Familial Hyperlysinemia with Lysine-Ketoglutarate Reductase Insufficiency

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ABSTRACT Fibroblasts grown in tissue culture from the skin of normal subjects have lysine-ketoglutarate reductase activity (lysine: α-ketoglutarate: triphosphopyridine nucleotide (TPNH) oxidoreductase (ϵ-*N*-[L-glutaryl-2]-L-lysine forming)). The activity of the enzyme is considerably reduced in the skin fibroblasts grown from three siblings with hyperlysinemia. The high concentrations of lysine in the blood of these patients, the previous demonstration in the intact subject of a reduction in the ability to degrade lysine, and the present demonstration of diminished lysine-ketoglutarate reductase activity, accurately define the metabolic defect and establish the saccharopine (ϵ-*N*-[L-glutaryl-2]-L-lysine) pathway as the major degradative pathway for lysine in the human.

INTRODUCTION

In 1964, a 4½ yr old girl was reported with mental retardation, excessive lysinuria, and hyperlysinemia (1). Since then two additional siblings and a first cousin have been discovered to have a similar metabolic abnormality. The metabolism of lysine has been studied in vivo in two of the siblings and evidence has been presented that a defect exists in the degradative pathway for lysine probably at the first metabolic step (2).

The recent demonstration of saccharopine (ϵ-*N*-[L-glutaryl-2]-L-lysine) formation from lysine in the rat (3, 4) and in the human (5), and the development in this laboratory of a microassay for the responsible enzyme, lysine-ketoglutarate reductase, (lysine: α-ketoglutarate: triphosphopyridine nucleotide (TPNH) oxidoreductase (ϵ-*N*-[L-glutaryl-2]-L-lysine forming)) have made it possible to investigate more specifically the enzyme defect. In the present study, it has been demonstrated that fibroblasts grown in tissue culture from skin biopsies obtained from normal subjects convert lysine to saccharopine. In comparison, the lysine-ketoglutarate reductase activity was considerably reduced in the skin fibroblasts of the three siblings with the disease. The results accurately define the enzyme defect in the disease and establish the saccharopine pathway as the major degradative pathway for lysine.

METHODS

Materials

L-lysine-U-^{14}C was purchased from New England Nuclear Corp., Boston, Mass. A trace radioactive contaminant with electrophoretic properties similar to saccharopine was removed by passage through a column of Rexyn 206 (OH^-), Fisher Scientific Company, New York. The resin was prepared by cycling with 3 N HCl and 1 N ammonium hydroxide and then was left in the hydroxyl phase. Under these conditions, lysine was eluted from the column with water. Cab-O-Sil, fumed silica, grade M5, was obtained from Cabot Corporation, Boston, Mass. Saccharopine was extracted from yeast by methods previously described (5).

Tissue culture

Skin biopsies were obtained from the three patients with hyperlysinemia and grown in monolayer in Waymouth's medium containing 10% calf serum and antibiotics (penicillin 50 U/ml, streptomycin, 50 μg/ml, and kanamycin 30 μg/ml). Foreskins obtained at circumcision of newborn infants were cultured as normal controls. Subcultures of the explants were permitted to grow to confluence in 4-oz glass bottles. At the time of assay, the normal cells had proliferated for 10–18 generations and the hyperlysinemics for 16–20 generations.

Enzyme assay

The fibroblasts from four bottles, representing a total of approximately 4 million cells, were harvested by replacing the nutrient medium with 5 ml of a 15: 85 v/v mixture of

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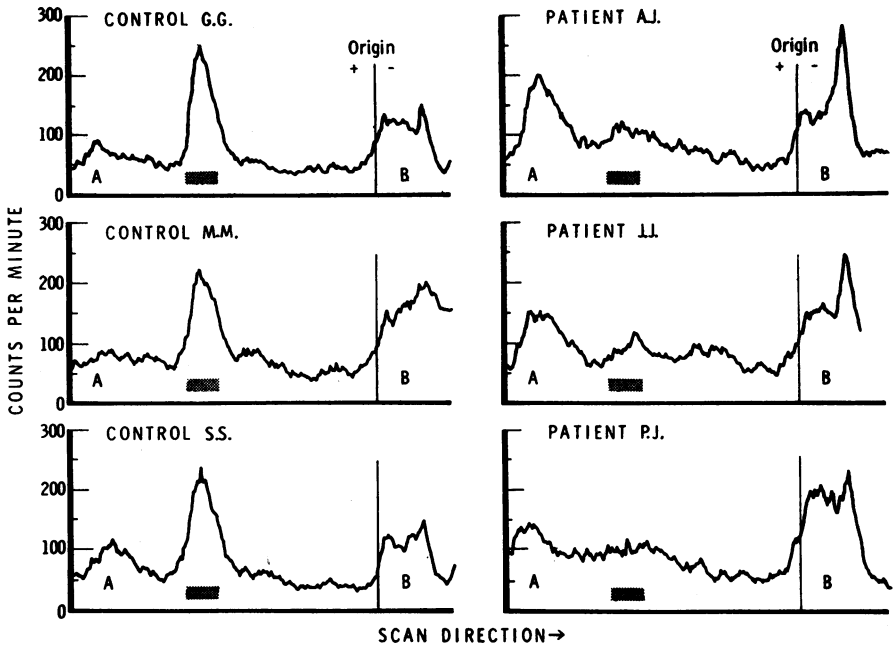

FIGURE 1 Radioactive pattern after high-voltage electrophoresis of incubation media. Disrupted skin fibroblasts were incubated with lysine-^{14}C. The incubation medium was applied to paper and subjected to high-voltage electrophoresis. Lysine migrates towards the cathode, and has been omitted from the radioactive scan. The control fibroblasts produce a radioactive peak corresponding in mobility to an internal standard of saccharopine (stippled bar), not evident after incubation with fibroblasts from hyperlysinemic subjects.

sodium phosphate, 0.11 mole/liter, pH 7.4, and 0.15 M NaCl, and removing the cells from the bottle with a rubber policeman. The suspension was centrifuged at 2000 *g* for 15 min, and the supernatant discarded. The cells were stored at −15°C for 4 days or more before testing.

For assay, the cells were thawed in a 23°C water bath and 0.15 ml of 0.05 M KCl was added. The fibroblasts were then disrupted by cycling five times between a dry-ice bath and the water bath over a period of 30 min, aided by occasional stirring with a glass rod. To each sample of disrupted cells was added the following incubation mixture: L-lysine-U-^{14}C, 0.5 μc, 1.0 μmole; $MgCl_2$, 0.05 μmoles; potassium α-ketoglutarate, 2 μmoles; potassium phosphate, pH 7.1, 10 μmoles; TPNH, 1.5 μmoles; and water to a final volume of 0.5 ml. Incubation was with agitation for 60 min at 30°C under a stream of nitrogen.

To terminate the incubation, 5 μmoles of saccharopine in 0.05 ml water was added to the incubation mixture and the reaction tubes were placed in a boiling water bath for 5 min. The tubes were centrifuged while hot, and the supernatant removed for analysis. The amount of protein in the precipitate was measured by dissolving the precipitate in 1.0 N NaOH and assaying the protein content by the method of Lowry (6). This provided an indication of the number of cells incubated.

Analysis

High voltage electrophoresis. 0.1 ml of the incubation medium was applied to Whatman 3MM paper and subjected to 2000 v for 3 hr in 0.05 M potassium phosphate buffer, pH 6.7. A lower pH was used than that previously reported (5) to better separate an unidentified radioactive peak (see Fig. 1, peak *A*) from saccharopine. The strip was surveyed in a Nuclear-Chicago Actigraph III strip counter. The saccharopine peak was located by removing a 2 mm wide strip from the paper and developing with ninhydrin. The remainder of the saccharopine area was removed, cut into small pieces with scissors, and eluted by shaking for 15 min in 5 ml of water at 60°C. The process was repeated and the eluates pooled, filtered, and evaporated to dryness under vacuum. The residue was dissolved in 1 ml of citrate buffer, pH 2.8.[1]

Column chromatography. Approximately 0.2 μmoles of methionine was added to the sample as a marker and the mixture applied to a Phoenix Micro Analyzer with a 0.9 × 15 cm column containing Spherix resin XX907-10-OPKU. Elution was with 0.2 M sodium citrate buffer, pH 3.25,[1] and a flow rate of 1 ml/min. The saccharopine peak appeared at 63 min.

Determination of radioactivity. The saccharopine peak was collected at the outflow of the colorimeter into scintillation counting vials in 1-min aliquots. The ninhydrin color was bleached by the addition of 0.1 ml 30% hydrogen peroxide to each vial, and the contents evaporated to dryness in a water bath at 60°C. The residue was dissolved in 0.5 ml water. To this was added 11 ml methanol, approximately 270 mg Cab-O-Sil (to produce a gel), and 9 ml of the scintillant mixture containing (per liter) : 2,5-diphenyloxazole

[1] The composition was that described by Hamilton (7) for buffer, pH 2.875, with the pH corrected by titration.

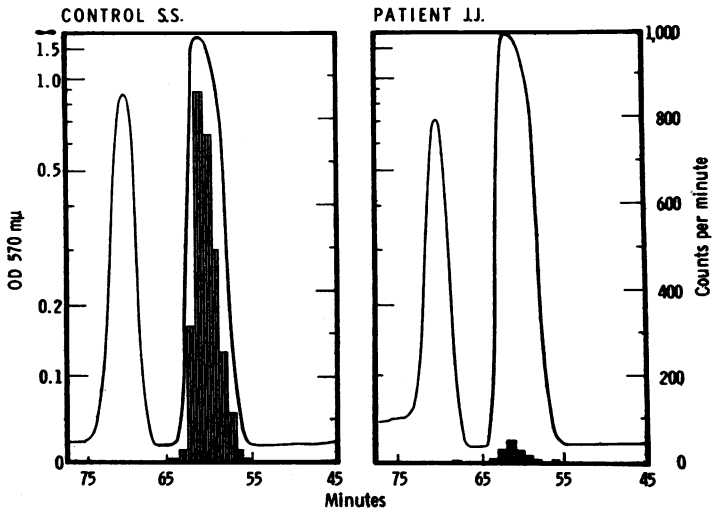

FIGURE 2 Column chromatography of presumed saccharopine. After high-voltage electrophoresis (Fig. 1), the saccharopine area was eluted and analyzed by column chromatography. The ninhydrin optical density (continuous line) is indicated by the left ordinate; radioactivity by the bar graph and the right ordinate. Methionine was added as an internal standard and appears to the left of the saccharopine peak. The radioactivity coincides with the saccharopine peak. Saccharopine-^{14}C synthesis by fibroblasts from the hyperlysinemic patient is greatly reduced. The results with the remaining normals and hyperlysinemics were essentially the same as those illustrated here.

(PPO), 15 g; dimethyl-POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene 0.4 g, and Triton X-100, 10 g. The vial contents were gelled by 5–10 sec treatment with a Model S 75 sonic generator, Branson Instruments, Inc., Stamford, Conn. Counting was with a wide window on the low energy channel usually used for tritium. Counting efficiency was 75%.

RESULTS

The radioactive patterns following high voltage electrophoresis are illustrated in Fig. 1. The saccharopine peaks are higher in the normal subjects than in the hyperlysinemics, whereas the reverse appears to be generally true of unidentified peak *A*.

After preliminary separation by high voltage electrophoresis, the only clearly defined radioactive peak on column chromatography coincided with saccharopine (Fig. 2). It was evidently considerably higher in the normal subjects than in those with hyperlysinemia.

The amount of synthesized saccharopine is presented in Table I. This figure was calculated by relating the amount of radioactivity recovered after column chromatography to the original specific activity of the radioactive lysine. The losses incurred during the analytical procedure were corrected for by comparing the final amount of saccharopine, as determined by ninhydrin, to the amount of carrier saccharopine added to the reaction flasks.

The amount of saccharopine synthesized by the three normal subjects averaged 21 mμmoles/mg protein. The hyperlysinemic patients synthesized 11% (A. J.), 10% (J. J.), and 7% (P. J.) of the average of the normals.

A few comments concerning the analytical methods may be useful to workers in this field. The mobilities of various metabolites of lysine on high-voltage electrophoresis and the elution times on ion exchange chromatography are presented in Table II. Both analytical pro-

TABLE I
Saccharopine Synthesis by Skin Fibroblasts

	Radioactivity*	Saccharopine synthesized
	counts/min	*mμmoles/mg protein*
Normals		
G. G.	2980	26
M. M.	1370	13
S. S.	2820	24
Hyperlysinemia		
A. J.	174	2.2
J. J.	144	2.1
P. J.	109	1.4

* Radioactivity contained in the six most active fractions under the saccharopine peak.

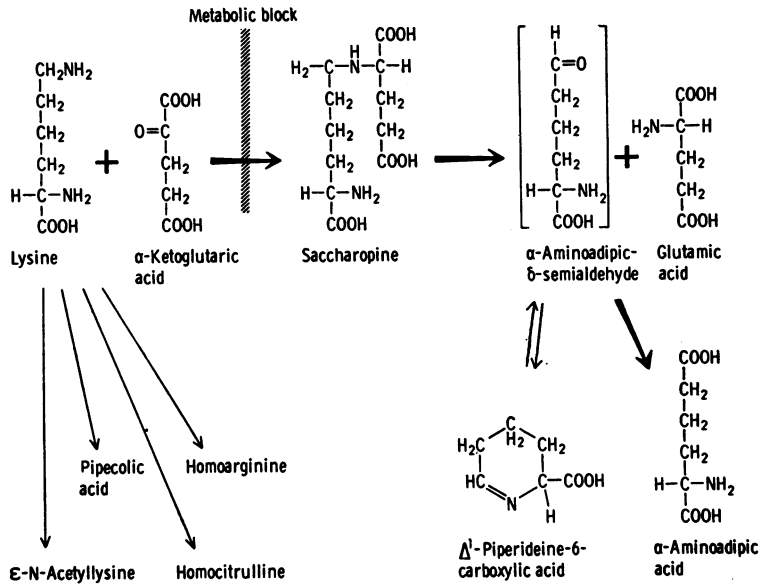

FIGURE 3 Degradative pathways of lysine.The major degradative pathway of lysine in man involves the formation of saccharopine, as established by the present studies. The remainder of the pathway is suggested by studies in lower organisms and has not been documented in mammals.

cedures were needed to isolate saccharopine. High-voltage electrophoresis separated saccharopine from lysine, pipecolic acid, homocitrulline, and unidentified radioactive peaks *A* and *B* produced by the hyperlysinemics. Column chromatography separated saccharopine from α-aminoadipic acid and glutamic acid. Cab-O-Sil proved extremely effective in incorporating water-soluble salts into a gel within the scintillants, maintaining good geometry and an efficiency in counting. This greatly facilitated the determination of radioactivity in aliquots of eluate after column chromatography.

DISCUSSION

Hyperlysinemia has now been recognized in all three children of a consanguineous marriage and in a first cousin of the children. The lysine levels in the parents are normal. The family lives in a relatively isolated community in which there is considerable intermarriage, so that the appearance of the defect in a cousin probably represents a high frequency of a recessive gene rather than a dominant trait. Mental retardation was a significant clinical feature in only the propositus (P. J.), and may reflect the method of case detection rather than damage as a result of the metabolic defect. The remaining affected individuals in the family appear somewhat slow mentally but, in the opinion of the examining psychologist, they conform with the general intellectual standards of the family and community. No definitive statement can be made as to the impact of hyperlysinemia on mental performance other than the amino acid imbalance does not appear to be as consistently or severely damaging as it is in phenylketonuria and in maple syrup

TABLE II
Chromatographic and Electrophoretic Characteristics of Some Lysine Metabolites

Compound	Column chromatography elution time	High Voltage electrophoresis mobility
	min	*mm*
Glutamic acid	32.5	+210
α-aminoadipic acid	42.0	+194
ε-*N*-acetyllysine	42.5	−50
Alanine	45.0	−50
Homocitrulline	53.5	−48
Valine	60.5	−50
Saccharopine	63.0	+123
Pipecolic acid	64.5	−61
Methionine	73.0	−50
α-*N*-acetyllysine	128.0	−48
Lysine	*	−231
Homoarginine	*	−219

High voltage electrophoresis was in 0.05 M potassium phosphate, pH 6.7, for 3 hr at 2000 v. Column chromatography was on a Phoenix Micro Analyzer with a 0.9 × 15 cm resin column, operated at 60°C, with 0.2 N citrate buffer, pH 3.25, pumped at 1 ml/min.
* Not eluted during the observed period of 135 min.

urine disease. It has not been possible to determine the effect of dietary reduction of the hyperlysinemia in these patients because of lack of cooperaton of the subjects and their parents.

In an earlier study from this laboratory, the metabolism of lysine by two of the children (A. J. and P. J.) was studied in vivo after the parenteral administration of radioactive lysine (2). As indicated by the amount of radioactive carbon dioxide produced, the hyperlysinemic subjects metabolized less than 10% of the lyine metabolized by normal subjects. If the normal subjects were previously loaded with lysine to make the lysine levels more comparable to that in the hyperlysinemic patients, the difference was even more striking, demonstrating that the normal subject had a considerably greater capacity for the degradation of lysine. The major radioactive compound in the urine of the hyperlysinemics was lysine, indicating that the site of the metabolic defect was probably at the first degradative step.

At the time of presentation of the above report, there was uncertainty as to the metabolic steps involved in the degradation of lysine. Most texts favored the pathway suggested by Rothstein and Miller (8) in which the α-amino group is removed by oxidative deamination, and the residual keto acid is cyclized and reduced to pipecolic acid. An alternative, suggested by Kim, Benoiton, and Paik (9) included prior acetylation of the ϵ-amino group to facilitate the removal of the α-amino radical. However, the demonstration of both ϵ-*N*-acetyllysine (10) and pipecolic acid [2] in the urine of the hyperlysinemics in relatively small amounts, as compared with the amounts of lysine excreted, indicates that these metabolites do not result from major metabolic activities. The recent report of Grove and Henderson (11) on the metabolism of D-lysine casts further doubt on the physiological importance of these pathways.

More recently, Saunders and Broquist have presented substantial evidence that saccharopine is an essential intermediate in the biosynthesis of lysine by neurospora (12). Higashino, Tsukada, and Lieberman have demonstrated that rat liver will catalyze the reverse reaction (3). This provided a mechanism for removal of the ϵ-amino group of lysine, rather than the α-amino group, as previously suggested. The mechanism differs from the more usual transamination to α-ketoglutarate in that a stable intermediate, saccharopine, is formed (Fig. 3). The enzyme responsible for the conversion of lysine to saccharopine, lysine-ketoglutarate reductase, is present in high activity in human liver suggesting that this enzyme may play a significant role in the degradation of lysine (5). Supportive but not conclusive evidence is provided by the recent description of a patient with saccharopinuria and moderate hyperlysinemia (13).

An assay for lysine-ketoglutarate reductase has been developed in this laboratory (5). Recognition of the requirement of TPNH as an essential cofactor has greatly increased the sensitivity of the assay and permitted the demonstration of lysine-ketoglutarate reductase activity in fibroblasts grown in tissue culture from the skin of normal subjects. This has made it possible to investigate the metabolic defect without resorting to liver biopsy.

The results (Table I) demonstrate considerable reduction in the ability of the skin fibroblast derived from hyperlysinemic subjects to convert lysine to saccharopine. This must reflect a generalized deficiency in lysine-ketoglutarate activity accounting for the high blood levels of lysine (10–23 mg/100 ml) and the inability to degrade lysine in vivo (2). In addition to defining the metabolic defect in these subjects, the saccharopine pathway is now established as the major degradative pathway for lysine in the human.

The relation of these patients to the patients with hyperlysinemia reported by others (13–16) may now be investigated definitely. The degree of hyperlysinemia in the present subjects is considerably greater than in the others suggesting either variations in the severity of the defect or the involvement of different metabolic steps.

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