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LYSINE-KETOGLUTARATE REDUCTASE IN HUMAN TISSUES

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Summary

Lysine-ketoglutarate reductase (saccharopine dehydrogenase ($NADP^+$, lysine-forming) EC 1.5.1.8) from human liver has been partially purified and characterized. A spectrophotometric assay is described. The Michaelis constants have been determined for lysine ($1.5 \cdot 10^{-3}$ M), α-ketoglutarate ($1 \cdot 10^{-3}$ M) and NADPH ($8 \cdot 10^{-5}$ M). The pH optimum is 7.8. The enzyme is product inhibited. The specificity of the enzyme, response to inhibitors, pH and thermal stability are reported. Lysine-ketoglutarate reductase is present in high concentration in liver and heart, to a lesser degree in kidney and skin and in trace amounts in several other tissues. Saccharopine dehydrogenase (saccharopine dehydrogenase (NAD^+, L-glutamate-forming) EC 1.5.1.9) was demonstrable only in liver and kidney. Lysine-ketoglutarate reductase reacts effectively with δ-hydroxylysine.

Introduction

The degradation of lysine in the human has been recently demonstrated to involve the transfer of the ϵ-amino group to α-ketoglutarate via the stable intermediate, saccharopine. Two enzymes are involved, lysine-ketoglutarate reductase (saccharopine dehydrogenase ($NADP^+$, lysine forming) EC 1.5.1.8) and saccharopine dehydrogenase (saccharopine dehydrogenase (NAD^+, L-glutamate-forming) EC 1.5.1.9) both of which can readily be demonstrated in human liver. Two genetic diseases have provided convincing proof of the importance of these two enzymes in the degradation of lysine. A deficiency of lysine-ketoglutarate reductase results in extreme elevations of serum lysine [1,2]; saccharopinuria [3,4] is reported to be associated with a deficiency of saccharopine dehydrogenase.

A previous publication from this laboratory partially characterized the human enzyme, lysine-ketoglutarate reductase, and described a radiochemical microassay [5]. The present report describes a spectrophotometric assay, a simplified enzyme purification and other extensions of the original studies.

Materials

Saccharopine was synthesized enzymatically in this laboratory [6]. Other biochemicals were obtained commercially, most of them from Sigma Chemical Company. Water was double-distilled in glass.

Substrates were stored at −20° C as 0.1 M solutions in water and were neutralized with HCl or NaOH. Lysine solution was adjusted to the experimental pH (usually 7.8) prior to incubation. NAD and NADPH solutions were freshly prepared in 0.01 M buffer.

Methods

Lysine-ketoglutarate reductase. This was assayed by incubation of enzyme with L-lysine 50 μmoles, sodium α-ketoglutarate 30 μmoles, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid(HEPES)—NaOH buffer, pH 7.8, 50 μmoles, NADPH 0.4 μmoles in a final volume of 1 ml. The test sample was preincubated with all constituents present except for α-ketoglutarate or lysine. The oxidation of NADPH was followed in a Beckman DU spectrophotometer equipped with a Gilford automatic sample changer and recorder, using a 1 cm light path at 340 nm. Incubation was at 23° C. A ΔA of 6.220 was accepted as equivalent to 1 μmole of NADPH oxidized and 1 μmole of saccharopine formed.

Glutamic dehydrogenase. This was assayed by substituting 5 μmoles NH_4Cl for lysine in the assay system described above. The assay was performed at the same pH as for lysine-ketoglutarate reductase although the pH optimum of glutamic dehydrogenase was determined as approximately 8.3.

Saccharopine dehydrogenase. The complete assay system contained saccharopine, 2 μmoles, *N*-tris-(hydroxymethyl)-methylglycine—NaOH pH 8.8, 50 μmoles, NAD^+ 3 μmoles and enzyme solution with a final volume of 1 ml. The reaction velocity was greater with this buffer than with Tris—HCl as used previously. An increase in *A* of 6.220 at 340 nm was accepted as equal to 1 μmole saccharopine cleaved [7].

Protein. Protein was determined by the method of Lowry [8], using bovine serum albumin as standard.

Enzyme preparation

Liver tissue was obtained at autopsy from young adults who had died of trauma. There was an interval of approximately 4—12 h between death and autopsy during which time the body was refrigerated. The tissue was stored at −20° C for 2 weeks before carrying out the following procedures at 0—4° C.

85 g of frozen liver was thawed and then homogenized in a Waring Blendor in 425 ml of 0.15 M NaCl. Homogenization was for 1 min, the blades were cleared of adherent tissue, and homogenization continued for another minute. Final pH was 6.0. Protamine sulfate solution was prepared by dissolving, at room temperature, 1.02 g of protamine sulfate in about 45 ml of 0.15 M NaCl, correcting the pH to 6 with 1 M HEPES—NaOH buffer, pH 7.8, and then diluting to 51 ml with NaCl solution. To 510 ml of the cold rapidly stirring homogenate was added dropwise all of the freshly prepared protamine solution.

TABLE I

ENZYME PREPARATION

Protamine sulfate solution was added to homogenate of human liver. The supernatant was fractionated by precipitation with increasing concentrations of sodium citrate. The 0.82—1.07 M fraction was used for characterizing lysine-ketoglutarate reductase.

Fraction	Volume (ml)	Protein (g)	Enzyme activity (μmoles/min)	Specific activity (μmoles/min/g protein)	Purification (fold)	Yield (%)
Protamine supernatant	510	91.8	82.6	0.90	(1)	(100)
0.82—1.07 M sodium citrate	36	0.61	24.3	40.1	44.6	29.4

Stirring was continued for an additional 2 h before centrifuging at 30 000 × *g* for 30 min.

To 400 ml of the rapidly stirring cold, clear red supernatant was slowly added 278 ml of 2 M sodium citrate resulting in a final concentration of 0.82 M. After 2 h, the solution was centrifuged. The 2 M sodium citrate was prepared by stirring the salt into solution at 50° C, adjusting the pH to 7 with concentrated HCl, correcting the volume and cooling to 25° C.

To 620 ml of supernatant was added 53 g crystalline sodium citrate· $2H_2O$, for a final citrate concentration of 1.07 M. The solution was stirred for 2 h and centrifuged. The precipitate was dissolved in 20 ml of 75 mM NaCl containing 50 mM HEPES—NaOH buffer, pH 7.8 and dialyzed twice against 2 l of the buffer for 12 h each time. There was a 2-fold increase in volume of the contents of the bag.

Approximately 70% of the activity of lysine-ketoglutarate reductase and saccharopine dehydrogenase was found in the 0.82—1.07 M fraction, and 25% in the 0—0.82 M fraction.

Information on preparation of lysine-ketoglutarate reductase is presented in Table I. The 0.82—1.07 M sodium citrate fraction was used for the following studies.

The saccharopine forming and cleavage enzymes were separated from glutamic dehydrogenase, but not from each other, on a Sephadex column. Sephadex G-200, 40—120 μM was suspended overnight in 50 mM NaCl contain-

TABLE II

SEPARATION OF SACCHAROPINE-FORMING AND CLEAVAGE ENZYMES FROM GLUTAMIC DEHYDROGENASE

Enzyme activities in nmoles/min/ml of eluate. The enzyme preparation (Table I) was fractionated on Sephadex G-200. Fraction 2 retains lysine-ketoglutarate reductase and saccharopine dehydrogenase activities with minimal amounts of glutamic dehydrogenase.

Fraction	Effluent collected	Lysine-ketoglutarate reductase	Saccharopine dehydrogenase	Glutamic dehydrogenase
1	0— 4	1.9	0	0
2	4— 6	39.0	24.0	0.95
3	6—12	14.5	9.0	57.0
4	12—17	2.85	2.4	6.0

ing HEPES, EDTA and 2-mercaptoethanol, 1 mM each (pH 7.8). The suspension was degassed by use of negative pressure, poured into a 9 mm diameter glass column and the excess buffer eluted under gravity at the rate of 1 ml/min, permitting the matrix to settle to a height of 120 mm. 1 ml enzyme solution diluted with 1 ml water was added to the top of the matrix and eluted with buffer (Table II).

Results

pH optimum

The standard lysine-ketoglutarate reductase assay was modified by the addition of *N*-(2-acetamido)-2-aminoethane sulfonic acid and glycylglycine, 50 μmoles each, to extend the buffering range. The pH of lysine and the medium were adjusted to the values indicated with HCl or NaOH.

The pH optimum for lysine-ketoglutarate reductase is 7.8 (Fig. 1). It has been suggested, in a previous report from this laboratory that a pH of 7.0 be used to obtain maximal yields of saccharopine on more prolonged incubation with radioactive substrate [5]. The lower pH appears to reduce the rate of metabolism of the accumulated product and its coenzyme. Alternatively the product can be protected by maintaining the coenzyme in its reduced form by recycling [5].

Specificity of reaction

Either lysine or α-ketoglutarate was replaced by an equimolar concentration of a series of analogues and the rate of reaction determined (Table III). In this series of studies lysine or lysine analogues were added at concentrations of 5 mM (63% of *V*); α-ketoglutarate or its analogues at concentrations of 10 mM (95% of *V*). Hydroxylysine consists of four isomers of which only the L-forms would be expected to react. The effective substrate concentration is

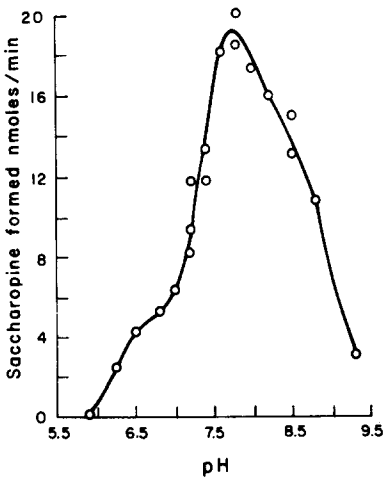


Fig. 1. Effect of pH on the activity of lysine-ketoglutarate reductase. The standard assay system was modified by the addition of *N*-(2-acetamide)-2-aminoethane sulfonic acid 50 μmoles and glycylglycine 50 μmoles.

TABLE III

SPECIFICITY OF LYSINE-KETOGLUTARATE REDUCTASE

Lysine and analogues were added at concentrations of 5 mM; α-ketoglutarate and analogues at 10 mM. The following compounds reacted with less than 2% the rate of an equimolar concentration of lysine: D-lysine, cadaverine, δ-aminovaleric acid, α-acetyl-L-lysine, α-amino-ε-hydroxycaproic acid, L-lysylglycine, L-2,4-diaminobutyric acid, DL-pipecolic acid, L-ornithine, ε-aminocaproic acid. The following compounds reacted with less than 2% the rate of α-ketoglutarate: α-ketoadipate, pyruvate, α-ketoisocaproate, α-ketoisovalerate, levulinate and α-hydroxyglutarate.

Test compound	Substituted for:	Reaction velocity*
DL-(plus DL-*allo*)-δ-Hydroxylysine	Lysine	18
S-2-Aminoethyl-L-cysteine	Lysine	47
DL-α,ε-Diaminopimelic acid	Lysine	4
Oxaloacetic acid	α-Ketoglutarate	7

* As per cent of velocity with equimolar concentrations of lysine or α-ketoglutarate.

therefore less than 5 mM, suggesting that the reaction velocity of hydroxylysine with lysine-ketoglutarate reductase is higher than indicated in Table III.

Lysine-ketoglutarate reductase reacted at a significant rate with only DL-(plus *allo*)-δ-hydroxylysine and with *S*-2-aminoethyl-L-cysteine. In both instances, new ninhydrin-reacting peaks appeared when the incubation mediums were subjected to ion-exchange chromatography on a Phoenix Micro Analyzer with elution times of 43 and 37 min, respectively. Under the same conditions glutamate and saccharopine are eluted at 32.5 and 63 min, respectively [5]. The identities of the new compounds were not further investigated.

Enzyme inhibitors

Several compounds related structurally or metabolically to lysine or α-ketoglutarate were added to the standard assay system, as well as several salts (Table IV).

A troublesome feature in our previous enzyme purification scheme was the strong inhibitory effect of ammonium ions introduced during fractionation with ammonium sulfate. This has also been noted by Fellows [11]. Lysine-ketoglutarate reductase activity was reduced 71% by 1 mM ammonium sulfate [5]. For that reason, sodium citrate has been used for salt fractionation in the present purification procedure. However, in this study the lysine-ketoglutarate reductase eluted from Sephadex 200 (fraction 2) was not inhibited by 40 mM ammonium chloride (see Table II). This preparation was essentially free of glutamic dehydrogenase activity, whereas the enzyme preparation described in the previous report was not. It appears that ammonium ion does not directly inhibit lysine-ketoglutarate reductase. The probable explanation is that the contaminating glutamic dehydrogenase reacts with the ammonium ion reducing the concentration of NADPH, which is common to both enzyme reactions.

Temperature stability

Enzyme was exposed to test temperatures for 10 min and then assayed for residual activity. For comparison, the closely associated enzyme, saccharopine dehydrogenase, was also studied. In both instances, activity rapidly falls at temperatures over 40° C (Fig. 2).

TABLE IV

ENZYME INHIBITORS

Compounds that inhibited less than 20% at 3 mM concentration were: D-lysine, pyrosaccharopine [19], α-amino-ε-hydroxycaproate, α-acetyl-L-lysine, ε-acetyl-L-lysine, glutamine, cystathionine, octopine, citrate, aspartic acid, α-aminovaleric acid, urea, sodium arsenate, iodoacetate, Na_2SO_4, NaF, LiCl.

Compound	Concentration (mM)	Activity remaining (%)
None	—	(100)
L-Glutamic acid	3	60
L-Ornithine	3	61
L-Homocitrulline	3	51
DL-Pipecolic acid	6	53
Cadaverine	3	74
L-Lysylglycine	3	75
Carbamylphosphate	3	75
Hydroxylamine	3	67
$CaCl_2$	3	72
$MgSO_4$	1	21
$ZnCl_2$	3	0
$CuSO_4$	1	13
$MnCl_2$	1	48
$HgCl_2$	0.01	11
$CoCl_2$	1	18

Storage stability

Approximately 50% of original activity remained after one month at −20° C. Fraction 2 (Table II) lost its activity after storage for 1 week.

pH stability

The pH of 0.5 ml of enzyme solution was reduced to the desired value

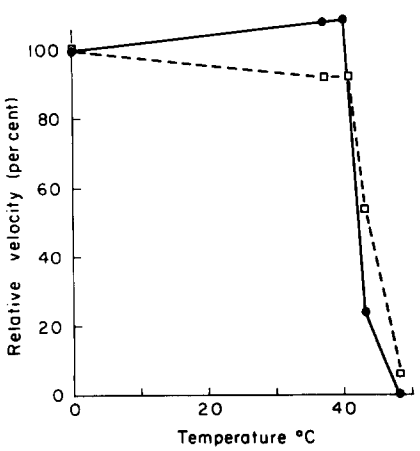


Fig. 2. Inactivation of lysine-ketoglutarate reductase and saccharopine dehydrogenase by heat. Aliquots of the enzyme were heated to the indicated temperatures for 10 min and then cooled and assayed for their respective activities under standard conditions. The equivalent of 0.03 ml of enzyme was used for each assay. Results are listed as percent of initial (unheated) activity.

TABLE V

pH STABILITY

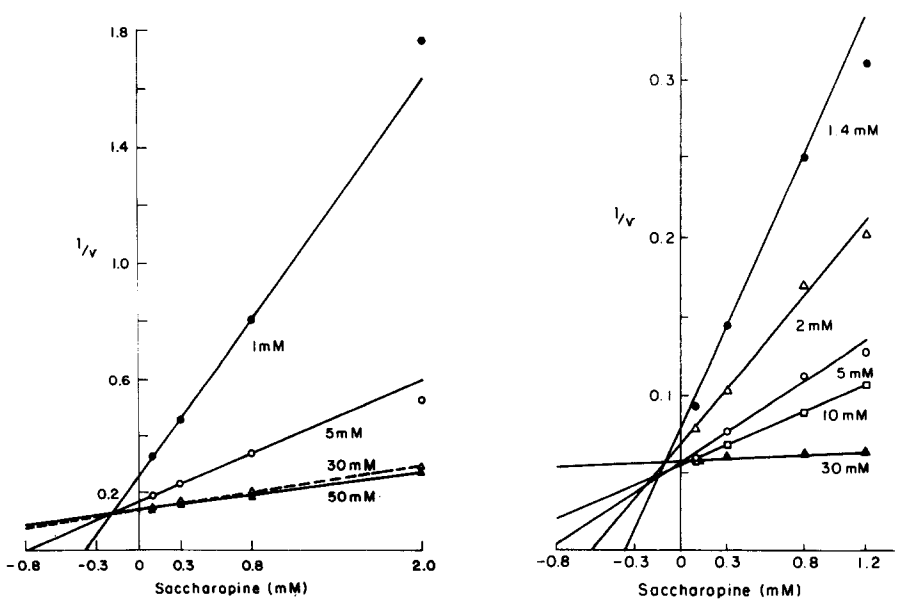
Enzyme solution was maintained at the indicated pH for 10 min and then assayed for residual activity. Presented as per cent of activity and protein concentration of original preparation.

pH	Lysine-ketoglutarate reductase (%)	Saccharopine dehydrogenase (%)	Protein (%)
4.9	100	100	69
4.6	100	100	55
4.4	49	58	36

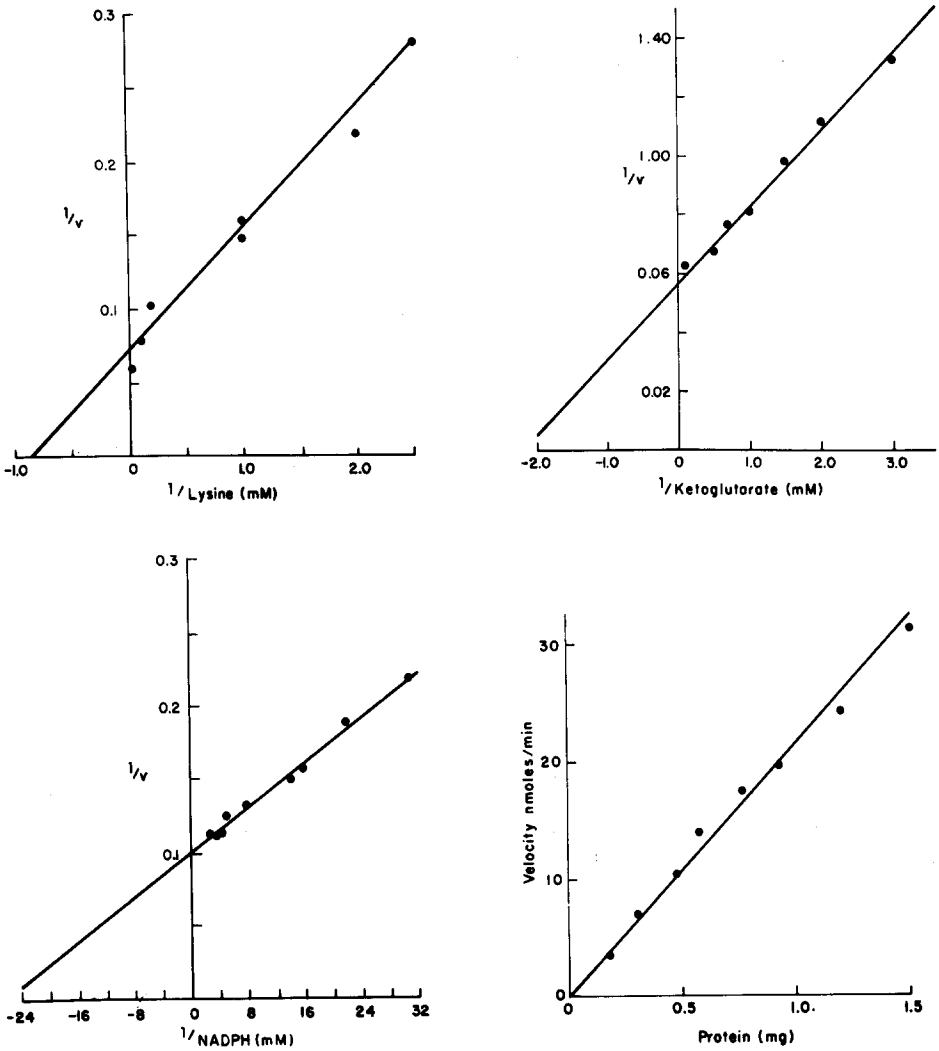
with 1 M HCl and agitated with a magnetic stirrer for 10 min. An equivalent amount of 1 M NaOH was added and denatured protein removed by centrifuging. Aliquots of neutralized enzyme, 0.06 ml, were assayed and compared to untreated but similarly diluted enzyme. The enzyme was stable at pH 4.6, so that this treatment increased the specific activity 2-fold (Table V).

Activation and SH-groups

Although the enzyme was shown to be sensitive to heavy metals suggesting the presence of SH groups, 1 mM cysteine, dithiothreitol or 2-mercaptoethanol did not activate the enzyme. A slight activation was noted with 1 mM EDTA or 1% Triton X-100. This effect was not consistent.



Figs 3 and 4. Dixon plots demonstrating that saccharopine competitively inhibits lysine (K_i = 0.2 mM) and α-ketoglutarate (K_i - 0.14 mM). The effect of the inhibition is shown for lysine (Fig. 3) and ketoglutarate (Fig. 4). Test conditions were as described for the lysine-ketoglutarate reductase assay.



Figs 5, 6 and 7. Lineweaver—Burk plots for lysine-ketoglutarate reductase with varied concentrations of lysine, α-ketoglutarate and NADPH. The concentration of the test substrate was varied as indicated.

Fig. 8. Proportionality of enzyme (protein) to reaction velocity.

Product inhibition

1 mM saccharopine inhibits lysine-ketoglutarate reductase by 30% in the standard assay system. Dixon plots [9] demonstrate that the inhibition is of the competitive type for both lysine and α-ketoglutarate (Figs 3 and 4). Product inhibition has previously been reported [12].

Enzyme kinetics

The Michaelis constants were derived graphically for lysine ($1.5 \cdot 10^{-3}$ M), α-ketoglutarate ($1 \cdot 10^{-3}$ M) and NADPH ($8 \cdot 10^{-5}$ M) (Figs 5, 6 and 7). The velocity is proportional to enzyme concentration (Fig. 8) and is linear with time for at least 10 min, but is subject to product inhibition as the reaction continues.

TABLE VI

TISSUE DISTRIBUTION OF ENZYME ACTIVITY

Enzyme activity in nmoles/min/g of wet tissue.

Tissue	Lysine-ketoglutarate reductase	Saccharopine dehydrogenase	Glutamic dehydrogenase
Liver	162	129	1201
Kidney	17	19	412
Heart	57	0	64
Muscle	Trace	0	5.9
Skin	8.3	0	43
Brain (white)	Trace	0	131
Brain (gray)	Trace	0	95
Spleen	Trace	0	24
Pancreas	0	0	0
Adrenal	0	0	50
Lung	0	Trace	9.5
Gut	0	0	44
Leukocytes	0	0	—
Erythrocytes	0	0	—
Skin fibroblasts	(58)*	—	—

* Based on average of three controls. The weight of skin fibroblasts was estimated from protein analysis, assuming a protein content of 16.7%.

Reverse reaction of lysine-ketoglutarate reductase

Incubation of enzyme with 2 mM saccharopine at pH 6.8, 7.8, 8.5, 9.3 and 9.5 failed to produce any detectable reduction of $NADP^+$. This contrasts with the enzyme isolated from *Neurospora* and yeast where reversible reactivity was easily demonstrable [10,11]. In the ox and human, a different enzyme, saccharopine oxidoreductase, catalyzes the conversion of saccharopine to lysine and α-ketoglutarate [12].

Tissue distribution in the human

Organs were obtained a few hours after the traumatic death of an apparently healthy white male, and stored at −20°C for up to 2 weeks before enzyme analyses. Approximately 2 g of each tissue were homogenized in a teflon pestle homogenizer in 18 ml of buffer: 0.15 M NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and 10 mM HEPES—NaOH, pH 7.8. Skin and intestine were diced with scissors and homogenized in a Virtis homogenizer. The homogenates were dialyzed against two changes of buffer and centrifuged 30 000 × *g* for 30 minutes. The supernatants were assayed for lysine-ketoglutarate reductase and saccharopine dehydrogenase activity (Table VI).

The tissue distribution of lysine-ketoglutarate reductase is similar to that previously reported on autopsy material for lysine-ketoglutarate reductase except for heart which is 8-fold higher [5].

Discussion

Saccharopine was first established as an important metabolite in the biosynthetic pathway of lysine of *Neurospora* and yeast [10,11]. Within a few

years, the saccharopine pathway was also demonstrated to be the major degradative pathway for lysine in the mammal, including man [1].

The present communication characterizes lysine-ketoglutarate reductase in human tissue, the enzyme that converts lysine to saccharopine. It differs in several respects from its counterpart in microorganisms [10,11]. The coenzyme in the human is NADPH rather than NADH. The function is degradative, rather than synthetic and the equilibrium of the reaction is strongly in the direction of saccharopine. A separate enzyme, saccharopine oxidoreductase, has been described in several mammals, including the human which catalyzes the cleavage of saccharopine to lysine and α-ketoglutarate [12].

A limited amount of information is also presented for saccharopine dehydrogenase, the second enzyme in the catabolism of lysine in man. This enzyme is also located in the mitochondrion [12], and accompanies lysine-ketoglutarate reductase throughout the purification procedure described in this report. However, its tissue distribution is confined to liver and kidney, differing from the more widely distributed lysine-ketoglutarate reductase.

An interesting but incidental observation was the effectiveness with which lysine-ketoglutarate reductase reacted with hydroxylysine, presumably with the formation of "hydroxysaccharopine." However, reports of patients with metabolic defects indicate that the major degradative pathway for hydroxylysine does not utilize lysine-ketoglutarate reductase [13—17]. Hyperlysinemia as a result of lysine-ketoglutarate reductase deficiency is not associated with hydroxylysinemia [18]. Conversely, patients with hydroxylysinemia, with an undefined enzymatic defect, do not exhibit lysinemia [13—17].

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