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PROLINE OXIDASES IN *HANSENULA SUBPELLICULOSA*

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ABSTRACT

LING, CHUNG-MEI (Illinois Institute of Technology, Chicago), AND L. R. HEDRICK. Proline oxidases in *Hansenula subpelliculosa*. J. Bacteriol. **87**:1462–1470. 1964—Cells of *Hansenula subpelliculosa* can use L-proline as a carbon and a nitrogen source after a 6- to 8-hr induction period. However, they cannot use L-glutamate as both nitrogen and carbon sources unless the induction period is of several days' duration. Two L-proline oxidases were demonstrated in the mitochondrial preparation of this yeast. One forms the product Δ′-pyrroline-2-carboxylic acid (P2C), which is in equilibrium with α-keto-δ-amino-valeric acid; the other forms the product Δ′-pyrroline-5-carboxylic acid (P5C), which is in equilibrium with glutamic-γ-semialdehyde. The first-mentioned enzyme is induced when L-proline is the carbon source; the second appears to be constitutive, and is probably associated with the use of L-proline as a nitrogen source. The P2C-forming enzyme is specific for the L isomer of proline, and is inactive against L-hydroxyproline. The enzyme activity is at its peak when the mitochondria are prepared from logarithmically grown cells, and is rapidly reduced after cells reach the stationary phase of growth. Kinetic studies with varying concentrations of substrate indicate a Michaelis-Menten constant of 2.45×10^{-2} M. Paper chromatographic studies, chemical tests with H_2O_2, sensitivity to freezing, and spectral measurements indicate that proline oxidase from *H. subpelliculosa* mitochondria forms a product from L-proline which is like, if not identical to, P2C formed by the action of sheep kidney D-proline oxidase upon DL-proline. The soluble portion of the cell extract contains NAD^+ enzymes which use either P2C (α-keto-δ-amino-valeric acid) or P5C (glutamic-γ-semialdehyde) as substrates. No glutamic dehydrogenase activity could be detected when L-glutamic acid and the nicotinamide adenine dinucleotide (NAD^+) cofactor were added to the supernatant solution with the yeast enzymes. The presence of a dehydrogenase NAD^+ enzyme for activity with P2C (α-keto-δ-amino-valeric acid) has not been previously reported.

Taggart and Krakaur (1949) found that L-proline was converted to glutamic acid in mammalian liver. Working with mammalian liver and kidney, Blanchard et al. (1944) reported that L-proline was oxidized to Δ′-pyrroline-2-carboxylic acid (P2C). The latter compound is also a product of D-proline oxidase of sheep kidney (Krebs, 1939). However, an L-proline oxidase in rat mitochondria was recently demonstrated to oxidize L-proline to Δ′-pyrroline-5-carboxylic acid (P5C; Johnson and Strecker, 1962). This latter compound is in chemical equilibirium with glutamic-γ-semialdehyde, and can be oxidized by beef liver mitochondria to L-glutamic acid (Strecker, 1960). The same pathway for conversion of L-proline to L-glutamic acid occurs in a mutant strain of *Escherichia coli* (Strecker and Mela, 1955). Meister (1954) showed that P2C is in equilibrium with α-keto-δ-amino-valeric acid.

In the study of L-proline metabolism in the yeast *Hansenula subpelliculosa*, we found that L-proline can serve as the sole nitrogen and carbon source, after a lag of 6 to 8 hr, whereas L-glutamic acid can supply the carbon and nitrogen needs only after an induction period of more than 3 days. Under this condition, moderate growth (optical density of 0.82) is attained with L-glutamate as both the carbon and nitrogen source. A diauxie-type curve is manifested after growth in a defined medium with excess L-proline and a limited (300 to 500 μg/ml) glucose supply. With ammonia as a nitrogen source, α-ketoglutarate or succinate will supply the needed carbon for growth.

The purpose of the work reported in this paper was to study the enzyme systems of *H. subpelliculosa* as related to L-proline metabolism, with emphasis on the characterization of one of the L-proline oxidases. Attempts were also made to elucidate the possible pathways of the metabolism of L-proline in this yeast, so that the afore-mentioned growth characteristics can be reasonably explained.

MATERIALS AND METHODS

The yeast studied was *H. subpelliculosa* Y 1683, a "slow" strain isolated in our laboratories. This

strain grows somewhat more slowly than does the normal one in L-proline as a carbon and nitrogen source; the growth rate in L-glutamic acid as a carbon and nitrogen source is much slower than that of the normal strain. Cells used as an inoculum were grown on a shaker in a medium consisting of $(NH_4)_2SO_4$, 300 μg of N per ml; glucose, 2 mg/ml; and the salts and vitamins used by Wickerham (1946). These cells were separated from the supernatant fluid by centrifugation, and were washed twice with sterile 0.001 M PO_4 buffer (pH 7.0). Packed cells stored at 4 C served as an inoculum for a period of 1 week. These cells were added to the experimental media so that the initial optical density was 0.1.

Two types of experimental media were employed: (A) L-proline as a carbon and nitrogen source for the induction of proline oxidase, and (B) proline as a nitrogen source with production of a low level of proline oxidase. The composition of these media is as follows: (A) glucose, 500 μg/ml, and L-proline, 2 mg/ml, in the salts and vitamin solution of Wickerham (1946); (B) glucose, 2 mg/ml, and L-proline, 2 mg/ml, in the salts and vitamin solution. The media were adjusted to pH 6 prior to autoclaving. Glucose was sterilized separately and added aseptically.

In either medium, after an inoculation so that the initial optical density was 0.1, cells were in the logarithmic growth phase after agitation on a shaker for 24 hr at 28 C. The shaker had a rotary speed of 180 rev/min. The wet weight yield in medium A was 4 g per liter; in medium B, it was 7 g per liter.

The nicotinamide adenine dinucleotide (NAD) coenzyme and cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, Mo. L-Proline was secured from Nutritional Biochemicals Corp., Cleveland, Ohio; DL-proline came from Mann Research Laboratory, New York, N.Y. Crystalline bovine serum albumin was obtained from Armour Laboratories. H. J. Strecker of Yeshiva University, New York, N.Y., kindly supplied the synthetic P5C.

Preparation of proline oxidase extract. Log-phase cells grown in medium A to induce proline oxidase were recovered immediately by centrifugation, and were washed three times with sterile phosphate buffer in a refrigerated centrifuge. The wet weight of the packed cells was determined, and 5 g of these cells were added to 25 ml of precooled ethylenediaminetetraacetic acid (EDTA) phosphate buffer (pH 7.3) in a 125-ml Erlenmeyer flask. Two types of buffer were used, one which contained lactose (0.25 M lactose, 0.001 M EDTA, and 0.01 M phosphate at pH 7.3), and one which contained KCl (0.115 M KCl, 0.01 M EDTA, and 0.02 M phosphate buffer at pH 7.3). Glass beads (4 mm) were added so that they filled the flask to the level of the surface liquid. The mixture was shaken at the rate of 300 rev/min for 4 hr at 4 C. This method of disrupting the cells was superior to the use of a Nossal disintegrator or a Hughes press.

Cellular fragments were removed by centrifugation at 1,370 $\times$ *g* for 10 min at 4 C. The mitochondrial fraction was centrifuged at 24,000 $\times$ *g* for 30 min at 4 C. A centrifugal sediment at 3,100 $\times$ *g*, after removing cellular fragments, showed the same specific activity of the proline oxidase as did the sediment at 24,000 $\times$ *g* of the supernatant fluid of 3,100 $\times$ *g*; therefore, the centrifugation at 3,100 $\times$ *g* was omitted throughout the remainder of the study. The proline oxidase is in the mitochondrial particles. The NAD enzymes which use the products of proline oxidase as substrates are in the supernatant fraction.

Rat liver mitochondria were prepared according to the method of Johnson and Strecker (1962). D-Proline oxidase was extracted from acetone powder of sheep kidney by the method of Krebs (1939).

The activity of L-proline oxidase from either the yeasts or the rat liver preparation was assayed in a Beckman DU spectrophotometer at 30 C, with cytochrome *c* or potassium ferricyanide used as the final electron acceptor, or in a Warburg apparatus at 26 C. The activity of D-proline oxidase, which does not involve the cytochrome system and is a one-step oxidation, were assayed only by the Warburg apparatus. The soluble enzymes of the supernatant fraction were assayed spectrophotometrically, with NAD^+ used as the cofactor. These enzyme fractions were generally dialyzed overnight at 4 C against 0.0004 M sodium pyrophosphate buffer (pH 8.6) to reduce endogenous reactions.

The oxidation product of L-proline by yeast mitochondria was prepared in 100 ml of the following suspension: mitochondria from 5 g of wet yeast cells, which had been grown in medium A for 21 to 24 hr; L-proline, 1.0 g; cytochrome *c*, 0.48 μM; catalase, 34 units (to remove any H_2O_2 formed); and potassium phosphate buffer (pH 7.3) to a final concentration of 0.05 M.

The suspension was shaken at 100 rev/min at 25 C for 5 hr. The concentration of the oxidation product, as revealed by oxygen consumption, was about 0.12 mg/ml. Then, trichloroacetic acid was added to a final concentration of 5% to precipitate the unwanted proteins, which were filtered off or removed by centrifugation. The filtrate or supernatant fluid was adjusted to pH 8.6 by KOH for enzymatic assays or paper chromatography.

Catalase unit is the Sigma unit: one unit decomposes 1 μmole of H_2O_2 per min at pH 7.0 and at 25 C, while the H_2O_2 concentration falls from 10.3 to 9.2 μmoles/ml of reaction mixture. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in optical density at 240 mμ.

The oxidation product of L-proline oxidase by sheep kidney was prepared in a similar way, except DL-proline was used rather than L-proline [sodium pyrophosphate buffer (pH 8.6, 0.01 M) instead of potassium phosphate buffer (pH 7.3); cytochrome *c* was omitted, and the digestion time was 2 rather than 5 hr]. The concentration of the final product was estimated to be 3 mg/ml. Both of the above oxidation preparations were designated as digests.

The protein of the soluble enzyme mixture was estimated by the method of Lowry et al. (1951), with crystallized bovine serum albumin used as the standard. The color developed was measured in a Klett-Summerson colorimeter with a no. 54 filter. Concentration of the oxidation digests by boiling to remove 90% of the water was necessary before spotting at least ten times in one place on filter paper for chromatography.

Paper chromatography, either radial or descending, was conducted on Whatman no. 1 filter paper with several solvent systems; all trials were conducted at room temperature. The amino acids on chromatograms were sprayed with a ninhydrin solution containing 0.25 g of ninhydrin in 2 ml of glacial acetic acid added to 90 ml of acetone. The detection of carbonyl compounds was carried out chromatographically according to Fincham (1953). For isolation of the oxidation products, the finished chromatograms were air-dried at room temperature; a strip of the filter paper was cut out from the chromatogram and sprayed with the ninhydrin solution as a guide to the positions of the bands, so that corresponding bands on the unsprayed portion of the chromatogram could be cut out for the elution with distilled water or sodium pyrophosphate for spectral determinations or enzymatic assays.

Ammonia determination was by the micro diffusion method (Conway and O'Malley, 1942).

The specific activity of the enzymes was calculated from the absorbancy and molar extinction coefficients in spectrophotometric assays, and from the oxygen consumption in Warburg experiments. Absorbancy changes for cytochrome *c* were read at 550 mμ, with potassium ferricyanide at 410 mμ, and with NAD^+ at 340 mμ. The concentration of reduced electron acceptors was determined with molar extinction coefficients, which are 1.8×10^4 for cytochrome *c* (the difference between the reduced and the oxidized form), 1.0×10^3 for ferricyanide, and 6.2×10^3 for reduced NAD (NADH).

RESULTS

Growth rates of the "slow" and normal strains of *H. subpelliculosa*, when cultured in the defined medium with L-proline or L-glutamic acid as the source of carbon and nitrogen, are given in Table 1. In this experiment, the inoculum was such that the initial optical density was about 0.02, which is much less than the optical density of 0.1 used for growth of cells for enzyme studies. With the latter size of inoculum, 500 μg/ml of glucose as in medium A, and L-proline as the principal carbon and nitrogen source, an optical density of 0.9 to 1.0 was attained within 24 hr.

Cells of the "slow" strain grown in medium A

TABLE 1. *Optical densities of "slow" and normal strains of Hansenula subpelliculosa**

Substrate	"Slow" strain					Normal strain				
	0†	24	48	72	90	0	24	48	72	96
L-Proline	0.02	0.42	0.74	1.0	1.0	0.01	0.5	0.8	1.2	1.2
L-Glutamic	0.02	0.24	—	0.37	0.82	0.03	0.18	—	0.77	0.92

* The strains were grown in a salts-vitamin medium, with either L-proline or L-glutamic acid as the carbon and nitrogen source, in the absence of added glucose. The flasks were on a rotary shaker at 28 C.

† Indicates number of hours the flasks were on shaker.

were disintegrated for enzyme studies. When cells were disrupted with glass beads in a cold room at 4 C, and the fragments were separated from the supernatant fluid by centrifugation, the inducible proline oxidases were located in the mitochondrial fraction. Evidence supplied in subsequent sections indicates that there are two proline oxidases in the yeast mitochondria: (i) the enzyme which is induced by use of L-proline as a carbon source and from L-proline forms the product P2C, which is in equilibrium with α-keto-δ-amino-valeric acid; and (ii) the enzyme which forms P5C, which is in equilibrium with glutamic-γ-semialdehyde. There was no difference in the mitochondrial fraction prepared in the EDTA phosphate buffers which contained either KCl or lactose. There was a one-third loss in activity when the mitochondria in EDTA lactose were washed once with 0.05 M potassium phosphate buffer (pH 7.3). No recovery of the lost activity was observed with the readdition of the washing fluid. When the mitochondria were stored at -15 C for 24 hr, there was a 50% reduction in the proline oxidase activity (Table 2; experiment 2). These two observations are in variance with that reported by Johnson and Strecker (1962), in that their enzyme preparations from rat liver were activated by freezing, and some of the loss of activity due to washing was restored by the addition of the washing fluid to the mitochondrial preparations.

The induced enzyme, proline oxidase, is most active after logarithmic growth of cells of *H. subpelliculosa* in medium A for 18 to 24 hr. Cells in the late stationary phase of growth have lost much of this proline oxidase activity. Attempts to stimulate the production of the enzyme within a 4-hr "induction period" were negative; in fact, shaking cells from a L-proline log phase in a nongrowth medium with L-proline resulted in reduction of the proline oxidase activity. Cells with a high proline oxidase activity release ammonium into the supernatant fluid on a stoichiometric basis; cells grown in a medium where L-proline is not the carbon source do not do so (Table 3).

Electron acceptors. The spectrophotometric measurement of the induced proline oxidase activity was the highest when the reaction was mediated with 0.04 mM cytochrome *c* as an electron acceptor. When ferricyanide (0.67 mM) was used as the electron acceptor, the addition of cytochrome *c* in the reaction mixture caused no added activity. In Warburg experiments, the omission of cytochrome *c* decreased the oxygen utilization by about 50%.

Inhibition and activation of yeast-induced proline oxidase. In Warburg studies with atmospheric oxygen as a terminal acceptor, KCN is an inhibitor. This fact permitted the use of KCN as an inhibitor in experiments with ferricyanide or

TABLE 2. *Effect of storage at -15 C upon activity of proline oxidase from Hansenula subpelliculosa mitochondria*

Expt no.*	N source	Concn†	C source	Concn†	Growth period	Specific activity‡			
						Age of mitochondria			
						0	1 day	1 week	2 weeks
		mg/ml		*mg/ml*	*hr*				
1	L-Proline	2	L-Proline	2	14		1.2	0.5	
2	L-Proline	2	L-Proline	2	17	12.9	6.5		1.0
3	L-Proline	2	L-Proline	2	48	3.5	2.2		
4	L-Proline	1	Glucose	2	28	0.9	1.0		
5	L-Proline	1	Glucose	2	28	0.6	0.6		
6	NH_3	0.5	Glucose	2	24	0.55	0.45		
7	NH_3	0.5	Glucose	2	24	0.63	0.40		
8	Rat liver mitochondria		Rat liver mitochondria			2.3	1.5		

* In experiments 1, 2, and 3, glucose (500 μg/ml) was added to decrease the lag period. Rat liver mitochondria was used for comparison. The results were obtained with a DU spectrophotometer. The concentration of reactants in 3 ml was: KCN, 3.3 mM; L-proline, 30 mM; cytochrome *c* as electron acceptor, 0.004 mM; and mitochondria, 0.3 mg; in 0.05 M K phosphate buffer (pH 7.3).

† Indicates concentration added to defined salts-vitamin growth medium.

‡ Expressed as $\times 10^{-3}$ μmoles of proline oxidized per min per mg of protein.

TABLE 3. *Effect of growth media upon the activity of L-proline oxidase and the liberation of ammonia*

Expt no.*	N source	Concn†	C source	Concn†	Specific activity‡	Age of cells	Nitrogen assimilated in cells (g per liter)	Nitrogen released as NH_3 in medium (g per liter)	Total N supplied
		mg/ml		*mg/ml*					
1	NH_3	0.5	Glucose	2	0.58×10^{-3}		—	—	—
2	NH_3	0.5	Glucose	2	0.6×10^{-3}		—	—	—
3	Proline	1	Glucose	2	0.58×10^{-3}		—	—	—
4	Proline	2	Proline	2	3.5×10^{-3}	48 hr stationary	—	—	—
5	Proline	2	Proline	2	13.7×10^{-2}	24 hr log	0.11	0.11	0.24
6	Proline	1	Glucose	2	—	24 hr log		0	0.26
7	Proline	2	Proline	2	—	24 hr log	0.08	0.42	0.51
	$+NH_3$	0.5							

* Experiments 1 and 2 are duplicates.

† Indicates concentration added to defined salts-vitamin growth medium.

‡ Specific activity of fresh mitochondrial fraction from *Hansenula subpelliculosa* cells, determined spectrophotometrically with cytochrome *c* as an electron acceptor. Expressed as μmoles of proline oxidized per min per mg of protein. Reaction mixtures as in Table 2.

cytochrome *c* in studies with the DU spectrophotometer.

Addition of amytal to a final concentration of 10^{-3} M does not inhibit the oxidation of L-proline. Because amytal is a specific inhibitor of NADH cytochrome *c* reductase (Smith and Hansen, 1962), electrons in the oxidation of L-proline do not flow through NAD enzymes to the cytochrome systems. This is in agreement with the report of Johnson and Strecker (1962) for the rat liver mitochondrial enzyme. The incorporation of catalase in the reaction mixture of the Warburg experiment increased the oxygen consumption more than twofold. This was very likely due to the elimination of hydrogen peroxide, which arose from the oxidation of L-proline, and which might be inhibitory to the proline oxidase.

Effect of substrate upon induced L-proline oxidase activity. Proline oxidase from *H. subpelliculosa* is specific for the L isomer of proline. The reduction of cytochrome *c*, as measured by the change in optical density at 550 mμ with DL-proline, was only one-half that for L-proline. Furthermore, L-hydroxyproline will not serve as substrate for this enzyme. The proline oxidase in rat liver is active against hydroxyproline (Johnson and Strecker, 1962).

In spectrophotometric measurements, a saturation of the enzyme by the substrate L-proline was attained when the substrate concentration was 30 mM or higher for 0.27 mg/ml of (crude extract) enzyme protein (Fig. 1). The Michaelis-Menten constant, estimated from a double reciprocal plot of reaction rates against the L-proline concentrations, is 2.45×10^{-2} M.

In the next few sections, evidence will be presented that P2C is the principal product of the oxidation of L-proline by mitochondria from *H. subpelliculosa* cells in the log phase of growth, with L-proline as the carbon source. As P2C has not been synthesized, experimental quantities were produced by the reaction of enzymes from sheep kidneys upon DL-proline (Krebs, 1939), and were isolated by paper chromatography.

Paper chromatography studies. For comparative studies, the products of the activity of proline oxidase from yeast and from sheep kidney were prepared. Mitochondrial enzymes from *H. subpelliculosa* were permitted to act upon L-proline, and the D-proline oxidase from sheep kidney was permitted to act upon DL-proline. Paper chromatograms were prepared for both of these reactions. Two identical spots appeared on each chromatogram; one spot was the substrate proline, which gave a yellow color surrounded by a pink ring, and the second spot, which was purple-pink in color, was P2C. The solvent used in this experiment was *n*-butanol–acetic acid–water (77.0:4.5:18.5). Synthetic P5C, treated similarily on paper chromatograms, has a bright pink color, and its rate of migration is faster than that of P2C.

Treatment of the purple-pink spots, representing P2C from either the yeast or sheep kidney enzymes activity, with hydrogen peroxide resulted in the disappearance of the spots. The P2C

presumably decomposed to aminobutyric acid and CO_2. Hydrogen peroxide does not decompose P5C. This is in accordance with the difference between P2C and P5C reported by Johnson and Strecker (1962). Areas corresponding to those with the purple-pink spots were eluted with pyrophosphate buffer for enzymatic and spectral studies.

The 2,4-dinitrophenylhydrazone of the enzymatic reactions was separated and detected by paper chromatography. With the synthetic P5C and the enzymatically prepared P2C as controls, the 2,4-dinitrophenylhydrazine derivative of the product of L-proline oxidase reaction from *H. subpelliculosa* mitochondria appears to be the dinitrophenylhydrazone of P2C (Table 4).

Paper chromatograms of the 2,4-dinitrophenylhydrazone of the residual carbonyl compound in medium A, after growth of the cells or in the total extract of disrupted cells, indicate that α-keto-glutaric acid is the only carbonyl substance present. This was verified by the use of two different solvent systems: (a) *n*-butanol saturated with ammonia and (b) *n*-butanol–acetic acid–water. In solvent a, the 2,4-dinitrophenylhydrazone derivative of α-keto-glutaric acid and that of the unknown had an R_F value of 0.17; in solvent b, both of these had an R_F of about 0.75.

Spectral studies. The reaction product of induced L-proline oxidase from *H. subpelliculosa* mitochondria has a maximal absorbancy at 270 mμ and a minimal absorbancy at 248 mμ, with little change in the peak and furrow due to aging of the compound (Fig. 2). This product was eluted from the paper chromatogram, and the absorbancy was determined with the use of a DU spectrophotomer. The spectrum of the freshly eluted D-proline digest from sheep kidney acetone powder had a peak corresponding to that of the yeast enzyme product, and there was some evidence of a furrow with a minimal value. However, when this compound had been aged on the chromatogram at room temperature for 1 week prior to elution, the spectrum of the product had the same maximal and minimal values as did the product of induced proline oxidase activity from *H. subpelliculosa*. This is comparable to the changes due to aging of P5C (Johnson and Strecker, 1962).

For comparative purposes, the spectrum was determined for freshly prepared and aged aqueous solutions of P5C. The fresh solution shows a nonspecific curve, with increasing absorbancy in

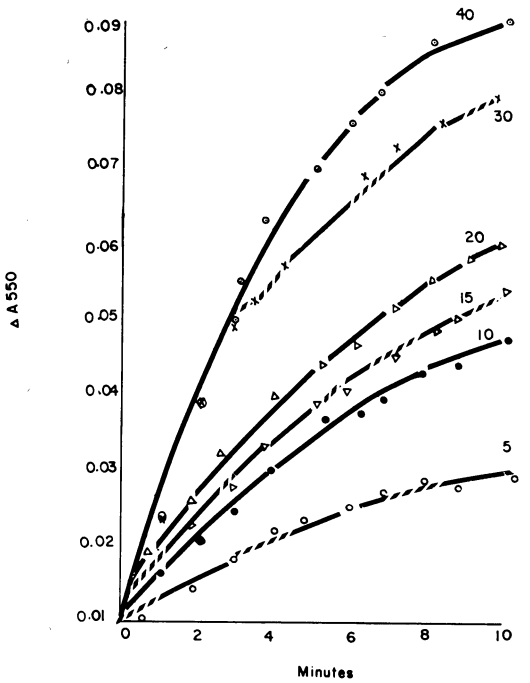

FIG. 1. *Kinetics of L-proline oxidase as a function of substrate concentration (mM of L-proline as indicated). The reaction mixture contained: cytochrome c, 0.02 mM; KCN, 0.33 mM; enzyme, 0.27 mg/ml; in potassium phosphate buffer, 0.05 M (pH 7.3).*

TABLE 4. *Chromatography of the 2,4-dinotrophenyl hydrazone of the products of L-proline oxidase activity**

Dinitrophenyl hydrazone of	Solvent	
	n-Butanol–acetic acid–water (77:4.5:18.5)	*n*-Butanol saturated with 6% ammonia
α-Ketoglutaric acid	0.64–0.82	0.16–0.18
Lactose	0.15–0.16	0
P5C (Δ′ pyrroline-5-carboxylic acid)	Front	0.46–0.48
Product of D-proline oxidase of sheep kidney, P2C(Δ′ pyrroline-2-carboxylic acid)	0.36–0.40	0.42
Product of L-proline oxidase of *Hansenula subpelliculosa*	0.34–0.38	0.41–0.43
2,4-Dinitrophenylhydrazine	Front	Front

* Conditions: room temperature, 5 hr; distance of migration of front, 6 cm. Results are expressed as R_F values.

the region of lower wavelengths. The spectrum of a solution aged at 4 C for 1 month has a peak at 280 mμ and a minimum at 260 mμ.

Reactions with NAD enzymes. The soluble fraction of cells of *H. subpelliculosa* has high activity for NAD^+ enzymes. These enzymes were active for the substrates, P2C from *H. subpelliculosa* and from the sheep kidney, and for the synthetic P5C. The soluble NAD^+ enzyme from this yeast was active upon the products of the yeast proline oxidase, when the oxidation digest was used directly without treatment (Fig. 3). However, the P2C from D-proline oxidase of sheep kidney must be isolated by paper chromatography before it can serve as substrate of the NAD^+ enzyme, because the digest appears to contain an inhibitor to this enzyme (Fig. 4). The graph for the activity of NAD^+ enzyme upon synthetic P5C is given in Fig. 5.

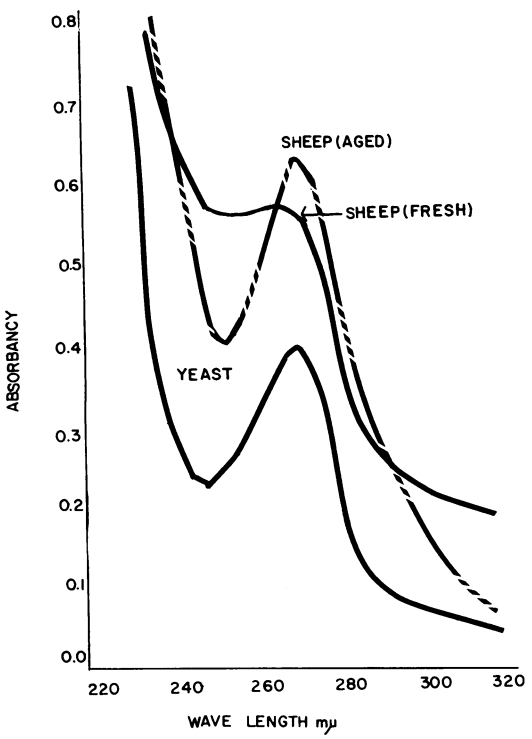

FIG. 2. *Spectra of the elutes of paper chromatography bands corresponding to the P2C products of sheep kidney* D-*proline oxidase or Hansenula subpelliculosa mitochondria* L-*proline oxidase activities on* DL-*proline and* L-*proline, respectively. The pH of the mixture was 8.6. Spectra of fresh and aged sheep kidney* D-*proline oxidase differ; the spectrum for yeast* L-*proline oxidase is the same for both fresh and aged preparations.*

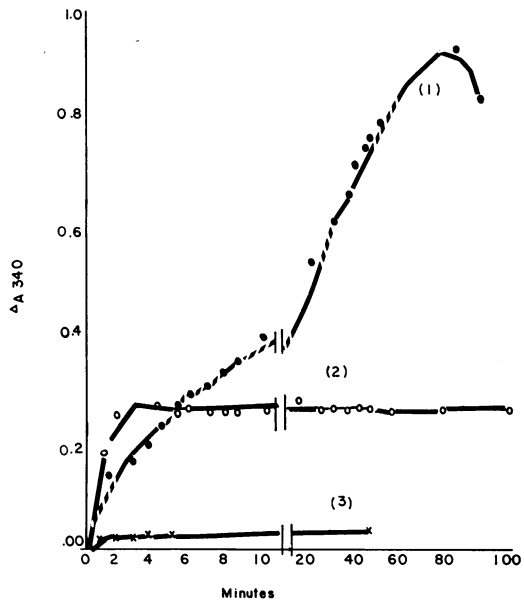

FIG. 3. *Soluble NAD^+ P2C-dehydrogenase of Hansenula subpelliculosa, with the use of (1) the product of the reaction of the* L-*proline oxidase from H. subpelliculosa as the substrate, (2) mitochondria control [same as (1), except no* L-*proline in the* L-*proline oxidase reaction]; and (3) endogenous reaction of the soluble enzyme. Reaction mixture in 1.0 ml: 0.3 mmoles of NAD^+; 8 μmoles of Na pyrophosphate buffer (pH 8.6); 0.092 mg of protein of the soluble enzyme; and about 0.7 μmoles of "oxidized* L-*proline" as the substrate. Specific activity = 0.49 μmoles of NADH per min per mg of protein.*

No glutamic dehydrogenases were detected under the same experimental conditions when L-glutamic acid and NAD^+ were supplied to the enzyme in the yeast extract.

DISCUSSION

The paper chromatographic experiments, chemical tests with H_2O_2, sensitivity to freezing, and spectral studies indicate that induced proline oxidase from *H. subpelliculosa* mitochondria forms a product from L-proline which is like, if not identical to, P2C formed by the action of D-proline oxidase from sheep kidney upon DL-proline.

Enzymatic studies with the substrate P2C from sheep kidney D-proline oxidase and synthetic P5C indicate that mitochondrial enzymes may

form either product, P2C or P5C. Furthermore, the soluble portion of the cell extract contains NAD^+ enzymes which use either P2C (α-keto-δ-amino-valeric acid) or P5C (glutamic-γ-semialdehyde) as substrates.

This study indicates that, with the conversion of P2C (α-keto-δ-amino-valeric acid) by NAD^+ enzymes to α-keto-valeric aldehyde, there is a corresponding release of ammonia. The aldehyde in the presence of the NAD^+ cofactor and soluble enzymes is converted to α-ketoglutaric acid. This, then, is available for use by the cell as a carbon source via the citric acid cycle.

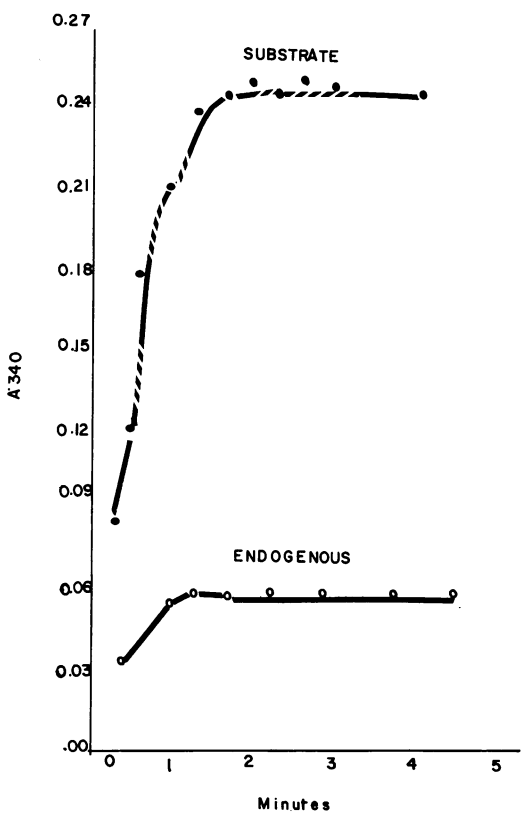

FIG. 4. *Soluble NAD^+ P2C-dehydrogenase, with P2C used as the substrate. P2C, the product of activity of sheep kidney* D*-proline oxidase on* DL*-proline, was isolated by paper chromatography. Corresponding isolates had spectra characteristic for the unaged product, as shown in Fig. 2. Assay mixture in 3.0 ml: 1.0 ml of the P2C eluate, 0.6 μmoles of NAD^+, 20 μmoles of sodium pyrophosphate buffer (pH 8.6), and 0.092 mg of the enzyme protein. Specific activity = 0.16 μmoles per min per mg of protein.*

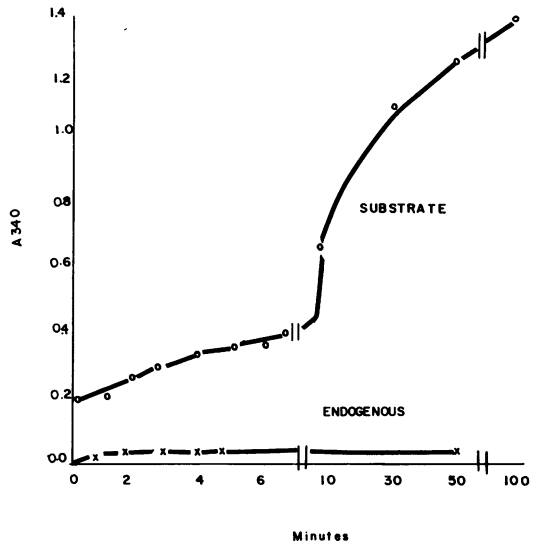

FIG. 5. *Soluble NAD^+ P5C-dehydrogenase of Hansenula subpelliculosa, with P5C as the substrate. Reaction mixture in 1.0 ml containing 0.23 mg of P5C: 8 μmoles of sodium pyrophosphate buffer (pH 8.6), and 0.3 μmoles of NAD^+. Specific activity = A_{340} 0.05 μmoles of NADH per min per mg of protein.*

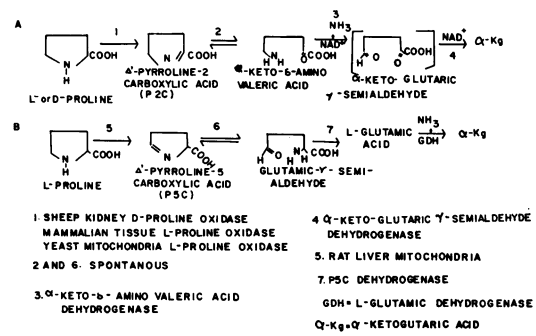

FIG. 6. *Diagrammatic sketch of postulated pathways for the use of* L*-proline by cells of Hansenula subpelliculosa. Pathway A: use of* L*-proline as carbon source. Pathway B: use of* L*-proline as nitrogen source.*

In the conversion of P5C (glutamic-γ-semialdehyde) by NAD^+ enzymes to L-glutamate, there is no release of ammonia. All the evidence accumulated in this and other studies in our laboratory indicate that the concentration of NAD^+ glutamic dehydrogenase is too low to convert glutamic acid into α-ketoglutaric acid and ammonia.

Other studies in our laboratory showed that the ratio of glucose carbon to ammonia nitrogen for assimilation and growth of this yeast strain is 40:1. Because the ratio of carbon to nitrogen in proline is 4:1, there is a great excess of nitrogen, which appears as ammonia.

We have demonstrated that cells of *H. subpelliculosa* grown upon either proline or ammonia as a nitrogen source have a very low level of proline oxidase (Table 3). This oxidase is most likely the P5C-forming kind, because the oxidase concentration remains low irrespective of the nitrogen substrate. The oxidase induced by use of L-proline as a carbon source is predominantly, if not entirely, the type which forms P2C.

This would suggest that the P5C-forming enzyme is constitutive and is associated with the use of L-proline as a nitrogen source, whereas the P2C-forming enzyme is only produced after the depletion of glucose or other available carbon source, and the yeast is thereby forced to employ proline as a carbon supply. This will account for the induction period of 2 to 3 hr in the diauxie type of growth curve when the glucose concentration is limiting.

Because the NAD^+ glutamic dehydrogenase concentration is not of sufficient concentration to split L-glutamic acid into ammonia and α-ketoglutaric acid, the ammonia released during the utilization of L-proline as a carbon source must come from the activity of α-keto-δ-amino-valeric acid dehydrogenase upon P2C (α-keto-δ-amino-valeric acid) to form ammonia and α-ketoglutaric acid. To our knowledge, this is the first report of this enzyme system.

A diagrammatic presentation of these pathways for the utilization of L-proline by cells of this yeast is given in Fig. 6.

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