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SACCHAROPINE CLEAVAGE BY A DEHYDROGENASE OF HUMAN LIVER

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SUMMARY

An enzyme catalyzing the cleavage of saccharopine (ε-*N*-(L-glutaryl-2)-L-lysine) has been purified 120-fold from human liver. The enzyme, (ε-*N*-(L-glutaryl-2)-L-lysine: NAD^+ oxidoreductase (L-2-aminoadipic-δ-semialdehyde and glutamate forming)) was purified by procedures which included $(NH_4)_2SO_4$ precipitation and chromatography on a DEAE-cellulose column.

The enzyme has a pH optimum of pH 8.8–9.0 and exhibits at least 50% of optimal activity between pH 7.8 and 9.7. The Michaelis constants for saccharopine and NAD^+ are $5 \cdot 10^{-4}$ M and $4 \cdot 10^{-4}$ M, respectively. While the crude enzyme appears to be stable at −20°, the purified fraction loses virtually all of its activity within 3 weeks. At 2°, the purified fraction loses 5–10% of its activity per week.

Incubation of the purified enzyme with [*glutaryl*-$^{14}C_5$]saccharopine resulted in the stoichiometric reduction of NAD^+ with the stoichiometric production of [^{14}C]glutamate.

INTRODUCTION

Convincing evidence has been recently presented[1] that the major degradative pathway of lysine in the human involves the initial formation of saccharopine (Reaction 1). Subsequent cleavage of saccharopine results in a unique transfer of the ε-amino group of lysine to α-ketoglutarate (Reaction 2). The sum of the two reactions is in effect, classical transamination (Reaction 3). Similar transformations have recently been demonstrated in *Neurospora* and yeast[2–5].

Reaction 1

$$\text{Lysine} + \alpha\text{-ketoglutarate} + \text{NADPH} + H^+ \rightarrow \text{saccharopine} + \text{NADP}^+ + H_2O$$

In the present report, a saccharopine dehydrogenase (glutamate forming) has been extracted from human liver which catalyzes the transformations indicated in Reaction 2.

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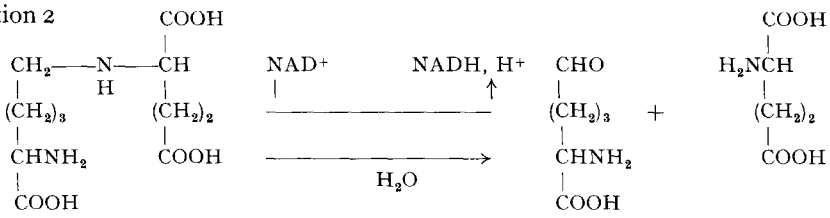
Reaction 2



Saccharopine (ε-*N*-(glutaryl-2)-lysine)

α-aminoadipic-δ-semialdehyde

Glutamic acid

Reaction 3

Lysine + α-ketoglutarate → aminoadipic-δ-semialdehyde + glutamate

METHODS

Materials

Saccharopine was purified from yeast[6]. The Rexyn ion exchange resins were purchased from Fisher Scientific Co., Springfield, N. J. Selectacel DEAE-cellulose, 88 mequiv/g, was obtained from Schleicher and Schuell Co., Kenne, N. H. DL-α-Amino-ε-hydroxycaproic acid was a gift from Dr. M. Bullock, American Cyanamid Company, Princeton, N.J.

Paper chromatography and electrophoresis

Chromatography was by ascending technique on Whatman number 1 paper for approx. 16 h. High voltage electrophoresis was performed on a Savant Instrument Co. apparatus having a 30 cm × 90 cm water cooled bed. The radioactive pattern on the paper strips was determined with a Nuclear Chicago Actigraph III scanner. Details of these procedures have been given[1,7]. Amino acids were located by parallel chromatography of standard compounds, or by removing a thin strip of the chromatography of standard compounds, or by removing a thin strip of the chromatogram. The compounds were made visible by spraying with a 0.5% solution of ninhydrin in *n*-butanol.

Synthesis of radioactive saccharopine

The incubation mixture contained potassium α-keto[$^{14}C_5$]glutarate, 50 μC, 25 μmoles (prepared by neutralizing α-keto[$^{14}C_5$]glutaric acid, Mallinckrodt Nuclear, St. Louis, Mo.); L-lysine, 40 μmoles; NADPH, 30 μmoles; and lysine: α-ketoglutarate reductase in 1 ml of phosphate buffer 7.0 (Fraction IV)[7]. Incubation was for 4 h at 30° in a nitrogen atmosphere.

Purification of product. The reaction was terminated by adding 30 μmoles of saccharopine and placing the solution in a boiling-water bath for 5 min. The denatured protein was removed by centrifuging. Chromatography of the reaction medium in *tert.*-butanol–formic acid–water (70:15:15, v/v/v), indicated 80% of the activity now had a mobility identical to that of saccharopine[7]. The incubation medium was placed on a column (2 cm × 8 cm) of Rexyn 206 (OH^- form), freshly prepared by cycling with 3 M HCl, water, 1.5 M NH_4OH, 1 M acetic acid and water. The column was subsequently washed with 60 ml water and eluted with 40 ml 3 M HCl. The eluate was taken

to dryness *in vacuo*, redissolved in 5 ml of water and placed on a Rexyn AG 50 column (H^+ form) prepared by cycling with 1.5 M NH_4OH, water, 3 M HCl and water. After placing the material on the column, it was washed with water and eluted with 1.5 M NH_4OH. The alkaline eluate was evaporated to dryness *in vacuo*[6].

The residue was dissolved in a minimal amount of water, neutralized with HCl and applied as a streak to a large piece of Whatman 3 MM paper. Chromatography in the above described solvent system was performed. The saccharopine area was cut from the chromatogram. Saccharopine was eluted by first dicing the paper and then shaking it for 15 min with 100 ml of water at 60°. Three changes of water were used. They were pooled, filtered and evaporated to dryness *in vacuo*.

The dry residue was dissolved in a minimal amount of water, transferred to a centrifuge tube and evaporated to approx. 0.4 ml with the aid of a stream of nitrogen and a 60° water bath. Crystallization was accomplished by adjusting the pH to 3.5 with ammonia and adding 8 volumes of absolute ethanol. Crystals were allowed to form for two weeks at 2°. They were harvested by centrifugation, washed with ethanol and dried. Overall yield was 28% based on radioactivity. No attempt was made to recover additional saccharopine from the mother liquor.

The product was pure as determined by high voltage electrophoresis in phosphate buffer, pH 6.7, 0.05 M and in formic acid buffer, pH 2.4, 0.2 M, and by ascending paper chromatography in *n*-butanol–acetic acid–water (12:3:5, v/v/v)[10]. The specific activity was reduced to desired levels by addition of saccharopine.

Assay for saccharopine dehydrogenase

Unless otherwise stated, the incubation mixture contained saccharopine, 2 μmoles; NAD^+, 3 μmoles; Tris–HCl buffer, pH 8.8, 40 μmoles; and enzyme solution in a final volume of 1 ml. The reduction of NAD^+ was followed at 340 mμ in a modified Beckman DU spectrophotometer equipped with a Gilford automatic sample changer and recorder (1-cm light path). Incubation was at room temperature (approx. 23°) unless otherwise noted. One enzyme unit is defined as that amount of enzyme which catalyzes the reduction of NAD^+ at the rate of 0.0001 absorbance unit per min. Protein was determined by the method of LOWRY *et al.*[8].

Enzyme purification

All procedures were carried out at temperatures below 5°. $(NH_4)_2SO_4$ with a low content of heavy metals was used. The solution was neutralized and filtered prior to use. The enzyme purification scheme is outlined in Table I.

Fraction 1: Crude extract. 165 g of human liver, obtained at autopsy and frozen, was thawed and homogenized in a Waring blendor with approx. 800 ml of 0.15 M NaCl which also contained potassium phosphate, pH 7.0, 0.1 M. The blendor was filled to capacity to prevent aeration. It was operated at low speed, stopping several times to free the blades of membranous tissue. The homogenate was diluted to 1100 ml, homogenized in a Potter–Elvehjem type glass homogenizer, then centrifuged at 22 000 × *g* for 35 min in a Lourdes refrigerated centrifuge. The precipitate was discarded.

Fraction 2: $(NH_4)_2SO_4$ 28–56%. Saturated $(NH_4)_2SO_4$ solution was slowly added with stirring to the supernatant to a final concentration of 15% satn. Mixing was continued for 40 min. The solution was centrifuged and the precipitate was dis-

TABLE I
PURIFICATION OF SACCHAROPINE DEHYDROGENASE

Step	*Volume (ml)*	*Total protein (mg)*	*Activity (units × 10^{-3})*	*Specific activity*	*Purification (-fold)*	*Yield (%)*
1. Crude extract	900	18 100	1440	79.6	(1)	(100)
2. $(NH_4)_2SO_4$, 28–56%	155	4 140	2260	546	6.9	157
3. Streptomycin and dialysis	188	4 140	2146	518	6.5	149
4. $(NH_4)_2SO_4$, 36–46%	40	860	894	1037	13	62
5. DEAE-cellulose eluate	330	77	590	7690	97	41
6. Central fraction of Fraction 5	48	17.3	168	9720	122	11.7

carded. The solution was taken to 28% satn. and the procedure repeated. The 28% supernatant solution was taken to 56% satn. The resulting precipitate was dissolved in 100 ml of the phosphate–NaCl buffer and dialyzed for 16 h against three changes of 4 l each of potassium phosphate buffer, pH 7.0, 0.05 M.

Fraction 3: Streptomycin precipitation. To the 155 ml of enzyme solution was added 12 g of streptomycin sulfate in 25 ml water. The solution was stirred, and the precipitated nucleic acids were removed by centrifuging. The supernatant was dialyzed as described above. The enzyme solution was stored overnight at −20°.

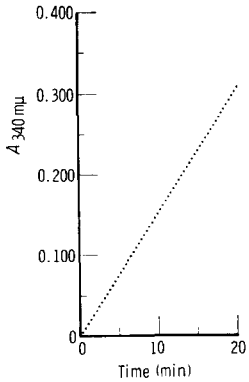
Fraction 4: Second $(NH_4)_2SO_4$ fraction 36–46%. The enzyme solution was thawed and brought successively to 30, 36, 41, 46 and 52% satn. with conc. $(NH_4)_2SO_4$, following the procedures already described. The precipitates obtained at 41 and 46% satn. contained most of the enzyme activity. Each of these fractions was dissolved in 20 ml of phosphate–NaCl buffer, and dialyzed.

Fraction 5: DEAE-cellulose chromatography. 70 g of the DEAE-cellulose was soaked overnight in 2 l of 0.5 M NaCl and then packed into a 45 mm × 350 mm column using air pressure delivered by a rubber bulb. The column was washed with 1.5 l of 0.04 M potassium phosphate buffer, pH 7.5. All the buffers used in this step contained EDTA 0.1 mM and 2-mercaptoethanol 1 mM. The 40 ml of dialyzed 36–46% saturation fraction was diluted to 60 ml with water and placed on the column. The column was eluted in sequence with potassium phosphate buffers, pH 7.5, as follows: 1100 ml of 0.05 M, 1400 ml 0.08 M, 500 ml 0.11 M and 800 ml 0.15 M. Flow rate was 8–10 ml/min. The eluate was collected in fractions and 0.2-ml aliquots were assayed for saccharopine dehydrogenase activity. Maximum activity was located in the 1487–1817 ml fraction (Fraction 5). The middle 48 ml of Fraction 5 was used to define the properties of the enzyme and is referred to as Fraction 6.

RESULTS

The reaction was linear with respect to time and enzyme concentration (Figs. 1 and 2).

pH optima and stability. The effect of pH on the reaction velocity of Fraction 6 is presented in Fig. 3. In each instance, the rate of reaction was maintained for at least 10 min, without evidence of enzyme inactivation, throughout the investigated pH range. The relatively alkaline pH optimum of 8.8–9.0 is a common finding in reactions involving the neutralization of a proton.



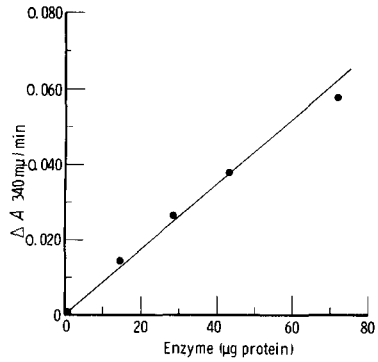


Fig. 1. Enzyme activity as a function of time. 400 ml of Fraction 6 containing 14.4 μg of protein was incubated with saccharopine and NAD^+. The reduction of NAD^+ was followed on a recording spectrophotometer. The reaction rate was maintained for 20 min or longer.

Fig. 2. Enzyme concentration *versus* velocity. Fraction 6, containing the amounts of protein indicated on the abscissa, was incubated under the conditions of the standard assay for 20 min. The resultant increases of optical density are indicated on the ordinate.

Substrate specificity. No close analogues of saccharopine were available for testing. Several compounds having a structural similarity to the precursors of saccharopine were incubated with the enzyme. These were: α-ketoglutaric acid, DL-α-aminoadipic acid, L-aspartic acid, L-lysine, ε-*N*-acetyl-L-lysine, DL-α-amino-ε-hydroxycaproic acid, δ-aminovaleric acid, ε-aminocaproic acid, and DL-pipecolic acid. A trace of NAD^+ reduction occurred when aminoadipic acid was incubated. The remaining compounds were nonreactive.

Temperature optima and stability. The optimal temperature approximates 30° (Fig. 4). Preincubation of Fraction 6 for 5 min at temperatures up to 37° caused no decrease in enzyme activity. At 44°, there was a 50% loss (Fig. 5).

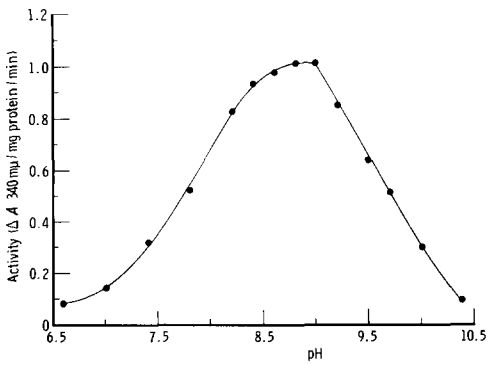
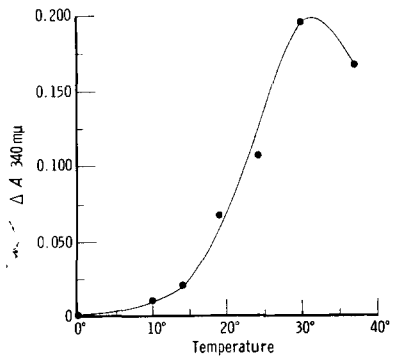


Fig. 3. Effect of pH on saccharopine dehydrogenase activity. The assay system contained *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and glycine as buffers, 40 μmoles each. The pH of the buffers was adjusted to the values shown by titration with HCl or KOH. In addition the complete system contained: saccharopine, 2 μmoles; NAD^+, 3 μmoles; 0.04 ml of enzyme (Fraction 6) and water in a final volume of 1 ml. This system had the same velocity of reaction at pH 8.8 as the standard assay system.



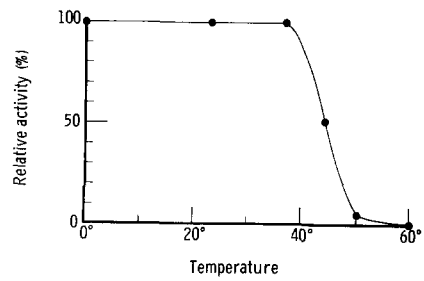


Fig. 4. Effect of temperature on enzyme velocity. Fraction 6 was incubated under the standard assay conditions for 20 min at each of the temperatures indicated .The change in absorbance is shown. The reduction in activity at temperatures above 37° probably reflects the instability of the enzyme.

Fig. 5. Stability of saccharopine dehydrogenase to heat. Aliquots of Fraction 6 (pH 7.5) were preincubated for 5 min at the indicated temperatures. Tris–HCl buffer, saccharopine, NAD^+ and water were added and the reaction velocity was measured at 23° in the standard way.

Stability upon storage. Saccharopine dehydrogenase was stable for several months at −20° when stored as the crude fraction (Fraction 2). However, the purified preparation (Fraction 6) was inactivated by 3 weeks storage at −20°. Storage of Fraction 6 at 2° resulted in losses of 5 to 10% of the activity per week.

Coenzyme requirements. The reaction velocity with NAD^+ was twenty-fold greater than with $NADP^+$. This differs strikingly from the effectiveness of the two coenzymes with the yeast enzyme (Table II).

Michaelis constants. The Michaelis constants for saccharopine and NAD^+ were $5 \cdot 10^{-4}$ M and $4 \cdot 10^{-4}$ M, respectively. Initial reaction rates were not maintained with

TABLE II

SACCHAROPINE ENZYMES FROM HUMAN LIVER AND FROM YEAST: THE RELATIVE EFFECTIVENESS OF NAD AND NADP AS COENZYMES

The enzyme activity with alternate coenzyme is presented as % activity of the preferred coenzyme.

Enzymatic transformation	*pH optima*	*Preferred coenzyme*	*Other coenzyme (relative rate of activity)*
Lysine + ketoglutarate → saccharopine			
Yeast[5]	7.0	NADH	NADPH (5)
Human[7]	7.0	NADPH	NADH (0)
Saccharopine → lysine + ketoglutarate			
Yeast[5]	approx. 10	NAD^+	$NADP^+$ (0)
Saccharopine → aminoadipic-semialdehyde + glutamate			
Yeast[4]	9.75	$NADP^+$	NAD^+ (80)
Human (see text)	8.9	NAD^+	$NADP^+$ (5)
Aminoadipic-semialdehyde + glutamate → saccharopine			
Yeast[4]	7.0	NADPH	NADH (10)

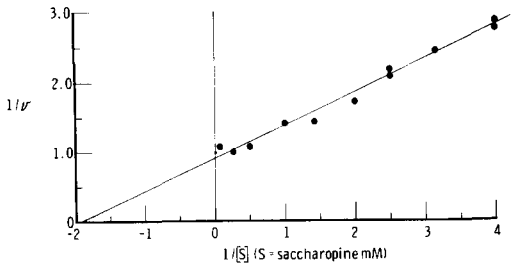


Fig. 6. Enzyme velocity *versus* saccharopine concentration. The reciprocal values of the molar concentration of saccharopine and of enzyme velocity have been graphed by the method of LINEWEAVER AND BURK[9]. For this determination each cuvette contained: NAD^+, 6 μmoles; Tris–HCl, pH 8.8, 40 μmoles; and saccharopine in the amounts indicated. The final reaction volume was 1 ml.

concentrations of NAD^+ of less than 0.5 mM. It is unlikely that this represented depletion of the coenzyme or an accumulation of reaction products. It suggests protection of the enzyme by the coenzyme.

A LINEWEAVER–BURK[9] plot is presented in Fig. 6.

Inhibition studies. The effect of a series of metal ions and metabolic inhibitors is presented in Table III. Notable are the strong inhibition by *p*-hydroxymercuribenzoate and the lack of inhibition by iodoacetate. This suggests that saccharopine dehydrogenase is a sulfhydryl enzyme, resembling that in yeast[4]. NH_4Cl has proven useful because it effectively inhibits a glutamate dehydrogenase present as an impurity in Fraction 6, at concentrations where the saccharopine dehydrogenase is little affected. Glutamate dehydrogenase, when active in the enzyme preparation, would confuse the results by deaminating glutamate, one of the reaction products, thereby increasing the reduction of NAD^+.

TABLE III

INHIBITORS OF SACCHAROPINE DEHYDROGENASE

The following compounds were not inhibitory at 10 mM: Sodium arsenate, KCN, EDTA K_2HPO_4 KCl, NaF, sodium azide, thiourea, diethyldithiocarbamide.

Inhibitor	*Concn. (mM)*	*Inhibition (%)*
p-Hydroxymercuribenzoate	0.01	33
p-Hydroxymercuribenzoate	0.1	96
$HgCl_2$	1.0	98
$CuSO_4$	1.0	96
$MgCl_2$	3.0	<5
$ZnCl_2$	1.0	65
Iodoacetate	10.0	25
NH_4Cl	1.0	5
NH_4Cl	3.0	8
NH_4Cl	10.0	40
Hydroxylamine	10.0	41
Sodium bisulfite	1.0	30
Sodium bisulfite	2.0	44
Dimedone	3.0	Slight activation

Identity of cleavage products. [^{14}C]Saccharopine was incubated with enzyme Fraction 6. NH_4Cl, 3 mM was added to the incubation mixture to inhibit any residual glutamate dehydrogenase remaining in the enzyme preparation. The reaction was monitored spectrophotometrically by following the reduction of NAD^+ and by measuring the formation of radioactive glutamate. A stoichiometric relation between the two products existed (Table IV). The glutamate was isolated from the incubation medium by high voltage electrophoresis, and further identified by ion exchange chromatography on a Phoenix precision micro analyzer[1]. The radioactive peak coincided with the ninhydrin peak of the carrier glutamate. The amount of glutamate synthesized was calculated by reverse isotope dilution methods. Additional confirmation of the identity of the glutamate was obtained by chromatography in *n*-butanol–acetic acid–water (12:3:5, v/v/v)[10].

TABLE IV

STOICHIOMETRY OF REACTION 2

[*Glutaryl*-$^{14}C_5$]saccharopine, 0.8 μmoles, 0.16 μC, was incubated for approx. 90 min in a cuvette with NAD^+, 6 μmoles; NH_4Cl, 3 μmoles; Tris–HCl, pH 8.8, 30 μmoles; Fraction 6, 0.1 ml; and water in a total volume of 1 ml. The reaction was followed optically at 340 mμ. NADH was assumed to have a mM extinction coefficient of 6.22. The reaction was terminated by adding 0.8 μmoles of glutamate and placing the incubation mixture on a boiling-water bath for 5 min. [^{14}C]Saccharopine and [^{14}C]glutamic acid were separated by high voltage electrophoresis at pH 6.7[1]. The two radioactive peaks were located by radioscanning and were in excellent alignment with the internal glutamate and saccharopine as determined by ninhydrin. The radioactive areas were cut out and measured quantitatively in a Packard liquid scintillation counter, in toluene *plus* scintillants. Separation by ascending paper chromatography in *n*-butanol–acetic acid–water[10] gave similar results. The data are consistent with the assumption that the cleavage of 1 mole of saccharopine requires 1 mole of NAD^+.

Substrate	*Counts/min*	*μmoles*	$\Delta A_{340\ m\mu}$
[^{14}C]Saccharopine, initially	3057	0.80	—
[^{14}C]Saccharopine, finally	2462	0.645	—
[^{14}C]Glutamate formed	432	0.113	—
NAD^+ reduced	—	0.119	0.741
Total recovery	2894	0.758	—
Percent recovery	94.6	—	—

DISCUSSION

There has been considerable uncertainty concerning the metabolic steps involved in the degradation of lysine. SCHOENHEIMER AND RITTENBERG[11] demonstrated that lysine does not participate extensively in transamination. ROTHSTEIN AND MILLER[12] suggested that the α-amino group of lysine was removed by oxidative deamination, and the residual keto acid was cyclized and reduced to pipecolic acid. Their evidence was based on the finding that DL-[ε-^{15}N]lysine was readily converted to pipecolic acid, while DL-[α-^{15}N]lysine was not. PAIK AND BENOITON[13] showed that the removal of the α-amino group of lysine could be facilitated by prior acetylation of the ε-amino group of lysine and suggested that this mechanism was a requisite first step of lysine catabolism in mammals.

The question of the major metabolic pathway for lysine degradation was re-

opened by TRUPIN AND BROQUIST[2] who demonstrated that saccharopine is an essential intermediate in the biosynthesis of lysine by *Neurospora* and yeast. HIGASHINO *et al.*[15] subsequently presented evidence that rat liver mitochondria converted trace amounts of [^{14}C]lysine to [^{14}C]saccharopine, and [^{14}C]saccharopine to α-[^{14}C] aminoadipate. HUTZLER AND DANCIS[7] partially purified an enzyme from human liver which catalyzed the transformation of lysine and α-ketoglutarate to saccharopine. They concluded that liver has sufficient enzyme activity, based on *in vitro* data, to convert dietary lysine to saccharopine in the quantities ordinarily ingested. The inference was that the initial steps in the degradation of lysine involve the transfer of the ε-amino group to α-ketoglutarate *via* the stable intermediate saccharopine. In contrast, *Achromobacter liquidum* completes the synthesis of lysine in the more usual fashion, with an epsilon aminotransferase dependent upon pyridoxal phosphate as coenzyme[16].

The fact that the major degradative pathway for lysine in the human involves the synthesis of saccharopine has recently been confirmed by the study of patients with genetic defects in lysine metabolism. Three siblings with hyperlysinemia and an inability to degrade lysine[17–19] have been shown to have a deficiency of lysine–ketoglutarate reductase (saccharopine forming) in fibroblasts grown by tissue culture from skin biopsies[1]. Detectable amounts of pipecolic acid and ε-acetyllysine are present in the urine of these patients suggesting that these compounds are products of minor pathways[17,18]. Pipecolic acid has recently been shown by GROVE *et al.*[20] to be primarily a catabolic product of D-lysine.

The present report concentrates on the next step in the degradation of lysine: the cleavage of saccharopine to α-aminoadipic-δ-semialdehyde and glutamic acid. An enzyme in human liver has been isolated, purified and characterized that reduces NAD^+ in the presence of saccharopine, with the formation of glutamate. The lysyl fragment has not been identified in this study, but is presumably α-aminoadipic-δ-semialdehyde[4,14,15]. *In vitro* data presented herein suggest that human liver has ample reserves of this enzyme to metabolize the saccharopine formed from ordinary quantities of dietary lysine.

A micro assay suitable for measuring saccharopine dehydrogenase in the limited quantities of liver obtainable by needle biopsy has been developed, based on these methods. A patient with mental retardation who excreted large amounts of saccharopine and lysine in the urine has recently been described by CARSON[21]. This represents the first report of saccharopine in human fluids and suggests a deficiency of saccharopine dehydrogenase or of another enzyme effecting the degradation of saccharopine.

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