Rhodotorulic Acid, a Diketopiperazine Dihydroxamic Acid with Growth-Factor Activity. I. Isolation and Characterization*

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ABSTRACT: A strongly iron(III)-binding compound with the properties of a secondary hydroxamic acid was isolated from supernatants of iron-deficient cultures of a red yeast, subsequently identified as *Rhodotorula pilimanae*. The name rhodotorulic acid was selected inasmuch as a number of *Rhodotorula* species were found to produce the compound in low-iron media. It was characterized by degradation, spectral properties, and synthetic experiments as LL-3,6-bis(*N*-acetyl-3-hydroxyaminopropyl)-2,5-piperazinedione, *i.e.*, the diketopiperazine of δ-*N*-acetyl-L-δ-*N*-hydroxyornithine, which amino acid is a constituent of ferrichromes, albomycins, and fusarinines. The analogous diketopiperazine of δ-*N*-acetyl-L-ornithine was synthesized and shown to be identical with a reduction product of rhodotorulic acid. Rhodotorulic acid has biological activity comparable with that of schizokinen in Lankford's *Bacillus* test system (Byers, B. R., Powell, M. V., and Lankford, C. E. (1967), *J. Bacteriol. 93*, 286). It also shows potent growth-factor activity in assays with *Arthrobacter* species, although lacking the antagonistic effect of other "sideramine" growth factors on albomycin inhibition of bacterial growth (Nüesch, J., and Knüsel, F. (1967), *in* Antibiotics, Vol. I, Gottlieb, D., and Shaw, P. D., Ed., Berlin, Springer-Verlag, p 499).

Numerous low molecular weight nonporphyrin iron-containing or iron-binding natural products have been discovered in recent years (Neilands, 1966, 1967; Sayer and Emery, 1968). Most of these compounds are hydroxamic acids and many have biological activity. The hydroxamic acids are described as *siderochromes*, and those with antibiotic and growth-factor activity as *sideromycins* and *sideramines*, respectively (Nüesch and Knüsel, 1967). We have found from a *Rhodotorula pilimanae* strain a new compound, rhodotorulic acid (RA),[1] which is structurally related to the ferrichromes, the fusarinines, and albomycin, and which has some of the biological properties of sideramines. This paper deals with the isolation, chemistry, and biological activity of RA; a subsequent paper[2] will deal with its distribution in *Rhodotorula* and related genera.

The original yeast strain used for the production of RA was isolated from a contaminated culture of *Ustilago sphaerogena*, which had been grown for production of deferri-ferrichromes. Addition of ferric salts to a supernatant of that culture had shown the presence of large amounts of what appeared to be hydroxamic acid, but which then could not be extracted with the ferrichromes. The complexing agent, RA, was crystallized from pure cultures of the yeast in low iron media and was characterized by the degradations and the synthesis of its deoxy form, the diketopiperazine or anhydride of δ-*N*-acetyl-L-ornithine (AOA), as shown in Figure 1.

Experimental Procedures

Materials and Methods. Crystalline ferrichrome and ferrichrome A were obtained from low-iron *U. sphaerogena* cultures (Garibaldi and Neilands, 1955). L-δ-*N*-Hydroxyornithine was obtained as the syrupy dihydrochloride or as the 2-nitro-1,3-indanedione salt from hydrolysates of deferri-ferrichromes (Emery and Neilands, 1961; Rogers and Neilands, 1963). The anhydrides of glycine and L-alanine were from this department's Emil Fischer collection of chemicals.

All melting points were measured in capillaries and are uncorrected. Microanalyses were performed by the Chemistry Department, University of California, Berkeley. The apparent pK_a's and neutral equivalents were determined in water solution at room temperature by titration with 1 N NaOH in the difunctional recording titrator (Neilands and Cannon, 1955). Spectra were measured with the following instruments: infrared of KBr pellets, Perkin-Elmer 257; visible and ultraviolet, Beckman DU-Gilford and Cary 14; proton magnetic resonance, Varian A-60; optical rotatory dispersion, Cary 60; and mass spectra, Associated Electrical Industries MS-12 and Varian M-66.

Electrophoresis was performed on Whatman No. 1 paper in a water-cooled apparatus at 20–30 V/cm, using the following solvents: I, 4% HCOOH; II, pyridine–acetic

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: RA, rhodotorulic acid; AOA, δ-*N*-acetyl-L-ornithine anhydride.

[2] H. J. Phaff, C. L. Atkin, and J. B. Neilands, manuscript in preparation.

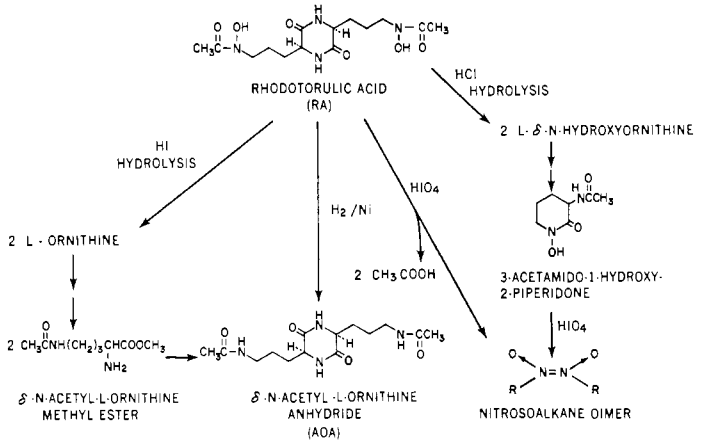

FIGURE 1: Degradations of rhodotorulic acid and synthesis of its reduction product.

acid–water, 7:5:465; III, 0.1 M pH 7 potassium phosphate; and IV, 0.1 M pH 10.4 sodium carbonate–bicarbonate. Chromatography on Whatman No. 1 paper employed these solvent systems: (V) *t*-butyl alcohol–butanone–water–diethylamine (10:10:5:1), (VI) methanol–water–diethylamine (20:5:1), (VII) *n*-butyl alcohol–acetic acid–water (4:1:1), (VIII) 80% methanol on paper impregnated with 0.01 M pH 7.0 potassium phosphate, (IX) *t*-butyl alcohol–4 mM HCl-saturated NaCl (2:1:1) on paper impregnated with acetone–water–saturated NaCl (6:3:1), (X) water–saturated *n*-butyl alcohol, and (XI) upper phase of *n*-butyl alcohol–ethanol–water (4:1:5). Developing sprays used were: 1% ninhydrin in acetone, tetrazolium for hydroxylamines (Snow, 1954), 1% $FeCl_3 \cdot 6H_2O$ in 0.05 N HCl for hydroxamic acids, and for detection of amides the Cl_2–starch–KI test of Rydon and Smith (1952), except that we chlorinated with a cotton-filtered Cl_2–CCl_4 solution prepared by extracting 50 ml of 6 N HCl plus 50 ml of 0.4% $KMnO_4$ with 100 ml of CCl_4.

Culture Methods and Production of RA. Our culture and all other yeast strains used were maintained on 4% Difco malt extract agar slants. The low iron medium for the isolation of RA was the same as that used for ferrichrome production (Garibaldi and Neilands, 1955). Small culture volumes were grown in culture tubes or erlenmeyers on a shaker at room temperature, while bulk batches were grown at room temperature in strongly aerated cultures in 50-l. carboys provided with filtered air and a dropping funnel for addition of Union Carbide SAG-471 antifoam. In either case, with a 5% inoculum, growth was complete in 3–4 days, and production of hydroxamate in 5–6 days. Culture supernatants were flash evaporated at 40° to about one-tenth volume and left to stand at 3°. Precipitation of RA was complete in several days, and the crystals were then collected by filtration on fluted disks of very coarse paper, such as H. Reeve Angel & Co. 835. The crude crystalline RA so obtained, although slightly colored with adhering residue, was identical in form, melting point, infrared spectrum, *etc.*, with recrystallized RA. Recrystallization was effected from boiling water or methanol after treatment with Norit at elevated temperature and filtration through diatomaceous earth on a fritted-glass filter. In a typical large batch, a 40-l. culture had evaporated due to aeration to 25–30 l. at the end of the growth period, and an assay showed about 2 g of RA/l. This ultimately gave about 20 g of dry, twice-recrystallized RA. A further yield of about 20 g of RA was obtained by extraction of the filtrate with 1:1 chloroform–phenol, reextraction into water by addition of ether, concentration, and crystallization.

RA produced as above contained 40–50% of the nitrogen in the medium. After producing large amounts of RA in this fashion, we found that cultures of both *Rhodotorula rubra* and our strain of *pilimanae* could be made to produce 3–4 g/l. by doubling the sugar and ammonium ion content; similar enrichment of *Ustilago* cultures repressed production of deferri-ferrichromes. Even small amounts of iron (1 mg/l.) essentially completely repressed RA synthesis.

Results

Properties of Rhodotorulic Acid. RA crystals are colorless, tasteless, nonhygroscopic, dichromic rhomboidal plates up to 1 mm in length, mp 217–218° with decomposition to a red residue. The substance is soluble in water at 25° to the extent of 1.05%; soluble in phenol, concentrated acids, aqueous solutions above pH 9, and boiling water or methanol; insoluble in camphor, chloroform, dimethylformamide, dimethyl sulfoxide, pyridine, and apolar solvents. RA gave negative ninhydrin and tetrazolium tests; red spots with the iron(III) spray; and a positive Abderhalden test for diketopiperazines (Katchalski *et al.*, 1946), in which an acethydroxamate control was negative. Titration of RA gave a neutralization equivalent of 172 ± 7, and a pK_a of about 9.3. The titration curve appeared skewed in comparison with that of acethydroxamic acid, and mathematical analysis (Albert and Serjeant, 1962) showed that the curve was incompatible with a single pK_a.

Electrophoresis of RA in systems I–III gave a neutral iron-positive spot, whereas in system IV it behaved as an anion. Paper chromatographic R_F values of RA in several systems were: V, 0.10; VI, 0.73; VII, 0.63; IX, 0.62; X, 0.42; and XI, 0.52.

TABLE I: Proton Magnetic Resonance of Diketopiperazines.[a]

Glycine Anhydride		L-Alanine Anhydride		AOA		RA		
τ[b]	H's[c]	τ	H's	τ	H's	τ	H's	Proton Assignment
		8.7	6.1	8.5	7.7	8.3	7.9	Side-chain methyl or ethylene
				8.0	6.1	7.9	6.2	Acetyl methyl
				6.9	4.3	6.4	4.0	Side-chain methylene adjacent to nitrogen
6.0	4.1	5.9	2.1	6.1	2.1	6.0	2.2	Ring α-carbon
2.2	1.9	2.2	1.8	2.2	1.8	2.1	1.6	Ring amide
				1.9	1.9			Side-chain amide

[a] Solutions (5–10%) in anhydrous trifluoroacetic acid at 25°. All peaks were unresolved singlets, except alanine anhydride showed the expected splittings. [b] $\tau = 10 -$ [parts per million downfield from tetramethylsilane (external standard)]. [c] Relative number of protons per molecule.

In the mass spectrometer RA gave only low molecular weight fragments, so a crude measure of the molecular size of RA was made by gel filtration (Laurent and Killander, 1964) on a Sephadex G-10 column using 0.1 M Na_2CO_3 as eluent. The elution volumes of blue dextran, RA, and glycine anhydride were plotted *vs.* the logarithm of the molecular weights. Assumption of an "exclusion molecular weight" of 700 for blue dextran led to a molecular weight of 400 for RA.

Table I summarizes the proton magnetic resonance data for RA, its reduction product, and simple diketopiperazines. The chemical shifts and peak integrations were completely consistent with the diketopiperazine structure. The *N*-hydroxyl groups of RA considerably deshielded the adjacent methylene protons, and the acetyl methyl protons showed the characteristically sharp peak. Because of their rapid exchange, the hydroxamic acid protons could not be seen anywhere upfield of the solvent peak near $\tau - 2$.

Principal absorption peaks of RA in the infrared region were at 3190, 3095, 2870, 1682, 1594, 1467, 1447, 1430, 1339, 1216, 1164, 969, 827, 797, and 777 cm^{-1}. The 1682-cm^{-1} peak was assigned as the amide I absorption. Characteristics of the *cis*-peptide bonds of the ring included a lack of N–H stretch bands higher than 3250 cm^{-1} (Tsuboi, 1949; Miyazawa, 1962), lack of any bands in the amide II region, and presence of the in-plane-bending vibration near 1450 cm^{-1} (Miyazawa, 1962), the latter being prominent in all the diketopiperazines studied. The strong 1594-cm^{-1} band was assigned to the hydroxamic acid carbonyls, although, perhaps because of hydrogen bonding, this is a very low wave number for C=O stretch. The carbonyl absorptions of acethydroxamic acid and deferri-ferrichrome were all found to be in the region 1630–1660 cm^{-1}.

The optical rotatory dispersion of dilute aqueous solutions of RA was, with the exception of a small new Cotton effect, similar to published spectra for other L-amino acid anhydrides (Balasubramanian and Wetlaufer, 1967; Schellman and Nielsen, 1967). Its principal features were, with decreasing wavelength, a levorotation increasing (in an absolute sense) to a negative maximum at 209 mμ, decreasing to zero rotation at about 200 mμ, then increasing in a positive direction to a much larger, positive maximum between 200 and 190 mμ. In more concentrated solutions a much smaller, new Cotton effect was observed near 240 mμ; it consisted of a small, negative, relative maximum at 250 mμ, and a negative relative minimum at 237 mμ. *Anal.* Calcd for $C_{14}H_{24}N_4O_8$ (344): C, 48.83; H, 7.025; N, 16.27. Found: C, 49.51; H, 7.18; N, 16.65.

Iron(III) Complex of RA. RA forms typical ferric hydroxamate complexes. Solutions of the complex are red at neutral pH and show a broad absorption centered about 425 mμ. With decreasing pH the color changes to red-purple or wine. In going from pH 7 to 1, the absorption maximum of monohydroxamate complexes shifts from near 425 to 500 mμ and beyond (Seifter *et al.*, 1960), while the ferrichrome spectrum shifts from 425 to 435 mμ (Emery and Neilands, 1960a). The RA complex showed an intermediate shift to 480 mμ.

The iron complex was readily extracted from neutral or slightly acidic solution into 1:1 chloroform–phenol, and precipitated or reextracted into water by addition of ether. All attempts to crystallize it have yielded only a red oil which solidified into a glass; it is doubtless a polynuclear complex, and behaved on Sephadex G-50 as something of molecular weight of several thousand.

Hydroxamate Assay. The standard spectrophotometric assay consisted of diluting aliquots containing up to 5 μmoles of hydroxamate, to a final volume of 3.0 ml with 5 mM $Fe(ClO_3)_4$–0.1 M $HClO_4$. This assay was found to be linear to an optical density of 1.5, and the RA complex was found to have an extinction coefficient, ϵ_{480}, of 1800 (calculated on total concentration of RA).

Hydrolysis of RA. RA (250 mg) was hydrolyzed with 3 ml of 6 N HCl for 12–16 hr at 100° in a sealed, evacuated Carius tube, and the hydrolysate was flash evapo-

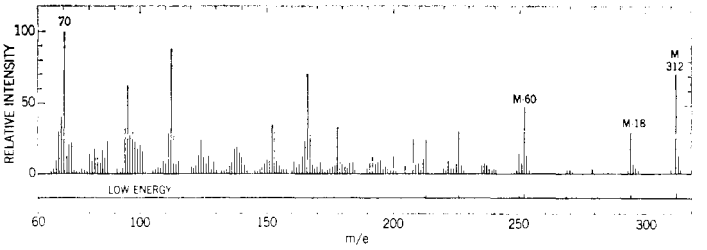

FIGURE 2: Mass spectrum in Varian M-66 apparatus of AOA from reduction of rhodotorulic acid. Probe temperature, 270°. Source energy (upper trace), 70 eV.

rated at 40° to an oil and diluted to 5 ml with water. The hydrolysate solution contained a single tetrazolium and ninhydrin-positive material which was identical with authentic L-δ-N-hydroxyornithine (Emery and Neilands, 1961; Rogers and Neilands, 1963) by the following criteria: coelectrophoresis in systems I and II; cochromatography in systems V, VII, and VIII; as the 2-nitro-1,3-indanedione salt by melting point and infrared spectra; $[\alpha]_D$ of a 2 N HCl solution of the amino acid filtered free of nitroindanedione; and by titration, which yielded 2 ± 0.1 equiv of protons at each of pK_a's = <3, 5, and 9 for each 344 g of RA hydrolyzed.

Reductive Hydrolysis. Hydrolysis of RA with freshly regenerated 50% HI (Foster and Nahas, 1946) was carried out as above for HCl. The residue from flash evaporation was vacuum desiccated and carefully heated with a heat lamp to sublime off most of the I_2. Similar hydrolysis of iron-free ferrichrome yields L-ornithine, and the RA hydrolysate behaves similarly as judged by these criteria: cochromatography with L-ornithine in systems V, VII, and VIII; coelectrophoresis in systems I–III; and specific ninhydrin test for ornithine (Chinard, 1952), incorporating the thiosulfate modification of Emery and Neilands (1961).

Periodate Oxidation of RA. Periodate oxidation of 0.005–0.05% solutions of RA in cuvets, after the method of Emery and Neilands (1962), gave a product with a sharp absorption band at 264 mμ; the optical densities of about 0.1–1 corresponding to a molar absorption, based on 344 as the molecular weight of RA, of about 5600. Oxidation of a total hydrolysate obtained with hot 6 N HCl, after removal of excess acid, gave an absorbancy of about 5900/344 g of RA. Emery and Neilands (1960b, 1962) identified this 264-mμ-absorbing product as a *cis*-nitrosoalkane dimer. They also obtained apparent molar absorptions of less than 10,000 (ϵ_{264} for nitrosomethane dimer) from several polyhydroxamic acids, presumably because polymerization to the dimer was incomplete, although release of the acyl moieties as carboxylic acids was found to be quantitative. Formation of the nitroso dimer in reasonable yield precluded the possibility that the hydroxylamino group is attached to the α-carbon of the amino acid, since the latter structure would have given N_2O, CO_2, and an aldehyde (Neilands and Azari, 1963).

A combination titration–periodate oxidation experiment was performed. A 5-ml solution of 10.65 mg (30.9 μmoles) of RA was titrated, acidified, and retitrated; equivalence for the pK_a of about 9 required 61.9 and 63.1 μequiv. To the stirred solution were added dilute HCl to neutrality, 1 ml of 0.1 M HIO_4, then several drops of ethylene glycol. The solution, upon immediate retitration, showed the appearance of 60 μequiv of a substance with pK_a of 4.8, and the complete loss of hydroxamic acid. The substance bearing the new titrating group was completely ether extractible at low pH. A precipitate, presumably the nitrosodimer, formed during the retitration. For comparison, the same experiment was done with a monomer of the same composition as RA, *e.g.*, 3-acetamido-1-hydroxy-2-piperidone.[8] The compound initially titrated with a pK_a of 8.9; after oxidation, a stoichiometric amount of an acid with pK_a = <4 appeared, and the hydroxamic acid was eliminated; the new acid was not extractible into ether.

Catalytic Reduction of RA to AOA. The hydroxamic acid moieties of RA were reduced to secondary amides by the method of Gipson *et al.* (1963), except that we used methanol solvent, hydrogenated 24 hr, and crystallized the product from boiling methanol. The Norit-decolorized, twice-recrystallized reduction product AOA was obtained in 70% yield and had these properties: crystalline form and solubilities (except in base) similar to those of RA; decomposed at about 265° to a red residue; negative ferric, ninhydrin, and tetrazolium tests and optical rotatory dispersion curve almost identical with that of RA. Mass spectral analysis (Figure 2) gave a strong molecular ion peak, M, at *m/e* 312. The intensities relative to M at M + 1 and M + 2 were 17.0 and 2.5%, respectively; the calculated intensity of M + 1 for $C_{14}H_{24}N_4O_4$ was 17.6% (Biemann, 1962). The major infrared absorption bands were at 3290 (N–H stretch of side-chain amide), 3200, 3095, 2960, 2875, 1675, 1629, 1545, 1444, 1369, 1334, 1289, 1197, 1134, 1124, 826, 796, and 774 cm^{-1}. *Anal.* Calcd for $C_{14}H_{24}N_4O_4$ (312): C, 53.83; H, 7.744; N, 17.94. Found: C, 54.39; H, 7.52; N, 18.28.

Synthesis of AOA. L-δ-*N*-Acetylornithine was synthesized (Greenstein and Winitz, 1961) and converted into its anhydride by the procedure used by Katchalski *et al.* (1946) for ε-carbobenzoxy-L-lysine anhydride. The recrystallized product was obtained in 14% overall yield, and in 37% yield from the free base of the methyl ester. The product was identical in every respect, including mass and optical rotatory dispersion spectra, with the reduction product of RA. *Anal.* Found: C, 52.98; H, 8.05; N, 17.71.

Biological Activity of RA. Ferrichrome or RA was added to 5-ml cultures of terregens-assay medium (Antoine *et al.*, 1964) prior to sterilization. The tubes

[8] Synthesis to be described in a later paper in this series.

were inoculated with a carefully washed suspension of *Arthrobacter* JG-9, which had been growing with sufficient ferrichrome, then agitated at 24° for 48 hr. Table II shows that half-maximal growth occurred

TABLE II: Growth Response of *Arthrobacter* JG-9.

	OD_{600} of 48-hr Culture[a]			
mμg/ml	Ferrichrome		RA	
0	0.030	0.032	0.035	0.032
0.1	0.082	0.095	0.035	0.072
1.0	0.581	0.555	0.358	0.364
10	0.835[b]	0.837	0.820[b]	0.820

[a] Duplicate cultures. [b] Maximum growth; no change with additional sideramine.

near 1 mμg of RA/ml. N. E. Morrison (personal communication) found a similar response to RA in *A. terregens* and *flavescens* (0.4 mμg/ml half-optimal for the latter). J. L. Arceneaux, B. R. Byers, and C. E. Lankford (personal communication) saw half-optimal growth at 0.2 mμg/ml for *Arthrobacter* JG-9 in a yeast extract medium, and also made the following observations in their *Bacillus* system (Byers *et al.*, 1967). RA was as active as schizokinen in reducing the inoculum-dependent initial growth lag of *Bacillus megaterium* Texas, but had no effect on the log-phase rate. RA was as active as schizokinen in the total growth response of the mutant *B. megaterium* SK^{-}_{300} (Arceneaux and Lankford, 1966). An inoculum-dependent lag could not be demonstrated in our *Rhodotorula* stain.

In antagonism tests *vs.* albomycin (Knüsel and Nüesch, 1965; Nüesch and Knüsel, 1967), RA showed no reversal of the antibiotic's activity against *E. coli* and *B. subtilis* grown on either nutrient or minimal agar, nor did RA show any effect of its own. When the plates were left 24 hr, however, RA showed some sort of *synergistic* effect with albomycin, as the high proportion of the inoculum which is naturally resistant to albomycin (about 10^{-4}) did not grow into colonies within the clear zone.

Classification of the Yeast and Generical Distribution of RA. Our RA-producing organism reproduced by budding. It was bright red-orange in color under all culture conditions, and a mixture of carotenoids was extractible after rupture in a French press. H. J. Phaff (personal communication) has identified the strain as *Rhodotorula pilimanae*. We have found production of hydroxamic acid in low-iron cultures of several other *Rhodotorula* and *Sporobolomyces* species, and these data will be reported elsewhere.[2]

Discussion

The characterization of RA was fairly straightforward because the previous work of Snow, Emery, Rogers, and Neilands on mycobactin and the ferrichromes provided methods for identification of the δ-*N*-hydroxyornithine and of hydroxamic acids. The acetyl groups required by the elemental analysis, titration, and proton magnetic resonance completed the list of constituents of RA, and their location could be assigned to the δ-nitrogen atoms because of the results of oxidation of RA with HIO_4. A monomer of the same empirical formula, 3-acetamido-1-hydroxy-2-piperidone, was very different from RA. A polyhydroxamic acid was indicated by both the complex nature of the titration curve and by the spectral shift of the iron complex with decreasing pH. The solubilities of RA and a crude measure of its molecular weight on Sephadex suggested the diketopiperazine structure, which was confirmed by synthesis of the reduction product AOA, the molecular weight of which was determined in the mass spectrometer. Analyses of infrared, optical rotatory dispersion, and proton magnetic resonance spectra of RA, AOA, and simple diketopiperazines were all consistent with the structure shown in Figure 1. To date we have not succeeded in a total synthesis of RA.

There have been numerous reports of natural diketopiperazines and closely related compounds from fermentations of molds, yeasts, and lichens. These products include albonoursine, aspergillic acids, echinulin, flavacol, gliotoxins, mycelianamide, picrorocellin, and pulcherriminic acid (*cf.* Gerber, 1967; Mitscher *et al.*, 1967; Neilands, 1966, 1967; Shibata *et al.*, 1964). It has been shown, however, that some of the simpler diketopiperazines can be isolated from various fresh media (Chmielewska *et al.*, 1967; Mitscher *et al.*, 1967). The question arises whether RA *per se* is synthesized by *Rhodotorula;* the following evidence indicates this is probably so. There are no direct precursors of RA, except acetate, in the medium. The low temperature and neutral to slightly acidic pH used in the isolation procedure are conditions not favorable for cyclization of even activated dipeptides. And lastly, using cultures which have never been above room temperature, electrophoresis in systems I and III of the supernatant itself and chromatography in several systems of an aqueous solution from $CHCl_3$–phenol extraction, shows the ferric-positive spots characteristic of RA.

As an addition to the catalog of natural hydroxamic acids, RA is interesting in several respects. It is isolated in higher yields than related compounds, and affords a convenient model in which to study certain of the biosynthetic reactions leading to this type of substance. RA is also an excellent source of L-ornithine. A wide variety of analogs is available, and a systematic study of these may allow determination of the structural basis for biological activity. Finally, complexation constants for hydroxamic acids (Anderegg *et al.*, 1963) indicate that for growth of our *Rhodotorula* under low iron conditions, iron and transition divalent metals are available only as rhodotorulate complexes, and calcium(II) and magnesium(II) may be at least partly sequestered as well. Experiments are now in progress to determine the structure and stability of such derivatives.

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