stimulated the reaction whereas another (ADP) was a relatively weak inhibitor. But together, the two compounds caused considerable inhibition. This apparent paradox may be explained by considering the equilibrium conditions favoring the formation of dead-end complexes EIM and EIMS. Figure 8 shows that when the experimentally determined kinetic constants were used in eq 1 to calculate the inhibition of reaction as a function of ADP in the presence and absence of As_i or P_i, the calculated response pattern bears a close resemblance to that actually observed (Figure 2). In the presence of As_i or P_i the amount ADP required for 50% inhibition is considerably decreased and the response of the system to small changes in ADP concentration in this region is greatly accentuated. These observations suggest the possibility of achieving a finer type of control of reverse electron flow than that provided by the effect of the phosphate potential alone (Chance, 1961; Klingenberg, 1961).

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Protonated Amino Acid Precursor Studies on Rhodotorulic Acid Biosynthesis in Deuterium Oxide Media†

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ABSTRACT: *Rhodotorula pilimanae* CBS 4479 growing in 99.8% D_2O with protonated sucrose as carbon source was given various additional protonated substrates, the incorporation of which into rhodotorulic acid was examined. It was found that L-ornithine and δ-*N*-acetyl-L-δ-*N*-hydroxyornithine are intermediates on the rhodotorulic acid pathway. Indirect evidence was found that O_2 rather than H_2O is the source of the hydroxylamino oxygen. The assembly of the amino acids into the cyclic peptide apparently is similar to the scheme which recently has been established for gramicidin and tyrocidine. Arginine metabolism in *R. pilimanae* and *Saccharomyces cerevisiae* appear to be regulated by similar mechanisms.

If *Rhodotorula pilimanae* and related yeasts are grown in a low-iron medium, large amounts of a diketopiperazine dihydroxamic acid called rhodotorulic acid are produced (Atkin *et al.*, 1970). Similar but more complex compounds from other organisms have been shown by Emery (1971a) to act as iron-transporting agents. Two types of these compounds (hydrox-

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amates and phenolates) collectively called siderochromes, have been described in a recent review (Neilands, 1972). Initial studies on the biosynthesis of rhodotorulic acid involved the addition of ^{14}C-labeled amino acids to cultures of *R. pilimanae* followed by measurement of their incorporation into the acid. Labeled glutamate, glutamine, proline, arginine, or ornithine were rapidly converted to rhodotorulic acid, and afforded little information on the initial precursor of the molecule. To overcome this problem the metabolic source of the individual hydrogens, rather than the carbon skeleton, was studied. For this purpose *R. pilimanae* was adapted to grow in 99.8% D_2O on protonated sucrose, and at approximately one-half to two-thirds maximum growth (initial phase of rhodotorulic acid synthesis) protonated substrates were added to the medium. After about 15 days rhodotorulic acid was purified and the relative protonation was determined at each position by proton magnetic resonance (pmr).

Using this technique a variety of intermediates of arginine metabolism were examined as precursors of rhodotorullic acid. A number of conclusions were drawn regarding regulation of the metabolism of this amino acid; also, the similarities between arginine metabolism in *R. pilimanae* and *S. cerevisiae* are discussed. The initial precursor was shown to be L-ornithine and δ-*N*-acetyl-L-δ-*N*-hydroxyornithine[1] was demonstrated to be an intermediate on the rhodotorullic acid biosynthetic pathway. The similarities between the assembly of the monomer amino acids into rhodotorullic acid and the accepted scheme of gramicidin and tyrocidine biosynthesis are noted. The isotopic method has the advantage over conventional radioactive metabolic studies in that the pmr spectrum distinguishes between different hydrogens, thus eliminating the need for chemical degradation of the product to determine the specific activity at each position. Since rhodotorullic acid is produced in high yields and is easily crystallized from water it is particularly suited for this type of biosynthetic study.

Methods and Materials

Stock cultures were maintained on malt-agar slants. Crystalline rhodotorullic acid was obtained from low-iron cultures of *R. pilimanae* (Atkin and Neilands, 1968). D_2O-adapted cultures were maintained as liquid cultures and were transferred monthly. D_2O (99.8 atom % D) was purchased from Bio-Rad Laboratories, Richmond, Calif. Proton magnetic resonance spectra were recorded at 220 MHz on a Varian HR220 spectrometer using 20-mg samples of rhodotorullic acid in 0.5 ml of D_6-dimethyl sulfoxide (Merck, Sharp and Dohme) and tetramethylsilane as internal standard.

ASKP Medium. The medium normally used for growing *R. pilimanae* contains acetate and citrate, both of which contain carbon-bound hydrogens. Therefore, the following medium was used for all studies performed in D_2O: 2 g of ammonium sulfate (enzyme grade), 4 g of anhydrous K_2HPO_4, and 20 g of sucrose per l. (designated ASKP medium). Trace metals and thiamine were the same as normally used for *R. pilimanae* (Atkin and Neilands, 1968) or *Ustilago spaerogena* (Garibaldi and Neilands, 1955). H_2O stock solutions of trace metals and thiamine were evaporated to dryness and then reconstituted with D_2O. D_2O cultures were adjusted to pD 6.8 using concentrated H_3PO_4. Autoclaving caused a slight precipitation of metal salts.

Determination of Deuterium Concentration. The deuterium concentration was determined spectrophotometrically (Crespi and Katz, 1961) using commercial 99.8% D_2O as a standard. With a hot autoclave and tightly cotton-plugged flasks, the deuterium concentration of the medium after autoclaving was 98.5%. At the end of a typical 2- to 3-week experiment when the rhodotorulic acid was harvested, the deuterium concentration dropped to 97.0–96.5%.

Hydroxamate Assay and Hydroxylamine Spray. The standard spectrophotometric hydroxamate assay (Atkin and Neilands, 1968) was employed, utilizing a Beckman DU spectrophotometer. Up to 3 μmoles of hydroxamate was diluted to 3 ml with 5 mM $Fe(ClO_4)_3$–0.1 M $HClO_4$. In these conditions AcOHOrn was found to have a molar extinction coefficient, A_{505}, of 935 (total AcOHOrn determined by a recording titrator) (Neilands and Cannon, 1955). Both the wavelength of maximum absorbance and the intensity of absorption agree with previous reports for monohydroxamates (Seifter *et al.*, 1960). The A_{505} is almost one-half and one-third of the absorbancy coefficients for rhodotorulic acid (Atkins and Neilands, 1968) and ferrichrome (Emery, 1967), respectively. Hydroxylamines were detected by the use of a tetrazolium spray (Snow, 1954).

Adaptation of R. pilimanae to D_2O. Two 10-ml cultures of ASKP medium were prepared, one using 100% (99.8%) D_2O and the other 50% D_2O and 50% H_2O; both were inoculated from a malt-agar slant. Growth was observed in the 50% culture in about 4 days. One drop of the 50% culture was added daily to the 100% culture starting with day 7. After about 20 days' growth was observed in the 100% culture. This culture then served as an inoculum for other 100% D_2O cultures. After several transfers deuterated *R. pilimanae* grew at about one-half the rate of protonated *R. pilimanae*. Cell volume was greater in deuterated cultures than in protonated, owing to an increase in cell size. On a volume basis deuterated cultures produce about 20% of the rhodotorulic acid of protonated cultures. Deuterated *R. pilimanae* often adheres to the sides of the culture flasks. Also, there is a change in some aspect of carotenoid biosynthesis since cultures more than 80% deuterated fail to turn the typical orange-red color of mature protonated cultures but instead remain a dark pink.

Synthesis of δ-N-Acetyl-L-δ-N-hydroxyornithine. Rhodotorulic acid (10 g) in 100 ml of 6 N HCl was sealed in two 2 × 50 cm evacuated Carius tubes and hydrolyzed overnight at 100° to δ-*N*-L-hydroxyornithine (Atkin and Neilands, 1968). The hydrolysate was flash evaporated at 40° to an oil, dissolved in 100 ml of H_2O, and the pH adjusted to 4.3 with pyridine. Acetic anhydride (1 equiv; 5.5 ml) was added in small portions over 1 hr while the solution was kept at 50°. Then 0.1-ml portions of acetic anhydride were added until the hydroxamate content reached a maximum as measured at 505 nm in the hydroxamate assay. The reaction mixture was concentrated, acidified with glacial acetic acid, and placed on a 3 × 50 cm Dowex 50 column, which was in the hydrogen form. The column was developed using a linear gradient between 2 l. of 2 N acetic acid and 2 l. of 2 N pyridinium–acetate (pH 5.0). L-AcOHOrn was distinguished from α,δ-*N*,*N*-diacetyl-δ-*N*-hydroxyornithine and δ-*N*-hydroxyornithine peaks by their elution sequence, electrophoretic mobility (0.1 M pyridinium–acetate, pH 4.8), and ninhydrin, hydroxamate, and tetrazolium tests. The L-AcOHOrn was acidified with HCl and the pyridinium–acetate removed by repeated addition and evaporation of water. After passing through 3.5 × 110 cm Sephadex G-10 column with water as eluent, 6.63 g (60% of theoretical) of L-AcOHOrn was recovered. The hydrochloride form was crystallized from water by the slow ad-

[1] Abbreviation used is: AcOHOrn, δ-*N*-acetyl-L-δ-*N*-hydroxyornithine.

dition of ethanol. The biological activity of AcOHOrn was tested using enb mutants of *Salmonella typhimurium* (Pollack *et al.*, 1970).

Synthesis of DL-*AcOHOrn.* Rhodotorulic acid (10 g) was hydrolyzed as above and the product racemized by refluxing for 2 hr in a solution containing 100 ml of glacial acetic acid and 50 ml of acetic anhydride. The resulting ninhydrin-negative material was decolorized with charcoal and flash evaporated to an oil. An attempt was made to resolve (Greenstein, 1957) a portion of this compound with porcine kidney acylase (Calbiochem); however, no ninhydrin-positive material was produced. The remainder of the diacetyl-DL-hydroxyornithine was hydrolyzed, acetylated, and purified by use of the same procedures applied to L-AcOHOrn. The method of Greenstein and Winitz (1961) was employed for the synthesis of δ-*N*-acetyl-L-ornithine. Argininosuccinic acid and L-α,γ-diaminobutyric acid·2HCl were purchased from Sigma Chemical Co. All other chemicals were from regular commercial sources.

Molecular Rotation of L-*AcOHOrn.* AcOHOrn (100 mg) in 2 ml of 50% HI was sealed in an evacuated Carius tube and hydrolyzed at 100° for 15 hr. The hydrolysate was flash evaporated at 40° to an oil and the HI and I_2 removed by repeated additions and evaporations of small amounts of 0.1 N HCl. A duplicate sample was prepared to contain 100 mg of a specimen of commercial ornithine which has previously been shown by optical rotatory dispersion to contain greater than 98% of the L isomer. The samples were diluted to 5.0 ml with 1 N HCl, the ornithine content determined (Chinard, 1952), and the optical rotatory dispersion spectrum recorded with a Cary 60 instrument. No racemization of L-ornithine was observed in this process. The ornithine from L-AcOHOrn was determined to be 0.856 L isomer. From these data the molecular rotation for the L isomer of AcOHOrn was determined to be $[M]^{20}_{400} = +130°$ and $[M]^{20}_{350} = +172°$ in 1 N HCl. Using these molecular rotations the partially racemized preparation of AcOHOrn was found to be 0.670 L isomer.

Purification of Deuterated Rhodotorulic Acid. The D_2O cultures were centrifuged and the medium flash evaporated at 40° to an oil. The oil was dissolved in 50 ml of water and extracted twice with 100 ml of $CHCl_3$–phenol (1:1 weight basis). The rhodotorulic acid was reextracted into water after the addition of about 500 ml of ether to the $CHCl_3$–phenol. The water phase was concentrated to about 5 ml, acidified with acetic acid, and applied to a 1.5 × 40 cm Dowex 50 column in the hydrogen form. Rhodotorulic acid was eluted with 2 N acetic acid, and hydroxamate-positive fractions were pooled and the acetic acid removed by repeated additions and evaporation of water. The rhodotorulic acid was dissolved in the least possible amount of water and placed on a 3.5 × 110 cm Sephadex G-10 column using water as an eluent. The rhodotorulic acid was dried *in vacuo* over P_2O_5. Using this procedure quantities as small as 35 mg were recovered.

Recovery of AcOHOrn. In the cultures containing exogenous AcOHOrn the rhodotorulic acid and unconsumed AcOHOrn were recovered by placing the acidified, concentrated culture medium directly on a 1.5 × 40 cm Dowex 50 column. The column was developed with a linear gradient between 250 ml of 2 N acetic acid and 250 ml of 2 N pyridinium–acetate (pH 4.8). Two hydroxamate-positive peaks were observed: the first to appear was rhodotorulic acid, while AcOHOrn was retarded. Both fractions were further purified by removing the pyridinium–acetate by repeated evaporation from water and gel filtration as described above for rhodotorulic acid.

General Procedures. In a typical experiment 100–170 ml of D_2O ASKP medium was inoculated with 1–2 ml of D_2O-adapted culture and placed on a rotary shaker at 30°. After about 4 days 1 ml of the culture was withdrawn and assayed for hydroxamate concentration. At the same time the protonated supplements were added. Enough supplement was added to make the culture 20 mM except for argininosuccinate, L-AcOHOrn, and DL-AcOHOrn which were added at a level of 5.0, 13.4, and 6.65 mM, respectively. After a total incubation period of 2–3 weeks the rhodotorulic acid was purified from the culture. The greatest amount of packed-cell volume with the smallest amount of rhodotorulic acid produced occurred by day 4.

The D_2O medium in tightly cotton-plugged erlenmeyer flasks was autoclaved using a preheated autoclave for 10 min at 120°. Using this procedure there was little protonation of the D_2O. The supplements were dissolved in about 10 ml of D_2O and the pD adjusted to 6–8 with concentrated H_3PO_4 or KOH (in D_2O) prior to sterilization. Samples of the amino acid supplements were analyzed by electrophoresis at pH 4.8 (0.1 M pyridinium–acetate), pH 7.0 (0.1 M phosphate), and pH 10.0 (0.1 M carbonate) before and after sterilization. Electrophoretograms were developed with ninhydrin to determine if any new amino-containing compounds were produced by the autoclaving procedure. In addition, samples of argininosuccinate before and after autoclaving were chromatographed in phenol–H_2O (100:20, w/w), a system which can resolve argininosuccinate and its anhydride form (Ratner, 1957). No new ninhydrin-positive products were observed.

Calculation of Data from Proton Magnetic Resonance Spectra. Proton magnetic resonance peak assignments for rhodotorulic acid have been reported previously (Atkin and Neilands, 1968). In reduced rhodotorulic acid's (Atkin and Neilands, 1968) pmr spectrum (Figure 1) the β- and γ-methylene hydrogens are resolved (Figure 1) and have been assigned (Llinás, 1971). Due to low yields in the reduction and limited solubility in dimethyl sulfoxide, the spectra of the reduced rhodotorulic acid species were not determined. Since the rhodotorulic acid samples were purified from water, and dimethyl sulfoxide was used as a solvent, the amide NH and hydroxamate NOH resonances appear as distinct peaks. However, due to the acidic nature of the hydroxamate group its resonance is broadened due to exchange. So, the amide NH area was used as a standard by defining it as one.

As the instrument's integrator was unreliable it was necessary to integrate the area under each peak by cutting it out and weighing the paper. Three expanded-scale copies of each spectrum were made and each paper cutout was weighed three times to the nearest 0.1 mg. Most weight measurements were in the 10- to 150-mg range. The average of the nine measurements for each peak was divided by the number of hydrogens of the type of rhodotorulic acid and normalized to the ring amide proton to give $[H]_{obsd}{}^{i}$ ($i = \alpha,\beta + \gamma,\delta$ or CH_3). Using this procedure and a protonated rhodotorulic acid sample, numbers within ±0.015 of the expected value of 1.000 were obtained. The variations in the weight (120 mg) of five 25 cm^2 samples of the paper used for recording the spectra was ±0.8 mg.

When an exogenous substrate was supplied a correction was required for the endogenous production of rhodotorulic acid. The fraction (X) of the rhodotorulic acid which resulted from exogenous substrate was determined by assuming the following. (1) The fraction of protonation ([H]) at each carbon for endogenously (endog) produced rhodotorulic acid is the same as that when no exogenous substrate is supplied (see Discussion); and (2) no H–D exchange for the non-α-hydro-

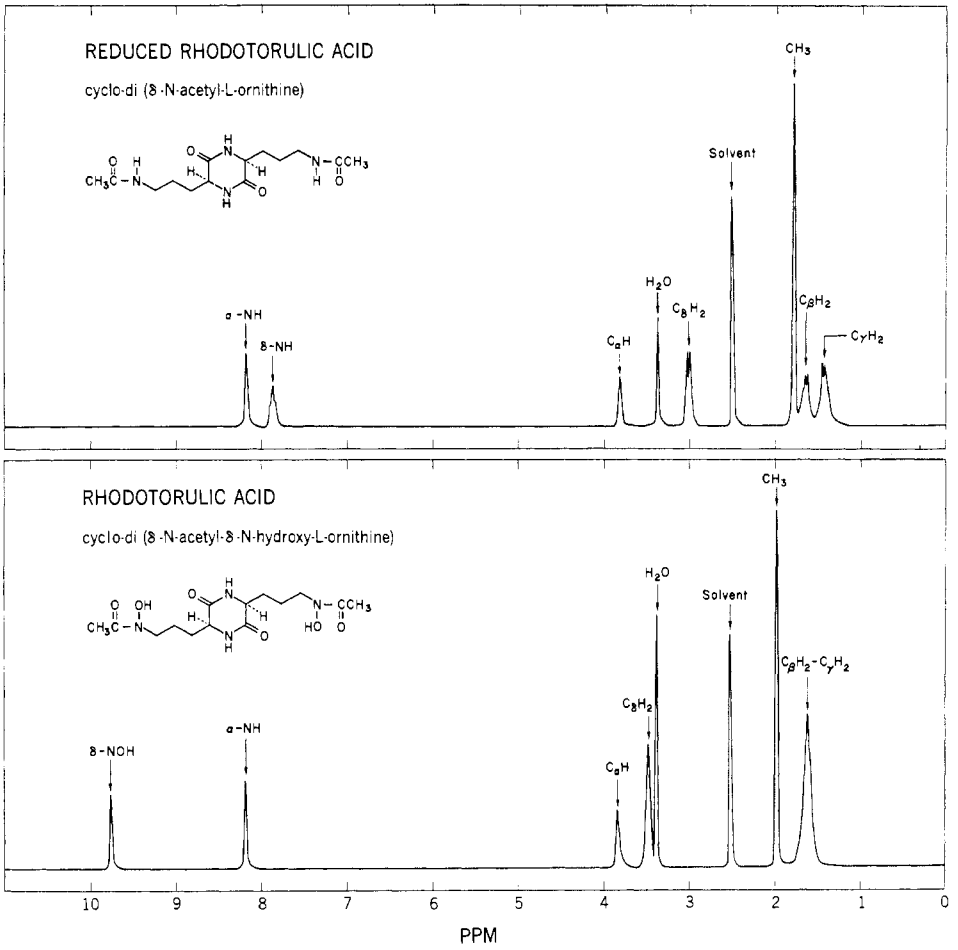

FIGURE 1: The proton magnetic resonance spectra of protonated rhodotorulic acid and reduced rhodotorulic acid in D_6-dimethyl sulfoxide. The ppm are relative to tetramethylsilane.

gen aliphatic hydrogens of the exogenous (exog) substrate, *i.e.*, $[H]^i_{exog} = 1.000$ for $i = \beta + \gamma,\delta, CH_3$. The experimental X^i values were then calculated

$$X^i = \frac{[H]^i_{obsd} - [H]^i_{endog}}{1.000 - [H]^i_{endog}}$$

The X^i values were averaged (see column A, Table I) to give X if the exogenous substrate had the hydrogens of the type considered. The $i = \alpha$ values were not considered in computing X. The $[H]_{exog}{}^i$ can be computed based on:

$$[H]^i_{exog} = \frac{[H]^i_{obsd} - [H]^i_{endog}(1 - X)}{X}$$

Results

Table I lists the relative retention (X) of protons from exogenous substrates furnished for the biosynthesis of rhodotorulic acid. The per cent of rhodotorulic acid derived from the exogenous substrate (see Methods), when present, is also listed. Corrections have been made for the endogenous production of rhodotorulic acid. The substrate for the L-AcOHOrn experiment was actually 85.6% L isomer while that for the DL-AcOHOrn experiment was 67.0% L isomer.

The relative protonation of the endogenous production of rhodotorulic acid is recorded in Table II. D-Alanine, D-ornithine, D-arginine, α-acetyl-L-glutamate, α-acetyl-L-ornithine, L-lysine, and argininosuccinate were also tried as exogenous substrates; however, they were not incorporated, as the relative protonation of the rhodotorulic acid recovered in these experiments was similar to the "none" values.

In the experiments involving AcOHOrn, the AcOHOrn that was not converted into rhodotorulic acid was recovered and its isomeric composition determined. The values observed are recorded in Table III. The AcOHOrn recovered from the DL-AcOHOrn experiment had a $[M]^{20}_{400}$ of $-140°$ indicating that it was completely D isomer. This D isomer was as active or more active on a molar basis than AcOHOrn that was 85.6% L isomer in supporting the growth of a number of siderochrome-requiring mutants of *Salmonella typhimurium*.

Protonated exogenous amino acids were added to aliquots of a protonated ASKP culture initially 0.94 mM in rhodotorulic acid. After 48-hr exposure to the exogenous amino acid the rhodotorulic acid concentration was again determined (Figure 2). The amino acid additives for this experiment were prepared in the same manner as the deuterated cultures except that H_2O was used instead of D_2O.

Discussion

Precursors of Rhodotorulic Acid. The data in Table I show that the two δ protons of exogenous arginine, citrulline, and ornithine both appear in the rhodotorulic acid produced when these substrates are present, indicating that one of these is the initial substance on the biosynthetic pathway to rhodotorulic

TABLE I: Relative Retention of Exogenous Protons into Rhodotorulic Acid.[a]

Exogenous Substrate	α	$\beta + \gamma$	δ	CH_3	A	%RA[b] from Exogenous Source
L-Glu	0.035 ± 0.005	1.000 ± 0.008	0.412 ± 0.020	0.010 ± 0.003	$\beta + \gamma$	25
L-Gln	0.050 ± 0.022	1.000 ± 0.030	0.290 ± 0.006	0.006 ± 0.006	$\beta + \gamma$	23
L-Pro	0.038 ± 0.009	1.000 ± 0.038	0.235 ± 0.049	−0.016 ± 0.034	$\beta + \gamma$	39
L-Cit	0.344 ± 0.015	1.010 ± 0.036	0.989 ± 0.051	0.061 ± 0.002	$\beta + \gamma,\delta$	106
L-Arg	0.547 ± 0.010	1.070 ± 0.005	0.938 ± 0.007	0.012 ± 0.002	$\beta + \gamma,\delta$	100
L-Orn	0.650 ± 0.009	1.036 ± 0.031	0.968 ± 0.024	−0.002 ± 0.004	$\beta + \gamma,\delta$	88
L-AcOHOrn	0.638 ± 0.012	0.984 ± 0.013	0.946 ± 0.009	1.066 ± 0.079	$\beta + \gamma,\delta,CH_3$	91
DL-AcOHOrn	0.596 ± 0.029	0.972 ± 0.005	1.012 ± 0.011	1.032 ± 0.013	$\beta + \gamma,\delta,CH_3$	88

[a] The relative incorporation into rhodotorulic acid of protons from the exogenous substrates listed. Values are relative to the proton type (or average of types) listed in column A. The figures have been corrected for endogenous production (blank values of Table II). The per cent of the rhodotorulic acid derived from the exogenous material, while present, is also shown. The L-AcOHOrn and DL-AcOHOrn were actually 85.6 and 67.0% L isomer, respectively. The tolerance figures listed are the standard deviation of the mean. These deviations are not meant to indicate the accuracy of the experiments, which is not greater than 1.5% (see Methods and Material section), but rather to show the precision of the measurements. [b] RA = rhodotorulic acid.

TABLE II: Relative Protonation of Rhodotorulic Acid.[a]

	i =			
Exogenous Substrate	α	$\beta + \gamma$	δ	CH_3
None (blank)	0.026 ± 0.001	0.076 ± 0.002	0.085 ± 0.002	0.079 ± 0.002
Acetate	0.199	0.064	0.078	0.113
α-Ketoglutarate	0.080	0.273	0.161	0.719

[a] The protonation of the rhodotorulic acid produced in deuterated cultures, relative to amide NH = 1.000. The deviations listed are the same type as in Table I.

acid. If proline, glutamine, or glutamate were the initial precursor of rhodotorulic acid one of the δ hydrogens from arginine, citrulline, or ornithine would be lost during the formation of glutamic semialdehyde and the second would be eliminated during the synthesis of glutamate.

The regular loss of α protons in the sequence citrulline → arginine → ornithine indicates that ornithine, rather than any other urea cycle intermediate, is the initial substance in rhodotorulic acid biosynthesis. This does not exclude the possibility of α-acetylornithine as the initial rhodotorulic acid precursor. However, three lines of evidence indicate that ornithine rather than α-acetylornithine is the initial precursor of rhodotorulic acid biosynthesis. (1) The possible distinction made in *R. pilimanae* between endogenous and exogenous ornithine (see below) with exogenous ornithine not available for arginine biosynthesis (or α-acetylornithine formation). (2) The interference of rhodotorulic acid formation by the ornithine analogs, lysine and α,γ-L-diaminobutyrate (Figure 2). (3) The reversibility of acetylornithine-δ-transaminase (Albrecht and Vogel, 1964), which would labilize one of the δ hydrogens.

Presumably, the α protons are lost due to Schiff-base formation with pyridoxal enzymes. However, ornithine and arginine decarboxylases would not labilize the α proton (Mandeles *et al.*, 1954). As AcOHOrn is a precursor of rhodotorulic acid (see below), the α-hydrogen data for ornithine and AcOHOrn (Table I) indicate that little if any α proton is lost at the ornithine level. A similar observation has been made with [α-^{15}N,α-^{2}H]lysine in intact rats (Clark and Rittenberg, 1951) indicating a noninvolvement of α,ω-diamino acids with pyridoxal enzymes. D-Ornithine has been reported on several occasions thus implicating an ornithine racemase in a number of organisms (Guinand *et al.*, 1969; Tsuda and Friedmann, 1970). An arginine racemase is known in *Pseudomonas graveolens* (Yorifuji *et al.*, 1971) but as this enzyme also catalyzes the racemization of ornithine it probably is not present in *R. pilimanae*. Initial Schiff-base formation of arginine or citrulline with pyridoxal is sufficient to promote exchange of the α proton independently of racemization or transamination (Snell and DiMari, 1970). The hope was to determine the relative retention of the α protons for all the urea cycle intermediates but, unfortunately, exogenously supplied argininosuccinate failed to appear in rhodotorulic acid. Using the α-proton values for citrulline and arginine it can be determined (exclusive of isotope effects) that a total of 37% of the exogenous citrulline loses the α proton at the citrulline and/or argininosuccinate level. Similarly, 16% of exogenous arginine α proton is lost before conversion to ornithine. The carbon skeleton of exogenous proline and glutamine reach ornithine *via* glutamate, at which point they are involved as cosubstrates for many transaminases, thereby losing the α proton. Labilization of the α proton might accompany active transport of

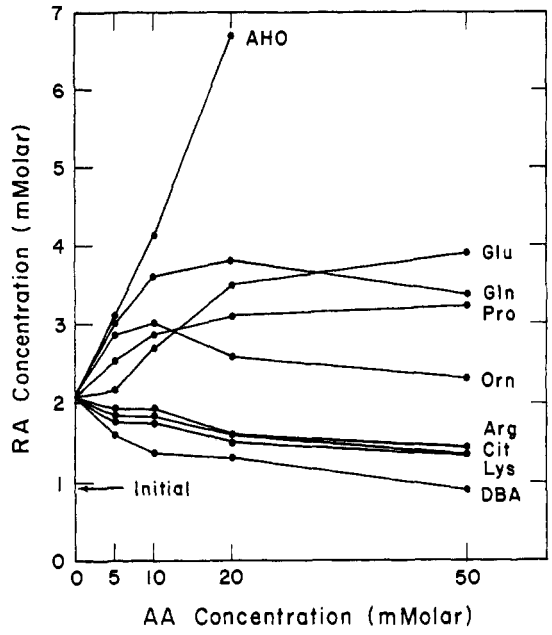

FIGURE 2: The effect of exogenous AA on the production of rhodotorulic acid. Exogenous AA were added to aliquots of a protonated culture initially 0.94 mM in rhodotorulic acid. After 48 hr the rhodotorulic acid concentration was again determined. All amino acids were the L isomer except AcOHOrn (AHO) which was 85.6% L isomer. DBA = α,γ-diaminobutyrate.

amino acids; however, this possibility has been excluded by Kessel and Lubin (1965).

As both of ornithine's δ protons are preserved in rhodotorulic acid, the possibility of a biosynthetic intermediate unsaturated at the δ carbon can be eliminated. This suggests that

TABLE III: Material Balance for Rhodotorulic Acid Production from AcOHOrn in D_2O.[a]

	AcOHOrn Expt "DL"	AcOHOrn Expt "L"
(A) Rhodotorulic acid present when AcOHOrn added	84	81
(B) AcOHOrn added (L isomer)	565 (378)	1140 (976)
(C) Total rhodotorulic acid present at end of experiment	578	1367
(D) AcOHOrn recovered (L isomer)	87 (0)	71 (11)
(E) Per cent of total rhodotorulic acid from exogenous AcOHOrn	75.0	79.8
Rhodotorulic acid from exogenous = (C)(E)	434	1090
AcOHOrn consumed = (B) − (D)	478	1069

[a] The material balance for rhodotorulic acid production from AcOHOrn in D_2O. In the experiments involving additions of AcOHOrn to deuterated cultures the unconsumed AcOHOrn was recovered. Figures in parentheses are amounts of L isomer of AcOHOrn. Percentages at row E are X values (see Methods and Materials). Figures are μmoles for and μmoles/2 for AcOHOrn.

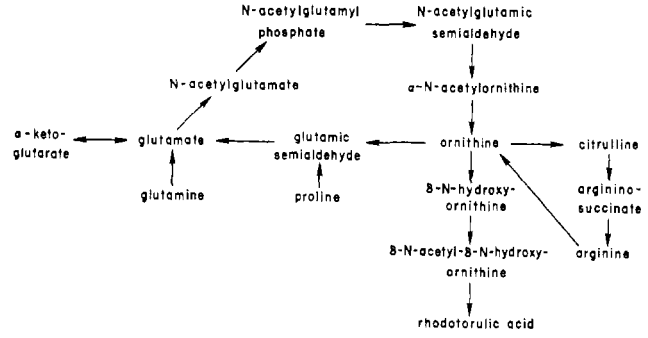

FIGURE 3: The accepted scheme of arginine metabolism. The proposed rhodotorulic acid biosynthetic pathway is shown.

O_2 rather than water is the source of the oxygen in the hydroxylamino group, a finding compatible with studies on the biosynthesis of hadacidin (Stevens and Emery, 1966).

As the $\beta + \gamma$, δ, and methyl protons of AcOHOrn are incorporated into rhodotorulic acid in a 1.00:1.00:1.07 ratio (average of two experiments) it can be concluded that AcOHOrn is not hydrolyzed to acetate and hydroxyornithine and then reassembled, but rather the hydroxamic acid containing amino acid (AcOHOrn) of rhodotorulic acid is synthesized before incorporation into the cyclic dipeptide. If AcOHOrn were hydrolyzed and reassembled the acetate would first mingle with endogenously produced deuterated acetate. As most of the rhodotorulic acid is protonated (~90% from exogenous AcOHOrn) the reassembled AcOHOrn would have a lower acetyl to hydroxyornithine protonation ratio than the unhydrolyzed case. Simultaneously, the acetate would have a higher than endogenous protonation and its condensation with oxaloacetate would eventually lead to ornithine with a higher than normal endogenous protonation at the γ position. Both of these effects would distort the 1:1:1 ratio observed. As more than 50% of the rhodotorulic acid was formed from exogenous AcOHOrn, rhodotorulic acid arises from identical monomers. Interestingly, 2,3-dihydroxy-*N*-benzoyl-L-serine is not a precursor of enterobactin, a representative of the other major type of iron-transport compound (Bryce *et al.*, 1971).

Emery (1966) studied the incorporation of AcOHOrn labeled with ^{14}C in the acetyl and hydroxyorthine portions, into ferrichrome compounds. He concluded that in ferrichrome, as in rhodotorulic acid, the hydroxamic acid portion is formed before the amino acids are cyclized. As Emery has shown that hydroxyornithine is a precursor of ferrichrome, the biosynthesis of rhodotorulic acid probably proceeds from ornithine to δ-hydroxyornithine to AcOHOrn, which is then assembled into the cyclic dipeptide (Figure 3).

The possibility that δ-*N*-acetyl-L-ornithine is a precursor of rhodotorulic acid and the report of the natural occurrence of this compound (Brown and Fowden, 1966) prompted the investigation of δ-*N*-acetyl-L-ornithine in this system; however, no incorporation of protons into rhodotorulic acid was observed. As this substance is a free amino acid, *R. pilimanae* should be able to accumulate δ-*N*-acetyl-L-ornithine. A possible reason incorporation is not observed is because *R. pilimanae* does not hydrolyze δ-*N*-acetyl-L-ornithine or convert it into AcOHOrn. Emery (1966) also concluded that this compound is not a precursor of the AcOHOrn groups in ferrichrome. He described (1971b) a δ-acetylating enzyme which uses δ-*N*-hydroxyornithine, not ornithine, as a substrate. The amide form was found not to be a precursor of hadacidin

(Stevens and Emery, 1966). However, MacDonald (1965) has reported N-hydroxylation of a diketopeperazine precursor of aspergillic acid, and ϵ-*N*-hydroxylysine does not appear to be an intermediate in the formation of mycobactins P and S (Tateson, 1970). Apparently, two methods of forming hydroxamic acids have evolved.

The lack of incorporation of α-acetylglutamate and α-acetylornithine into rhodotorulic acid is not sufficient evidence to indicate that *R. pilimanae* does not use these intermediates in the biosynthesis of ornithine. Other organisms have been reported which lack the ability to transport these substances but still use them as intermediates (Prozosky, 1967; and references therein). The failure of argininosuccinate to be incorporated may be due to similar reasons, as argininosuccinate lyase mutants of *Proteus mirabilis* are unable to grow on argininosuccinate (Prozosky, 1967).

The high level of protonation of the δ position from exogenous glutamate, glutamine, or proline is unexplained since glutamate and glutamine do not originally have δ hydrogens and those of proline are lost in conversion to glutamate. Little if any glutamic semialdehyde is converted to ornithine *via* ornithine-δ-transaminase, an essentially irreversible reaction (Strecker, 1965). Also, the α hydrogen of proline is preserved in this conversion. If *R. piliminae* is considered to use the accepted acetylated pathway for ornithine synthesis (see Figure 3 and below) one of the hydrogens originates during a transamination and the second from NADPH. Although Dunathan (1970) has observed transfer of the α hydrogen to the amino acceptor during transamination (δ on rhodotorulic acid), a more probable source of the δ protonation is from NADPH arising from metabolism of proline, glutamine, or glutamate, as α-ketoglutarate produces rhodotorulic acid with more than endogenous protonation (Table II).

Exogenous acetate appears to a greater extent in the acetyl groups than in the γ position (*via* citrate synthetase and ornithine synthesis). The acetate consumed in the citric acid cycle may be segregated from the acetate used in the acetylation step of rhodotorulic acid biosynthesis. The high level of protonation of the α position may be due to the formation of protonated NADPH during the metabolism or the exogenous acetate. An examination of the methyl column in Table I shows that there is little perturbation when exogenous substrates are present, *i.e.*, the values are near zero with non-acetyl-containing exogenous substrates. This indicates there is no significant change in the metabolic source of the acetyl groups, justifying the assumption made above (see methods and materials).

In *Rhodotorula glutinis* it is known that pyruvate will only be utilized after glucose is completely exhausted (Medrano *et al.*, 1969). A similar situation with acetate would explain the low level of incorporation of acetate into rhodotorulic acid.

Arginine Metabolism and Regulation in R. pilimanae. The pathways shown in Figure 3 involving arginine metabolism (excluding the δ-*N*-hydroxyornithine branch) have been partially investigated and thought to exist in a number of fungal genera, namely, *Saccharomyces* (Middelhoven, 1964; DeDeken, 1962), *Aspergillus* (Piotrowska *et al.*, 1969), *Candida* (Middelhoven, 1963), *Blastocladiella* (Smith and Holmes, 1970), *Neurospora* (Castañeda *et al.*, 1967), and *Ustilago* (Prieur, 1971). They are assumed also to be the major pathways of arginine metabolism in *R. pilimanae*.

Arginine is known to regulate its own biosynthesis in a number of organisms (Prozosky, 1969; Udaka, 1966; DeDeken, 1962). A similar situation probably exists in *R. pilimanae*, since when arginine is present in the medium all the rhodotorulic acid synthesized comes from exogenous arginine (Table I). This would be the case if exogenous arginine prevented the endogenous synthesis of arginine, as the only source of ornithine would be from the breakdown of exogenous arginine.

When citrulline is present all of the rhodotorulic acid is synthesized from the exogenous material (Table I). This could be due to intracellular conversion of exogenous citrulline to arginine, as citrulline does not regulate α-*N*-acetylglutamic reductase in *S. cerevisiae* (DeDeken, 1962). The arginine produced from citrulline could now prevent the endogenous production of ornithine from glutamate. This suggests that argininosuccinate synthetase and argininosuccinate lyase are not repressed by arginine, but that the regulation of arginine biosynthesis in *R. pilimanae* may be by feedback inhibition rather than *via* the coordinate operon control model. Middelhoven (1969) observed that argininosuccinate lyase in *S. cerevisiae* was not repressed by exogenous arginine. Unfortunately, argininosuccinate synthetase was not examined.

Decrease in synthesis of rhodotorulic acid (Figure 2) when arginine or citrulline is supplied exogenously could be explained by a decrease in the availability of ornithine, as in *S. cerevisiae* (Middelhoven, 1970) and *Aspergillus nidulans* (Piotrowska *et al.*, 1969) ornithine transaminase and arginase are coordinately induced by high levels of arginine. Ramos *et al.* (1970) found that the internal ornithine concentration in *S. cerevisiae* is lower when arginine is added to their ammonium-containing medium. Citrulline was not tested.

When the ornithine analogs lysine or α,γ-L-diaminobutyrate are added to *R. pilimanae* cultures (Figure 2) there is a decrease in the production of rhodotorulic acid. In *S. cerevisiae* high levels of exogenous lysine decrease the internal ornithine level (Ramos *et al.*, 1970), explaining the lower rhodotorulic acid production. Another possibility is that lysine and α,γ-L-diaminobutyrate, inhibit the initial hydroxylation enzyme since the acetylation enzyme is known to use either ϵ-hydroxylysine or δ-hydroxyornithine as a substrate (Emery, 1971b).

As there is no loss of α protons at the ornithine level for exogenous ornithine (see above), it can be concluded that a significant portion of the exogenous ornithine is not touring the urea cycle to be re-formed into ornithine. If this were the case α protons would be lost at the arginine and citrulline and/or argininosuccinate stage. This could be due to the repression of ornithine transcarbamylase by high levels of exogenous ornithine, which is known to occur in *S. cerevisiae* (Ramos *et al.*, 1970). Another possible explanation is that for the synthesis of rhodotorulic acid a distinction is made between endogenous and exogenous ornithine. Compartmentalization of ornithine is known in *Neurospora crassa*, as exogenous ornithine is degraded by ornithine transaminase while endogenous ornithine (possibly mitochondrial) is used mainly for the synthesis of arginine (Davis, 1968). A distinction between the two types of ornithine would suggest that the arginine biosynthetic enzymes are segregated from the initial enzyme on the rhodotorulic acid pathway.

When 20 mM exogenous ornithine is supplied to deuterated *R. pilimanae* cultures 88% of the rhodotorulic acid produced is derived from the exogenous ornithine (Table I). The remaining rhodotorulic acid is derived from endogenous ornithine, whose synthesis was not prevented by this high level of exogenous ornithine. Again, a similar observation has been made in *S. cerevisiae*, where *N*-acetylglutamic acid reductase is feedback inhibited by arginine but not by ornithine. Arginine is known to repress the synthesis of this enzyme in *S.*

cerevisiae, but ornithine was not examined (DeDeken, 1962). In general, the regulation of arginine metabolism appears to be the same in *R. pilimanae* and *S. cerevisiae*.

Similarly, since endogenous AcOHOrn is produced in the presence of 13.4 mM exogenous AcOHOrn it is concluded that the biosynthesis of AcOHOrn is not regulated rigidly by AcOHOrn. The high level of production of rhodotorulic acid from AcOHOrn (Figure 2) indicates that the rate-determining step in rhodotorulic acid biosynthesis precedes AcOHOrn formation. When cultures are shaken vigorously they produce more rhodotorulic acid and hydroxylation may be the rate-limiting step. Another possibility is that the enzymes in the pathway are sequentially induced and a high level of AcOHOrn induces the cyclization enzymes.

The appearance of protons in the acetyl group of rhodotorulic acid when α-ketoglutarate was the exogenous substrate (Table II) can be explained by the reversal of the citric acid cycle. The two protons originally on the γ position of α-ketoglutarate would appear in the methyl group of acetyl-CoA by using the citrate cleavage enzyme (Atkinson, 1969). Since this enzyme exists extramitochondrially (Srere, 1959) and since the acetylating enzyme uses acetyl-CoA as a substrate (Emery, 1971b), the high incorporation of the protons of α-ketoglutarate into the acetyl portion of rhodotorulic acid, and the possibility of two ornithine pools suggests that the biosynthesis of rhodotorulic acid does not occur in the mitochondria.

AcOHOrn Racemase. Table III shows that *R. pilimanae* preferentially consumed the L isomer of the AcOHOrn in the biosynthesis of rhodotorulic acid. However, since there is not enough L-AcOHOrn initially present in the exogenous substrate to account for the amount of rhodotorulic acid produced, some D isomer must have been converted into rhodotorulic acid. The rhodotorulic acid produced under these conditions is possibly an LD- or DD-diketopiperizine. But, as three chromatography solvent systems (Kopple and Ghazarian, 1968) known to separate DL- and LL-diketopiperizines failed to resolve the rhodotorulic acid from either the L- or DL-AcOHOrn experiment, *R. pilimanae* apparently has the capacity to racemize AcOHOrn. Ferribactin, from *Pseudomonas* fluorescens, contains the D form of δ-*N*-hydroxyornithine (Maurer *et al.*, 1968). The presence of an AcOHOrn racemase would explain the observed labilization of the α hydrogen at the monomer level. Because other D-amino acids were not incorporated into rhodotorulic acid, *R. pilimanae* may not have a specific D-amino acid transporting system. However, D-AcOHOrn may enter *R. pilimanae* as a metal hydroxamate *via* a transport system that does not specifically examine the configuration at the α carbon, as D-AcOHOrn was observed to satisfy a siderochrome requirement of mutant *Salmonella typhimurium*.

Cyclic Peptide Formation. The incorporation of D-AcOHOrn and the labilization of the α hydrogens are consistent with recent developments in the synthesis of the cylic decapeptides gramicidin and tyrocidine (Kleinkauf *et al.*, 1971; and references therein). The initial enzyme for tyrocidine assembly has been shown to be an ATP-dependent racemase, which can activate either D- or L-phenylalanine. In addition, aminoacyl-pantotheine intermediates have been shown to be involved in the assembly process. The labilization of the α hydrogen of thio esters is well known (Bruice and Benkovic, 1966). By analogy to the cyclic decapeptide scheme the rhodotorulic acid assembly system should consist of an initial enzyme which is capable of activating L- or D-AcOHOrn. On this enzyme the D-AcOHOrn is labilized. The second rhodotorulic acid assembly enzyme (cyclizer) will also activate AcOHOrn and should contain a pantothenic acid moiety that is involved in amide-bond formation. The α hydrogens of two AcOHOrn monomers which are assembled into a single rhodotorulic acid molecule have both been shown to be labile at some phase in the biosynthesis (H. Akers and J. B. Neilands, manuscript in preparation), an observation consistent with the above predictions, as both monomers are thio esters at one stage of biosynthesis. Work is in progress to isolate the enzyme system involved in the synthesis of rhodotorulic acid and the cyclic hexapeptide ferrichrome.

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Adsorbents for Affinity Chromatography. Use Of *N*-Hydroxysuccinimide Esters of Agarose†

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ABSTRACT: Procedures are described for the preparation of the *N*-hydroxysuccinimide ester of succinylated aminoalkyl agarose derivatives. These active ester derivatives of agarose are stable for months when stored in dioxane. The reaction of the active ester of agarose with various amino acids and with trypsin has been studied in detail. Stable amide linkages are formed very rapidly (at 4°) with the unprotonated form of primary aliphatic or aromatic amino groups in the pH range of 6–9. Of the amino acid functional groups tested, only sulfhydryl groups compete effectively with free amino groups for reaction. The *N*-hydroxysuccinimide ester derivative of agarose can be used by very simple and mild procedures to immobilize proteins and complex amino group containing ligands to agarose. The coupled products are separated from the matrix backbone by lengthy hydrocarbon extensions. Similar procedures can be used to attach ligands and proteins to porous glass beads. These studies in addition demonstrate that agarose beads tolerate quite well certain organic solvents such as dioxane and methanol. The activated agarose derivatives, after lyophilization to remove dioxane, are quite stable in the powder form and readily swell and react with amino groups when suspended in aqueous medium.

Various procedures have been described for the preparation of selective agarose adsorbents for use in affinity chromatography of proteins (Cuatrecasas *et al.*, 1968; Porath *et al.*, 1967; Cuatrecasas, 1970, 1972; Cuatrecasas and Anfinsen, 1971a,b). There is abundant evidence that successful purification of enzymes frequently depends on utilizing an adsorbent in which the ligand is spatially separated from the matrix backbone (reviewed by Cuatrecasas, 1972). This is generally best accomplished by linking the ligand to agarose derivatives which contain lengthy hydrocarbon extensions terminating in a functional moiety such as a primary amino group or a carboxyl group. A variety of chemical reactions have been used to couple ligands to such "long-armed" agarose derivatives (Cuatrecasas, 1972). However, no generally applicable procedure is available for coupling ligands such as amino acids which contain more than one functional group without using complicated blocking and deblocking procedures of one or more of these groups. Furthermore, it has heretofore been very difficult to covalently attach proteins to any solid support which contains functional groups (*e.g.*, amino, carboxyl, diazonium, bromoacetyl) without coupling through the tyrosyl or histidyl residues of the protein or without introducing intermolecular protein cross-links and polymerization.

This report describes in detail the preparation and use of active carboxylic acid esters of long-armed agarose derivatives which are stable in anhydrous media over prolonged periods of time. Ligands and proteins which contain free amino groups can be coupled very rapidly through amide linkage to these activated derivatives under mild conditions (aqueous

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