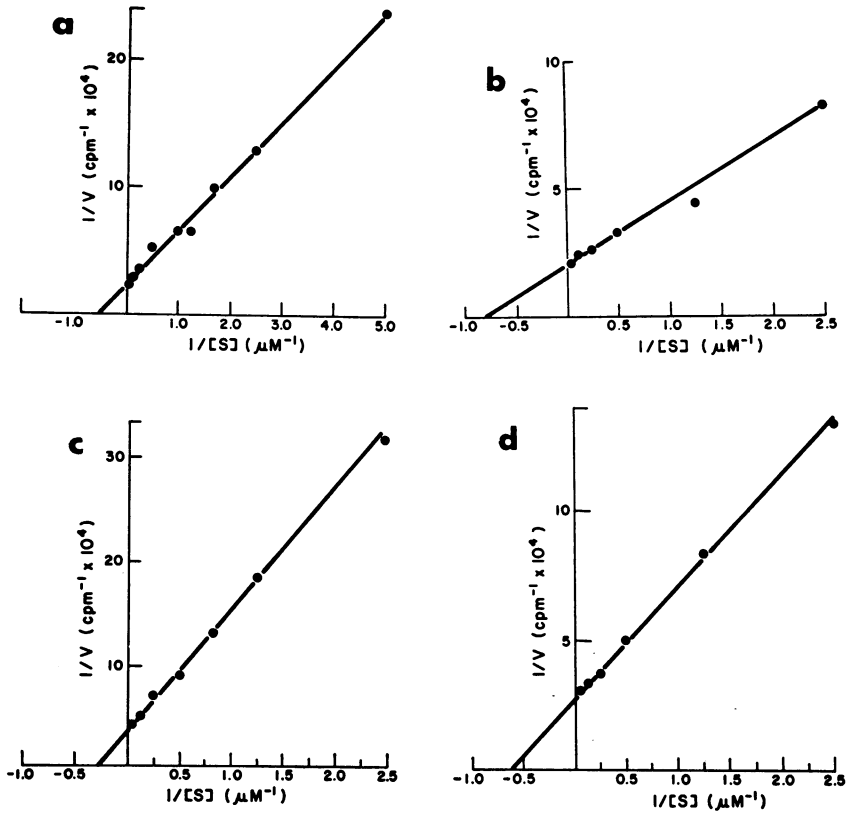

FIGURE 1 Determination of K_m between cysteine and cysteinyl-tRNA synthetase. Each 0.05-ml reaction mixture contained the following components in addition to the common components listed in Methods: [^{14}C]cysteine as indicated on the abscissas of the figure; 0.5 A_{260} units of human placental tRNA; and one of the cysteinyl-tRNA synthetase preparations. i.e., normal (2.4 μg), infantile-1 (3.4 μg), infantile-2 (6.2 μg), or adolescent (9.4 μg). The curves were fit by eye and the K_m's were determined at the intercept of the line with the abscissa. Source of cysteinyl-tRNA synthetase: (*a*) normal cell, (*b*) adolescent cystinosis, (*c*) infantile cystinosis-1, and (*d*) infantile cystinosis-2.

200 μM for both normal and infantile-1 cell lines. The K_m's for $tRNA_{Cys}$, using human placental tRNA as the source of $tRNA_{Cys}$, were 1.7 μM in the infantile-1 cell line and 1.9 μM in the normal cell line.

DISCUSSION

In metabolic diseases it is important to consider that normal levels of enzyme activity do not prove that the enzyme is normal. Enzyme activities are often determined at unphysiologic levels of substrate concentration. Defects of enzymes in which there is a decreased affinity of the enzyme for the substrate would be missed under these conditions. Such a defect would be reflected in an increased intracellular level of substrate (16). An interesting example of an altered enzyme substrate affinity which led to a requirement for increased intracellular amino acid levels has been described by Roth and Ames (8). In the hisS1520 mutant of *Salmonella typhimurium,* it was found that the histidyl-tRNA synthetase had a 50-fold decreased affinity for histidine and required an increased intracellular level of histidine for normal growth. These authors demonstrated that the concentration of histidyl-tRNA of the cell appeared to regulate the level of histidine biosynthetic enzymes. In human disease, there have been recent examples of enzyme-cofactor complexes which exhibit a decreased affinity. Those metabolic diseases which require an increased vitamin supplement for correction have been reviewed recently (17, 18).

These experiments were designed to test the possibility that, in cystinotic cells, a defect might exist in the ability of the Cys-tRNA synthetase to accept cysteine. The K_m of the aminoacyl-tRNA synthetase for its substrates: cysteine ATP, and tRNA were similar in cystinotic and normal cells. Our experimental results indicate that amino acid activation is not defective. It is possible, however, that K_m differences which are smaller than we are able to detect could still have important physiologic consequences.

The results of our amino acid analysis indicated that cysteine levels, as well as cystine, are elevated in the infantile form of cystinosis. This finding will require further substantiation since Schulman, Schneider, Bradley, and Seegmiller (3) have concluded that cysteine levels are not increased in infantile cystinosis. Our data also indicate that the infantile form is somewhat different than the adolescent form, since the cysteine/cystine ratio is lower in the adolescent form of the disease. The labeling of a mixed culture indicates that "complementation" by growing normal cells with abnormal cells will not lower the cyst(e)ine content in the cystinotic cells, as is seen with mucopolysaccharide disease (15).

Although the amino acid-activating mechanisms appear to be normal for cysteine in cystinotic cells, the protein-synthesizing system should be considered as a potential source of an inborn error of metabolism in other diseases. Since the activating mechanism is specific for a single amino acid, a single abnormal amino acid synthetase could lead to high amino acid levels if its K_m were altered. This would especially be true if the intracellular level of the amino acid is regulated by the level of charged tRNA. It is also possible that nonessential amino acid biosynthetic enzymes and transport enzymes are regulated by charged tRNA, as is illustrated in the hisS mutants of *Salmonella typhimurium* (8, 9).

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REFERENCES

1. Seegmiller, J. E., T. Friedmann, H. E. Harrison, V. Wong, and J. A. Schneider. 1968. Cystinosis: combined clinical staff conference at the National Institutes of Health. *Ann. Intern. Med.* **68**: 883–905.
2. Schneider, J. A., K. H. Bradley, and J. E. Seegmiller. 1968. Transport and intracellular fate of cysteine-^{35}S in leukocytes from normal subjects and patients with cystinosis. *Pediatr. Res.* **2**: 441–450.
3. Schulman, J. D., J. A. Schneider, K. H. Bradley, and J. E. Seegmiller. 1971. Cystine, cysteine, and glutathione metabolism in normal and cystinotic fibroblasts *in vitro,* and in cultured normal amniotic fluid cells. *Clin. Chim. Acta.* **35**: 383–388.
4. Patrick, A. D. 1962. The degradative metabolism of L-cysteine and L-cystine *in vitro* by liver cystinosis. *Biochem. J.* **83**: 248–256.
5. Tietze, F., K. H. Bradley, and J. D. Schulman. 1972. Enzymic reduction of cystine by subcellular fractions of cultured and peripheral leukocytes from normal and cystinotic individuals. *Pediatr. Res.* **6**: 649–658.
6. Hummeler, K., B. A. Zajac, M. Genel, P. G. Holtzapple, and S. Segal. 1970. Human cystinosis: intracellular deposition of cystine. *Science (Wash. D. C.).* **166**: 859–860.
7. Schulman, J. D., K. H. Bradley, and J. E. Seegmiller. 1969. Cystine: compartmentalization within lysosomes in cystinosis leukocytes. *Science (Wash. D. C.).* **166**: 1152–1154.
8. Roth, J. R., and B. N. Ames. 1966. Histidine regulatory mutants in *salmonella typhimurium.* II. Histidine regulatory mutants having altered histidyl-+RNA synthetase. *J. Mol. Biol.* **22**: 325–334.
9. Littaner, U. Z., and H. Inouye. 1973. Regulation of tRNA. *Annu. Rev. Biochim.* **42**: 439–470.
10. Goldman, H., C. R. Scriver, K. Aaron, E. Delvin, and Z. Canlos. 1971. Adolescent cystinosis: comparisons with infantile and adult forms. *Pediatrics.* **47**: 979–988.

11. Schulman, J. D., W. Y. Fujimoto, K. H. Bradley, and J. E. Seegmiller. 1970. Identification of heterozygoud genotype for cystinosis *in utero* by a new pulse-labeling technique: preliminary report. *J. Pediatr.* **77**: 468–470.
12. Yang, W. K., and G. D. Novelli. 1971. Analysis of isoaccepting tRNA's in mammalian tissues and cells. *In* Methods in Enzymology. K. Moldave and L. Grossman, editors. Academic Press, Inc., New York. **20**: 44–55.
13. Delihas, N., and M. Staehelin. 1966. The preparation of rat-liver soluble reibonucleic acid. *Biochim. Biophys. Acta.* **119**: 385–391.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
15. Kresse, H., and Neufeld, E. F. 1972. The Sanfillippo A corrective factor. Purification and mode of action. *J. Biol. Chem.* **247**: 2164–2170.
16. Scriver, C. R. 1967. Commentary. *In* Amino Acid Metabolism and Genetic Variation. W. Nyhan, editor. McGraw-Hill, New York. **1**: 225.
17. Rosenberg, L. E. 1970. Vitamin-dependent genetic disease. *Hosp. Pract.* **5**: 59–66.
18. Mudd, S. H. 1971. Pyridoxine-responsive genetic disease. *Fed. Proc.* **30**: 970–976.