

Effects of Supersuppressor Genes on Enzymes Controlling Lysine Biosynthesis in *Saccharomyces*¹

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Received for publication 24 October 1969

Yeast supersuppressor genes capable of masking the effects of several lysine mutant genes (ly_{1-1} , ly_{9-1} , ly_{2-1}) were studied with respect to their effects on the respective enzymes (saccharopine dehydrogenase, saccharopine reductase, and α -amino-adipic acid reductase). In all strains tested, the supersuppressors functioned by allowing enzyme synthesis not found in the unsuppressed mutant. Studies by optical methods of saccharopine dehydrogenase and saccharopine reductase extracted from suppressed ly_{1-1} and ly_{9-1} cells, respectively, revealed that the K_m values for these enzymes were significantly greater than those found in wild type. Saccharopine dehydrogenase from suppressed ly_{9-1} cells was found to have K_m values similar to wild type. These findings are consistent with the inference that a supersuppressor may act by enabling nonsense codons to be read, producing altered enzyme protein. Recent findings that lysine degradation in mammals may involve saccharopine and that the human diseases, hyperlysinemia and saccharopinuria, may be due to metabolic blocks in this route of lysine degradation suggest the ly_{1-1} and ly_{9-1} yeast mutants as models for the human condition and its possible euphenic treatment.

Since the discovery of supersuppressors in yeast (9), the evidence supporting their similarity to the bacterial nonsense suppressors has been largely inferential and indirect (16). Recent direct findings (6), however, have demonstrated the comparability of the two systems.

Several theories proposed for the mechanism of nonsense suppression have been reviewed by Gorini and Beckwith (7). The principal theoretical model assumes a nonsense codon in the supersuppressible allele, leading to chain termination and an incomplete mutant protein. The supersuppressor gene is assumed to have mutated to form an altered species of transfer ribonucleic acid (tRNA) capable of reading the nonsense codon and of inserting an amino acid at the nonsense site, enabling chain completion (1).

The observation that supersuppressor genes in yeast are dominant, allele specific, and locus nonspecific (9) is consistent with this model.

The current study describes the isolation and characterization of two supersuppressors in yeast. These were recognized in terms of their effects on

several nonallelic mutant lesions (ly_{1-1} , ly_{9-1} , ly_{2-1}), controlling three different enzymes after α -amino-adipic acid (3) (α -AAA) in the lysine biosynthetic pathway (Fig. 1). This, at first, made us suspect the existence of an alternate pathway between α -AAA and lysine, but a more comprehensive genetic study (A. Roshanmanesh, Ph.D. Thesis, Southern Illinois University, Carbondale, 1968) demonstrated that the suppression was caused by supersuppressors rather than pathway suppressors. The effects of these supersuppressors at the level of enzyme was the subject of the present study. The properties of α -AAA reductase (ly_{2-1}), saccharopine reductase (ly_{9-1}), and saccharopine dehydrogenase (ly_{1-1}) have been compared in wild-type, mutant, and super-suppressed mutant strains.

The findings that saccharopine and α -AAA are involved in mammalian lysine degradation (8, 11) and the reports that certain cases of hyperlysinemia in humans are based on a lesion in the lysine-saccharopine conversion (27) suggest the ly_{1-1} mutant in yeast as an experimental model for the human condition.

MATERIALS AND METHODS

Yeast strains. The strains used in the current study were derived principally from the Berkeley and Carbondale collections. The supersuppressor genes used in this study are designated as S_I and S_{II} since

¹ A summary of this paper was presented at the 68th Annual Meeting of the American Society for Microbiology, 5-10 May 1968. Taken from a thesis submitted by Thorsten A. Fjellstedt in partial fulfillment of the requirements for the Ph.D. degree in Microbiology, Southern Illinois University, 1969.

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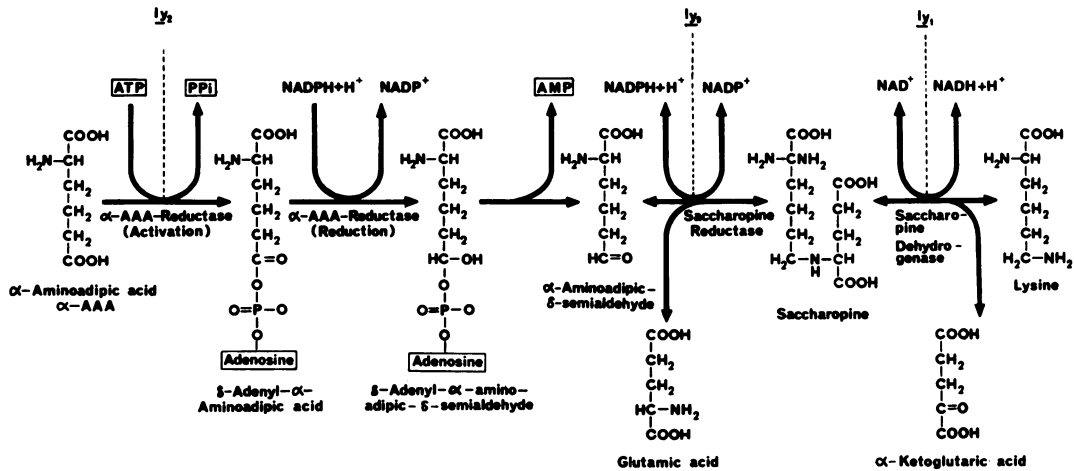


Fig. 1. Proposed pathway of α -amino adipic acid conversion to lysine.

they were independently isolated and have not been compared by us with the supersuppressor classification of Hawthorne and Mortimer (10). They appear to be class I supersuppressors (R. Gilmore, *personal communication*). All other genetic symbols correspond to the nomenclature adopted at the 1961 Carbondale Yeast Genetics Conference (24).

Yeast strains include: F_2 , *LY* (a diploid wild-type isolate of Fleischmann's Yeast); S288C-24, α ly_{1-1} ; S720B, a ly_{1-1} tr_4 hi_2 ; S856C, a ly_{2-1} ad_1 ; 19B, α ly_4 ; 50078, α ly_4 S_{II} ; JB101, α ly_{9-1} ad_3 ; X1012-1D, a ly_{9-1} ad_2 ad_1 ; MO-49-14D, α ly_{2-1} (a segregant of a cross between JB101 and S856C); BM-6-MO-49-14D, α ly_{2-1} S_I (a revertant clone isolated on lysineless media containing α -AAA as sole N source); MO-49-16B, a ly_{9-1} ad_2 ; MO-51-7A, a ly_4 S_{II} (a segregant of a cross between 50078 and S856C); MO-59-13A, α *LY*, and MO-59-13B, a ly_{1-1} S_{II} (segregants of a cross between MO-51-7A and S288C-24); MO-72-20D, a ly_{1-1} S_I tr_4 (a segregant of a cross between BM-6-MO-49-14D and S720B); MO-73-18A, α ly_{9-1} S_I , and MO-73-18C, a ly_{9-1} S_I ad_2 (segregants of a cross between MO-49-16B and BM-6-MO-49-14D); MO-76-5C, a ly_{1-1} S_I , and MO-76-5D, a ly_{1-1} S_I ad_2 tr_4 (segregants of a cross between MO-72-20D and JB101). The phenotypes of all strains used are indicated in Table 1.

Culture media. Liquid culture media were prepared as indicated below. When a solid medium was required, 25 g of washed agar (Difco) was added prior to dilution to 1 liter.

Complex (COMP): glucose, 10 g; peptone, 3.5 g; KH_2PO_4 , 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; Dry Yeast Extract, 5.0 g, per liter.

Complete synthetic (CS): glucose, 10 g; KH_2PO_4 , 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1.0 g; inositol, 1 mg; nicotinic acid, 0.4 mg; calcium pantothenate, 0.4 mg; pyridoxine hydrochloride, 0.4 mg; thiamine hydrochloride, 0.4 mg; *p*-aminobenzoic acid (PABA), 0.4 mg; biotin, 0.002 mg; $CaCl_2$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 0.25 mg; $MnSO_4 \cdot 4H_2O$, 0.04 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.018 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.088 mg; $CuSO_4$,

$5H_2O$, 0.04 mg; $ZnSO_4 \cdot 7H_2O$, 0.31 mg; KI, 10 mg; choline chloride, 4.0 mg; adenine sulfate, 40 mg; L-arginine hydrochloride 30 mg; L-alanine, 30 mg; L-aspartic acid, 30 mg; L-cysteine hydrochloride, 30 mg; glycine, 30 mg; L-histidine monohydrochloride, 30 mg; L-isoleucine, 30 mg; L-leucine, 30 mg; L-phenylalanine, 60 mg; L-lysine, 30 mg; L-methionine, 30 mg; L-proline, 30 mg; L-serine, 300 mg; L-threonine, 300 mg; L-tryptophan, 30 mg; L-tyrosine, 30 mg; L-valine, 300 mg; and L-glutamic acid, 100 mg, per liter. The pH was adjusted to 5.8 with KOH.

CS-*ly*: same as above except L-lysine was omitted.

CS-*ly* + α -AAA: same as above except that 2 g of DL- α -amino adipic acid was added.

Minimal (MIN): same as CS except all amino acids were omitted.

Minimal minus nitrogen (M - N) + α -AAA: same as MIN except $(NH_4)_2SO_4$ was also omitted and 2 g of DL- α -AAA was added per liter as principal N source.

All media except those containing α -AAA were sterilized by autoclaving. The latter, adjusted to pH 6, was sterilized separately by filtration and added aseptically.

Labeled and unlabeled substrates. Labeled and unlabeled substrates and cofactors were obtained from either Calbiochem (Los Angeles, Calif.) or Sigma Chemical Co. (St. Louis, Mo.) and were of the highest purity available. A generous sample of saccharopine was made available by H. Broquist.

Cell growth conditions. Cells were generally grown to stationary phase (36 to 48 hr) in 100 ml of COMP contained in a 500-ml Erlenmeyer flask on a reciprocating shaker at 30 C.

When large cell populations were required, cells were grown in 10 liters of COMP medium in a VirTis Fermentor under comparable conditions. Revertant checks were made on all cell populations at the end of the growth phase by plating triply washed cell samples on COMP and CS-*ly* agar media. In no case was the revertant frequency greater than 10^{-6} .

Enzyme preparations. Cells were harvested and washed three times in 0.07 M potassium phosphate

TABLE 1. Phenotypes of yeast strains

Strain	Genotype	Growth response			
		MIN	CS	CS-ly	CS-ly + α -AAA
F ₂	LY	+	+	+	+
S288C-24	ly ₁₋₁	-	+	-	-
S720B	ly ₁₋₁ tr ₄ hi ₂	-	+	-	-
S856C	ly ₂₋₁ ad ₁	-	+	-	-
19B	ly ₄	-	+	-	+
50078	ly ₄ S _{II}	-	+	-	+
JB101	ly ₉₋₁ ad ₂	-	+	-	-
X1012-1D	ly ₉₋₁ ad ₂ ad ₁	-	+	-	-
MO-49-14D	ly ₂₋₁	-	+	-	-
BM-6-MO-49-14D	ly ₂₋₁ S _I	+	+	+	+
MO-49-16B	ly ₉₋₁ ad ₂	-	+	-	-
MO-51-7A	ly ₄ S _{II}	-	+	-	-
MO-59-13B	ly ₁₋₁ S _{II}	+	+	+	+
MO-59-13A	LY	+	+	+	+
MO-72-20D	ly ₁₋₁ S _I tr ₄	-	+	+	+
MO-73-18A	ly ₉₋₁ S _I	+	+	+	+
MO-73-18C	ly ₉₋₁ S _I ad ₂	-	+	+	+
MO-76-5C	ly ₁₋₁ S _I	+	+	+	+
MO-76-5D	ly ₁₋₁ S _I ad ₂ tr ₄	- ^a	+	+	+

^a Neither the ad₂, hi₂, or tr₄ alleles used were affected by S_I.

buffer (pH 6.8). Dilution of the cell mass to approximately 10¹⁰ cells/ml in buffer preceded sonic treatment. A 12-ml sample of the cell suspension was sonically treated in a 25-ml stainless-steel centrifuge tube by using a Branson Sonifier (model S-110) with a flat horn tip. The cells were sonically treated for 4 min at a function setting of 7 in a stirred crushed-ice bath. The crude sonic-treated material was cleared of cell debris and large particles by centrifugation at 20,200 × g for 40 min in the cold by using an RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with the SS34 rotor.

Ammonium sulfate fractionation. Partial enzyme purification was achieved by the following ammonium sulfate fractionation procedure: the sonically treated supernatant fluid was brought to 40% of saturation with a cold saturated ammonium sulfate solution, equilibrated in the cold for 1 hr, and centrifuged at 20,200 × g for 40 min. The supernatant fluid was brought to either 60 or 80% of saturation with ammonium sulfate to precipitate the desired fractions.

After centrifugation, the pellets were redissolved in 0.1 M potassium phosphate buffer (pH 6.8) and either used directly (partially purified enzyme) or after dialysis for 24 hr in the indicated buffers (dialyzed, partially purified enzyme).

Incubation of enzyme preparations with radioactive substrates. Enzymatic conversion of either labeled α -AAA or labeled lysine to saccharopine was studied by adapting a system previously described (18).

A 30- μ liter sample of enzyme was incubated with 0.5 μ c of either L-lysine-6-¹⁴C (specific activity, 2.58 mc/mmmole) or DL- α -AAA-6-¹⁴C (specific activity, 3.48 mc/mmmole) and 10 μ liters of a solution of the appropriate cofactors. Control incubations with no

cofactor substituted 10 μ liters of buffer for the cofactor solutions.

For the lysine to saccharopine conversion, the stock cofactor solution contained 10 mg of reduced nicotinamide adenine dinucleotide (NADH) and 10 mg of α -ketoglutaric acid (α -KG) per ml, and, for the conversion of α -AAA to saccharopine, the stock cofactor solution contained 10 mg of ATP per ml. After incubation of 2 to 4 hr, the reactions were sampled and spotted directly in 5- μ liter portions on 589 Orange Ribbon paper strips (Schleicher & Schuell Co., Keene, N.H.) for descending chromatography. After chromatography, the strips were dried and the locations and intensities of radioactive areas were determined by scanning with a Radiochromatogram Strip Scanner (Actigraph III; Nuclear-Chicago Corp., Cleveland, Ohio). The chromatographic migrational direction is from left to right on all figures with the base line and solvent front indicated, respectively, by B.L. and S.F.

Counting parameters were as follows unless otherwise indicated: high voltage, 950 v; scan speed, 60 cm/hr; background suppression, none; full scale, 3,000 counts/min; time constant, 10 sec; collimator slit width, 1.5 mm. Known standards of lysine, saccharopine, and α -AAA were included as references in each solvent system.

Incubation of whole cells with radioactive substrates. Various strains of *Saccharomyces* were grown in COMP medium, harvested, and washed 3 times in 0.07 M monobasic potassium phosphate and finally in 0.07 M monobasic potassium phosphate containing 3% glucose.

Approximately 625 mg of packed cells was incubated for 6 hr at 30 C with 2.0 μ c (specific activity, 3.48

mc/mole) of α -AAA- $6\text{-}^{14}\text{C}$ and 0.5 ml of 0.07 M potassium phosphate containing 3% glucose.

Cells and media were then separated by centrifugation. The cells were washed once in distilled water and resuspended to the original volume in distilled water for hot-water extraction of the amino acid pool. Control experiments demonstrated that pool extraction at autoclave temperatures (121 C) destroyed a significant part of the α -AAA, whereas 20 min in a boiling water bath extracted the α -AAA quantitatively without significant destruction. The centrifuged hot-water extract was spotted in 20- μ l portions on paper strips for descending paper chromatography.

Spectrophotometric assay of saccharopine dehydrogenase and saccharopine reductase. Saccharopine dehydrogenase and saccharopine reductase activities were estimated by slight modification of the optical assays of Saunders and Broquist (22) and Jones and Broquist (14). Our incubation mixtures for saccharopine dehydrogenase assay contained: NADH, 2 μ moles; α -KG, 50 μ moles; L-lysine, 50 μ moles, in a total volume of 3 ml. These concentrations gave somewhat enhanced sensitivity and were capable of detecting 1% of the wild-type enzyme level. For saccharopine dehydrogenase, the assay involved following the oxidation of NADH at 339 nm in the presence of substrates (α -KG and L-lysine) and enzyme (Fig. 1). Saccharopine reductase activity was assayed by following the reduction of nicotinamide adenine dinucleotide phosphate (NADP) at 339 nm in the presence of saccharopine and enzyme (14).

Substrates and cofactors used in the saccharopine dehydrogenase assays were dissolved in 0.1 M potassium phosphate buffer (pH 6.8). Substrates and cofactors used in the saccharopine reductase assays were dissolved in 0.1 M glycine-NaOH buffer (pH 9.5).

A 2000 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with auxiliary offset control was used for spectrophotometric assays.

Protein concentrations, where indicated, were determined by the method of Waddell (25).

RESULTS

Isolation of the S_I and S_{II} supersuppressors.

The S_I suppressor gene was isolated in one of six revertant clones which arose after 7 days from 10^8 cells of strain MO-49-14D spread on a plate of M-N + α -AAA. The presence of a suppressor was indicated by the cross of this revertant (BM-6-MO-49-14D) to a known wild type, with the recovery, in recombinant tetrads, of the original ly_2 mutant lesion. Additional crosses then characterized S_I as an unlinked, dominant gene capable of suppressing the ly_{1-1} , ly_{2-1} , ly_{9-1} , tr_{5-48} , ar_{4-17} , and hi_{5-2} lesions (A. Roshanmanesh, Ph.D. Thesis, Southern Illinois University, Carbondale, 1968). S_I was also confirmed as a supersuppressor by R. Gilmore.

S_{II} was found in strain 50078 of the Carbondale breeding stocks. Since the ly_4 lesion in

TABLE 2. Chromatographic R_F values of compounds relating to lysine biosynthesis from α -amino adipic acid in yeast

Radioactive intermediates	Solvent systems ^a			
	<i>n</i> -But	<i>n</i> -Prop	<i>t</i> -But	M,E,K
α -Amino adipic acid...	0.28	0.15	0.49	0.50
Lysine.....	0.08	0.36	0.16	0.18
Saccharopine.....	0.08	0.05	0.22	0.23
Unknown.....	0.42	0.03	0.52	
α -AAA- δ -CHO ^b	0.12	0.29	0.34	

^a Solvent systems: *n*-But, *n*-Butyl alcohol-acetic acid-water 4:1:1, vol/vol; *n*-Prop, *n*-Propanol: ammonia 70:30, vol/vol; *t*-But = *t*-Butyl alcohol-formic acid-water 70:15:15, vol/vol; M,E,K = *t*-Butyl alcohol-methylethyl ketone-formic acid-water 40:30:15:15, vol/vol.

^b α -Amino adipic acid- δ -semialdehyde.

strain 50078 was not suppressible by S_{II} , realization of the presence of this suppressor depended on outcross to strains carrying supersuppressible lesions. In a series of such crosses, S_{II} was confirmed as a supersuppressor gene closely resembling S_I (A. Roshanmanesh, Ph.D. Thesis, Southern Illinois University, Carbondale, 1968).

Metabolism of labeled lysine. Incubation of L-lysine- $6\text{-}^{14}\text{C}$ with NADH and α -KG in the presence of a yeast enzyme preparation produced labeled saccharopine. The identity of the saccharopine was confirmed by paper chromatography in five solvent systems against a saccharopine standard active in the saccharopine dehydrogenase and reductase assays.

The lysine conversions to saccharopine in 2 hr achieved by enzyme preparations from three strains are compared in the three radiochromatogram scans represented in Fig. 2. The top scan, that of wild-type strain F₂ shows extensive conversion of lysine to saccharopine in 2 hr. The bottom scan, that of an unsuppressed ly_{1-1} mutant strain (S720B) shows no conversion of lysine to saccharopine, indicating that the block at saccharopine dehydrogenase in this strain is apparently total. The middle scan, that of a supersuppressed ly_{1-1} mutant strain (MO-76-5C), exhibited a small saccharopine peak after 2 hr of incubation. Comparison of the rates of saccharopine formation by wild-type and supersuppressed enzyme incubations, sampled at a series of time intervals, indicated that the supersuppressed enzyme preparation probably functioned at a rate less than 10% that of wild type.

Ability to convert lysine to saccharopine by a number of yeast strains chosen as examples is summarized in Table 3. All ly_1 strains tested

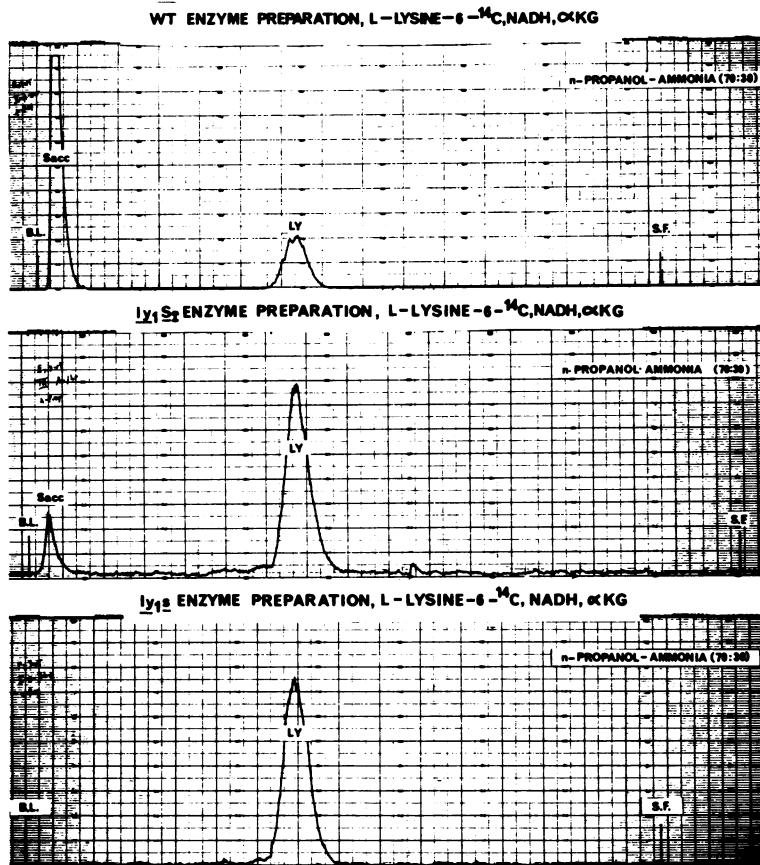


FIG. 2. Radiochromatogram scans of 1-hr incubations of *L*-lysine-6-¹⁴C, added cofactors, and yeast enzyme. Top, wild type (F_2); middle, supersuppressed ly_{1-1} (MO-76-5C); bottom, unsuppressed ly_{1-1} (S720B). Samples, 5 μ liter. Abbreviations: LY, lysine; Sacc, saccharopine; α -KG, α -ketoglutarate; B.L., base line; S.F., solvent front.

TABLE 3. Effects of S_I and S_{II} suppressors on the growth and lysine to saccharopine conversion of lysine auxotrophs^a

Strain	Genotype	Phenotype	
		Growth on CS-ly	Saccharopine production
F_2	LY	+	+
MO-49-14D	ly_{2-1}	-	+
X1012-1D	ly_{9-1}	-	+
S720B	ly_1	-	-
S288C-24	ly_{1-1}	-	-
MO-76-5C	$ly_{1-1} S_I$	+	+
MO-76-5D	$ly_{1-1} S_I$	+	+
MO-59-13B	$ly_{1-1} S_{II}$	+	+

^a Complete incubation system consisted of: sonically treated yeast supernatant fluid, 30 μ liters; *L*-lysine-6-¹⁴C, 10 μ liters; and NADH + α -KG cofactor stock solution, 10 μ liters. Incubation time, 2 hr.

which bore no suppressor failed to produce saccharopine in the labeled assay, whereas all ly_{1-1} strains tested bearing either the S_I or S_{II} suppressors exhibited reduced but significant saccharopine production. Enzyme preparations from lysineless mutants with lesions at other sites (ly_2 , ly_9) behaved like wild type in the lysine-to-saccharopine assay.

Metabolism of labeled α -AAA. Incubation of DL- α -AAA-6-¹⁴C with an undialyzed wild-type yeast enzyme preparation yielded labeled saccharopine. The undialyzed system required ATP, but after dialysis reduced NADP Mg²⁺ and adenosine triphosphate were required.

There was no evidence in our wild-type labeled incubation mixtures for the production of any intermediates between α -AAA and saccharopine. Indirect evidence has previously been presented for an adenylated intermediate (19) and for an α -amino adipic acid- δ -semialdehyde (28) as precursors of saccharopine. Evidence for inter-

mediates between α -AAA and saccharopine was, however, found in our studies by using enzyme preparations extracted from mutants blocked before saccharopine.

The α -AAA conversion to saccharopine was compared in enzyme preparations from three strains. The first, a wild-type strain (F_2), showed extensive conversion of α -AAA to saccharopine. Since we employed the DL- α -AAA as substrate and assuming that only the L- α -AAA is converted to saccharopine, the wild-type conversion in 4 hr appeared to be close to 100%. The second, an unsuppressed ly_{9-1} mutant strain (X1012-1D), showed no conversion of α -AAA to saccharopine, suggesting that the block in saccharopine reductase in this ly_9 mutant strain is total. The solvent systems employed gave clear demonstration that no radioactive material resembling sac-

charopine is produced by this mutant strain. It was evident, however, that small amounts of radioactive material appeared at other sites on the chromatograms and as a shoulder on the α -AAA peak. The third strain, a supersuppressed ly_{9-1} mutant (MO-73-18C), exhibited considerably reduced saccharopine production in 4 hr compared to wild type. These results were confirmed in four different solvent systems.

The accumulation of labeled intermediates between α -AAA and saccharopine was most clearly demonstrated by using the unsuppressed ly_{9-1} mutant (blocked before saccharopine) when the incubation mixture was chromatographed in a *n*-butyl alcohol-acetic acid-water solvent system. Two new peaks were differentiated, neither of which was saccharopine. Figure 3 compares the results of incubation of

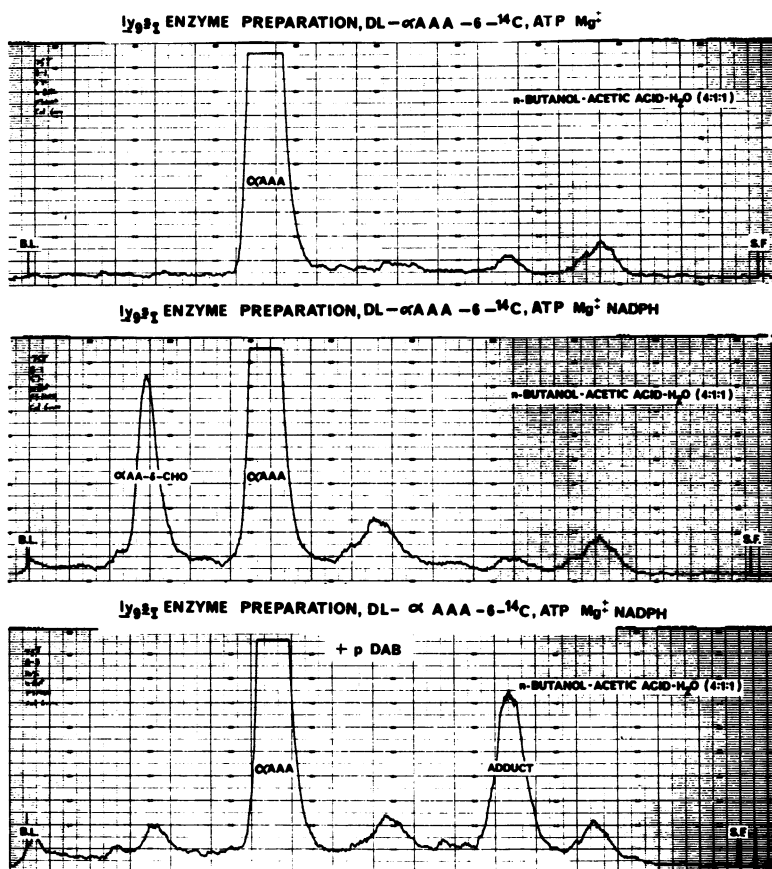


FIG. 3. Radiochromatogram scans of 4-hr incubations of DL- α -aminoadipic acid-6- 14 C, added cofactors, and a dialyzed unsuppressed ly_{9-1} (X1012-1D) yeast enzyme (40 to 60% ammonium sulfate fraction). Top, with ATP and $MgCl_2$; middle, with ATP, $MgCl_2$, and NADPH; bottom, same as middle scan except mixed after incubation with an equal volume of 4% dimethylaminobenzaldehyde, sealed in a capillary tube, and immersed in a boiling water bath for 5 min. Top and middle scans represent 5- μ liter samples; bottom scan represents a 10- μ liter sample to keep the number of counts comparable. Scanning parameters are as indicated in Materials and Methods with the following exceptions: full scale, 1,000 counts/min; collimator, 6 mm. Abbreviations: p-DAB, p-dimethylaminobenzaldehyde; α -AA- δ -CHO, α -aminoadipic- δ -semialdehyde.

labeled α -AAA with a dialyzed, partially purified preparation of the unsuppressed ly_{9-1} mutant enzyme. Comparison of the top and middle scans reveals the dependence of the dialyzed enzyme on NADPH for a reductive step in the metabolism of α -AAA. The middle scan reveals two new peaks positioned on either side of α -AAA in this solvent system. The large peak to the left of α -AAA has an R_F value close to saccharopine in this solvent system. The fact that it is clearly not saccharopine is revealed in other solvent systems and by its reaction with *p*-dimethylaminobenzaldehyde demonstrated in the lowest scan. The reaction produced an orange adduct which migrated at a new site on the chromatogram. These observations taken together with previous work (20, 21) suggest that the intermediate peak migrating to the left of α -AAA is the δ -semialdehyde of α -AAA or its cyclized form (δ^1 -piperidine-6-carboxylate). The smaller peak migrating to the right of α -AAA in this system has not yet been identified.

These two new peaks were also observed, but to a lesser extent, in supersuppressed ly_{9-1} incubations. It is clear that only in the reduction or complete absence of saccharopine reductase activity do the steady state concentrations of these intermediates increase to discernible levels.

Table 4 compares the production of labeled saccharopine from α -AAA-6- ^{14}C by supersuppressed and that by unsuppressed enzyme preparations. The top three strains (LY , ly_4 , and $ly_{1-1} S_I$) are wild type with respect to this portion of the pathway and, as expected, are able to

convert α -AAA to saccharopine. The two different supersuppressed ly_{9-1} strains (MO-73-18A and MO-73-18C) also demonstrated a low level of saccharopine production, indicating that the effect of S_I was to relieve the ly_{9-1} mutant lesion and allow synthesis of saccharopine reductase. Comparison of the rates of saccharopine formation by wild type and supersuppressed enzyme incubations, sampled at 1, 2, and 4 hr, indicated that the supersuppressed enzyme preparation probably functioned at a rate less than 10% that of wild type. The finding that the unsuppressed ly_{2-1} strain tested was also unable to accumulate labeled saccharopine is in agreement with localization of the ly_2 metabolic block immediately after α -AAA in lysine biosynthesis (13) and indicates that this block is probably total. The inability of supersuppressed ly_{2-1} to form any labeled product, however, is inconsistent with its ability to grow on NH_4^+ in the absence of lysine or α -AAA and suggested the possibility that the supersuppressed ly_{2-1} enzyme might be labile under the condition employed.

To test this possibility, a whole-cell incubation system was used to assay the amino acid pool for intermediates produced from labeled α -AAA by a supersuppressed ly_{2-1} strain.

A wild-type control strain (MO-59-13A) showed considerable labeled saccharopine and lysine production from α -AAA, whereas an unsuppressed mutant strain (MO-49-14D) gave no indication of any conversion of labeled α -AAA. Whole-cell incubations of a supersuppressed ly_{2-1} strain (BM-6-MO-49-14D) revealed a small amount of labeled saccharopine production, thus firmly establishing a consistent pattern regarding supersuppression of the ly_{2-1} , ly_{9-1} , and ly_{1-1} mutant lesions.

Spectrophotometric assays for saccharopine dehydrogenase and saccharopine reductase. For kinetic studies, continuous optical assays are more convenient than discontinuous radioactive assays. To study the specific effect of a suppressor gene (S_I) on saccharopine dehydrogenase and saccharopine reductase, spectrophotometric assays (14, 22) were utilized. Table 5 compares the specific activities of supersuppressed and wild-type saccharopine dehydrogenase. Depending on the mode of expression of the results, the supersuppressed activity was found to be between 6 and 10% of wild-type activity, confirming the preliminary estimate made with labeled lysine.

Since the reduced levels of enzyme activity in the supersuppressed mutant might have been due either to a reduced rate of synthesis of the wild-type enzyme or to an enzyme alteration, or both, a study was made of the Michaelis

TABLE 4. Effects of S_I and S_{II} suppressors on the growth of α -AAA to saccharopine conversion of lysine auxotrophs^a

Strain	Genotype	Phenotype	
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F ₂	<i>LY</i>	+	+
19B	<i>ly</i> ₄	-	+
MO-59-13B	<i>ly</i> ₁₋₁ S_I	+	+
MO-49-14D	<i>ly</i> ₂₋₁	-	-
BM-6-MO-49-14D	<i>ly</i> ₂₋₁ S_I	+	- ^b
X1012-1D	<i>ly</i> ₉₋₁	-	-
MO-73-18A	<i>ly</i> ₉₋₁ S_I	+	+
MO-73-18C	<i>ly</i> ₉₋₁ S_I	+	+

^a Complete incubation systems consisted of: sonically treated yeast supernatant, 30 μ liters; α -AAA-6- ^{14}C , 10 μ liters; and ATP cofactor stock solution, 10 μ liters. Incubation time, 4 hr.

^b Saccharopine production demonstrated in whole cells.

TABLE 5. Comparison of supersuppressed and wild-type saccharopine dehydrogenase activity

Parameter	A, super-suppressed (<i>ly₁₋₁ S_I</i>)	B, wild type	Ratio A/B × 100
ΔA_{339} /min/mg of protein crude preparation	0.00807	0.1246	6.48
ΔA_{339} /min/mg of protein 40% to 60% fraction	0.02997	0.2865	10.46
ΔA_{339} /min/10 ⁸ cells	0.0557	0.7920	7.03

constants of wild-type and supersuppressed saccharopine dehydrogenase (Table 6). The values obtained for wild-type saccharopine dehydrogenase from our wild-type strain compare favorably with those reported previously (22). It is significant to note that in all cases, the K_m values for the supersuppressed enzyme were considerably greater than the comparable values for the wild type.

A similar study of the effect of S_I on saccharopine reductase in a *ly₉₋₁ S_I* strain produced the results indicated in Table 7. Here again, the K_m value for each substrate of supersuppressed saccharopine reductase was greater than the comparable value for the wild-type enzyme. A previous report of K_m values for a more extensively purified saccharopine reductase preparation again compares favorably (14).

The demonstration that S_I alters both saccharopine reductase and saccharopine dehydrogenase in *ly₉₋₁ S_I* and *ly₁₋₁ S_I* strains, respectively, suggested a control experiment to check whether the saccharopine dehydrogenase in a strain bearing a supersuppressor-altered saccharopine reductase (*ly₉₋₁ S_I*) would be wild type or altered.

Saccharopine dehydrogenase extracted from *ly₉₋₁ S_I* cells was found to yield K_m values closely comparable to wild-type enzyme (Table 6), indicating that S_I produced no detectable effect on properties of this enzyme when the gene controlling saccharopine dehydrogenase was wild type.

DISCUSSION

The general model for nonsense suppressors (7) has been enlarged, in part, by proposals

TABLE 7. Comparison of Michaelis constants for saccharopine reductase

Michaelis constants (M)	Supersuppressed (<i>ly₉₋₁ S_I</i>)	Wild type
K_m Saccharopine	$4.0 \pm 1.0 \times 10^{-3}$	$3.4 \pm 0.2 \times 10^{-4}$
K_m NADP	$1.0 \pm 4.0 \times 10^{-3}$	$1.2 \pm 0.2 \times 10^{-5}$

arising from the study of supersuppression in yeast (2, 5, 23). These proposals suggest that there are sets of redundant tRNA types, each member of a set representing a unique molecular species under separate genetic control with the codon and amino acid specificity shared by all members of a set. A supersuppressor is believed to arise when alteration in a gene controlling a single member of a set modifies the tRNA it controls, enabling it to introduce an amino acid at the site of a nonsense codon preventing premature chain termination and enabling the completion of a protein. Direct evidence of single amino acid replacement in the completed protein (cytochrome *c*) of a supersuppressed mutant has been provided (6). Most gene products are still beyond the reach of primary-structure analysis, but information relevant to the mechanism of supersuppression can be obtained by studying the properties of enzymes in supersuppressed strains. Convincing evidence consistent with the present model is provided if the suppressed strain exhibits restoration of activity of the mutant enzyme, particularly if it can be shown to occur with a significant alteration of physical properties.

The suppressors examined in the current study seemed interesting at first as possible pathway suppressors, because our initial observations that they suppressed three loci in lysine biosynthesis after α -AAA (*ly₂₋₁*, *ly₉₋₁*, *ly₁₋₁*) but did not suppress certain loci before α -AAA (e.g., *ly₄₋₁*) suggested that there might be an alternate biosynthetic route from α -AAA to lysine. A careful study, however, indicated that they were not pathway suppressors but, in fact, supersuppressors affecting a number of alleles in other biosynthetic pathways as well. Our results indicate that the S_I supersuppressor restored saccharopine dehydrogenase and sac-

TABLE 6. Comparison of Michaelis constants for saccharopine dehydrogenase

Michaelis Constants (M)	Supersuppressed (<i>ly₁₋₁ S_I</i>)	Wild type	Supersuppressed (<i>ly₉₋₁ S_I</i>)
K_m lysine.....	$8.8 \pm 3.0 \times 10^{-2}$	$4.2 \pm 1.0 \times 10^{-3}$	$2.6 \pm 1.0 \times 10^{-3}$
K_m α -ketoglutarate.....	$9.3 \pm 4.0 \times 10^{-3}$	$1.4 \pm 0.3 \times 10^{-4}$	$1.0 \pm 0.4 \times 10^{-4}$
K_m NADH.....	$3.1 \pm 0.2 \times 10^{-4}$	$6.6 \pm 0.8 \times 10^{-5}$	

charopine reductase activity to ly_{1-1} and ly_{9-1} mutants, respectively. The altered Michaelis constants for these enzymes in the suppressed strains, compared to those of the same enzymes in wild-type strains, are consistent with the model for supersuppression. Although the effects of nonsense suppressors are still not clearly delimited with respect to normal chain termination, it is interesting that supersuppressed ly_{9-1} strains exhibited an altered saccharopine reductase but an apparently unaltered saccharopine dehydrogenase.

We have thus far been unable to demonstrate restoration of function of α -AAA reductase in cell-free preparations of supersuppressed ly_2 mutants but have succeeded in making the comparable demonstration in whole cells. This suggests that the amino acid replacement in the altered α -AAA reductase produced an enzyme which is labile to the enzyme isolation procedures thus far employed.

Recent findings have demonstrated the similarity between the lysine biosynthetic route in yeast (Fig. 1) and the major route of lysine degradation in humans. Higashino, Tsukada, and Lieberman (11) have demonstrated that rat liver will catalyze the conversion of lysine to saccharopine and suggested that this may be the initial step in the major lysine degradative pathway in mammals. Studies in humans have indicated the probable relationship between certain cases of elevated blood lysine level (hyper-lysinemia) and an apparently heritable disease in humans (26). The demonstration of a metabolic block at an early step in lysine degradation (27) and the indication that saccharopine is a direct product of lysine conversion in humans (12) make the ly_{1-1} yeast mutant an attractive experimental model for this human condition.

Accumulation of high levels of saccharopine in patients believed to carry another inborn error in lysine metabolism [saccharopinuria (4)], indicating an inability to degrade saccharopine suggests the ly_{9-1} yeast mutant as a model for this human condition.

The possibilities of a general approach to the treatment of genetic disease by "euphenic" means (15) and of the medicogenetic application of gene products of specific suppressors (17) have been considered.

ACKNOWLEDGMENTS

We are grateful to R. Gilmore for providing several super-suppressible markers and for performing independent checks confirming S_I as a supersuppressor.

We also wish to thank H. P. Broquist for a generous sample of saccharopine and for his personal encouragement.

This work was supported by the American Cancer Society grant E981.

LITERATURE CITED

1. Benzer, S., and S. P. Champe. 1962. A change from nonsense to sense in the genetic code. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1114-1121.
2. Bergquist, P. L. 1966. Degenerate transfer RNAs from brewers yeast. *Cold Spring Harbor Symp. Quant. Biol.* **31**:435-447.
3. Broquist, H. P. 1964. ϵ -N-(L-glutaryl-2)-L-lysine, a novel intermediate in the aminoacidic pathway of lysine biosynthesis. *Proc. Sixth Int. Cong. Biochem.* **5**:353-364.
4. Carson, N. A., B. G. Scally, D. W. Neill, and I. J. Carre. 1968. Saccharopinuria: a new inborn error of lysine metabolism. *Nature (London)* **218**:679.
5. Gilmore, R. A., and R. K. Mortimer. 1966. Super-suppressor mutation in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **20**:307-311.
6. Gilmore, R. A., J. W. Stewart, and F. Sherman. 1968. Amino acid replacements resulting from super-suppression of a nonsense mutant of yeast. *Biochem. Biophys. Acta.* **161**:270-272.
7. Gorini, L., and J. R. Beckwith. 1966. Suppression. *Ann. Rev. Microbiol.* **20**:401-422.
8. Grove, J., and L. M. Henderson. 1968. The metabolism of D- and L-lysine in the intact Rat, perfused liver and liver mitochondria. *Biochim. Biophys. Acta* **165**:113-120.
9. Hawthorne, D. C., and R. K. Mortimer. 1963. Super-suppressors in yeast. *Genetics* **48**:617-620.
10. Hawthorne, D. C., and R. K. Mortimer. 1968. Evidence for chain termination by super-suppressible mutants in yeast. *Genetics* **60**:719-733.
11. Higashino, K., K. Tsukada, and L. Lieberman. 1965. Saccharopine, a product of lysine breakdown by mammalian liver. *Biochem. Biophys. Res. Commun.* **20**:285-290.
12. Hutzler, J., and J. Dancis. 1968. Conversion of lysine to saccharopine by human tissue. *Biochim. Biophys. Acta* **158**:62-69.
13. JONES, E. E., and H. P. Broquist. 1965. Saccharopine, An intermediate of the aminoacidic acid pathway of lysine biosynthesis. II. Studies in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **240**:2531-2536.
14. Jones, E. E., and H. P. Broquist. 1966. Saccharopine, an intermediate of the aminoacidic acid pathway of lysine biosynthesis. III. Aminoacidic semialdehyde-glutamate reductase. *J. Biol. Chem.* **241**:3430-3434.
15. Lederberg, J. 1963. Molecular biology, eugenics and euphenics. *Nature (London)* **198**:428-429.
16. Magni, G. E., and P. P. Puglisi. 1966. Mutagenesis of super-suppressors in yeast. *Cold Spring Harbor Symp. Quant. Biol.* **31**:699-704.
17. Ogur, M., 1969. Outline for an experimental attack on the treatment of certain varieties of genetic disease. *Perspect. Biol. Med.* **12**:471-474.
18. Piediscalzi, N., T. Fjellstedt, and M. Ogur. 1968. A glutamic- α -ketoacidic transaminase in *saccharomyces*. *Biochem. Biophys. Res. Commun.* **32**:380-384.
19. Sagisaka, S., and K. Shimura. 1960. Mechanism of activation and reduction of α -aminoacidic acid by yeast enzyme. *Nature (London)* **188**:1189-1190.
20. Sagisaka, S., and H. Shimura. 1962. A method for the quantitative determination of dehydropiperidine carboxylic acid, a reduction product of α -aminoacidic acid by yeast enzyme. *J. Biochem. (Tokyo)* **51**:27-31.
21. Sagisaka, S., and K. Shimura. 1962. Studies in lysine biosynthesis. IV. Mechanism of activation and reduction of α -aminoacidic acid. *J. Biochem. (Tokyo)* **52**:155-161.
22. Saunders, P. P., and H. P. Broquist. 1966. Saccharopine, an intermediate of the aminoacidic acid pathway of lysine biosynthesis. IV. Saccharopine dehydrogenase. *J. Biol. Chem.* **241**:3435-3440.
23. Soll, D., J. Cherayil, D. S. Jones, R. D. Faulkner, A. Ltempel, R. M. Bock, and H. G. Khorana. 1966. sRNA Specificity for codon recognition as studied by the ribosomal binding

- technique. Cold Spring Harbor Symp. Quant. Biol. **31**:435-447.
24. Von Borstel, R. C. (ed.). 1963. Carbondale yeast genetics conference. Microbiol. Genet. Bull. **19** (supplement).
25. Waddell, W. J. 1956. A simple UV spectrophotometric method for the determination of protein. J. Lab. Clin. Med. **48**:311-314.
26. Woody, N. C. 1964. Hyperlysinemia. Amer. J. Dis. Child. **108**:543-553.
27. Woody, N. C., J. Hutzler, and J. Dancis. 1966. Further studies of hyperlysinemia. Am. J. Dis. Child. **112**:577-580.
28. Yura, T., and H. J. Vogel. 1959. An ω -hydroxy- α -amino acid dehydrogenase of *Neurospora crassa*: partial purification and some properties. J. Biol. Chem. **234**:339-342.