A Hydroxamic Acid Present in *Rhodotorula pilimanae* Cultures Grown at Low pH and Its Metabolic Relation to Rhodotorulic Acid†

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ABSTRACT: *Rhodotorula pilimanae* CBS 4479, when grown at pH 2.8 in iron-limited media, produced rhodotorulic acid and a second hydroxamic acid which was characterized as 1-hydroxy-3(*S*)-amino-2-piperidone (HAP). The latter, which was not converted to rhodotorulic acid, was shown to arise from cyclization of δ-*N*-hydroxyornithine. The addition of iron to growing cultures effected a parallel depression of formation of HAP and rhodotorulic acid; iron also repressed the conversion of δ-*N*-acetyl-δ-*N*-hydroxyornithine to rhodotorulic acid. Cultivation of *Rhodotorula pilimanae* at low pH apparently interdicts the biosynthesis of rhodotorulic acid at the stage of δ-*N*-hydroxyornithine; the latter amino acid is excreted to the medium where it cyclizes spontaneously to HAP.

We have previously reviewed the features of rhodotorulic acid which render it the substance of choice for biosynthetic studies in the siderochrome (iron transport compound) series (Akers *et al.*, 1972). Rhodotorulic acid was thought to arise from δ-*N*-hydroxyornithine (OHOrn)[1]; however, due to the instability of OHOrn at pH values above the hydroxylamine pK_a of 5.5 we were reluctant to examine this compound as a precursor. In an attempt to overcome this problem we adapted *Rhodotorula pilimanae* to grow at pH 2.8. A hydroxamic acid produced at this pH differed from rhodotorulic acid in that it was retained by a cation exchange resin.

This paper deals with the purification, characterization by comparison with synthetic material, and origin of 1-hydroxy-3(*S*)-amino-2-piperidone (HAP). The results indicate that at low pH OHOrn, a direct precursor of rhodotorulic acid, is diverted into the medium where it is cyclized nonenzymatically to HAP.

Materials and Methods

R. pilimanae cultures adapted to low pH were maintained as liquid cultures on low pH ASKP medium (see below). Crystalline rhodotorulic acid was obtained from low-iron cultures of *R. pilimanae* (Atkin and Neilands, 1968). OHOrn was from HCl hydrolysates of rhodotorulic acid (Atkin and Neilands, 1968). δ-*N*-Acetyl-δ-*N*-hydroxyornithine (AcOHOrn) was synthesized from OHOrn (Akers *et al.*, 1972).

Medium. R. pilimanae was cultured for 10–14 days at 30° on a rotary shaker in the ASKP medium previously described (Akers *et al.*, 1972). The pH was adjusted to 2.8 ● 0.1 using concentrated H_3PO_4. Iron (as ferric ammonium sulfate) was not added unless specifically stated.

Hydroxamates were determined spectrophotometrically (Atkin and Neilands, 1968). A tetrazolium spray was used for the detection of hydroxylamines (Snow, 1954). Periodate oxidation of hydroxamic acids was by the method of Emery and Neilands (1962). OHOrn was reduced with tin to ornithine (Klages and Sitz, 1959). Hydrolysis of HAP with 6 N HCl was performed on a steam bath for 30 min. HI hydrolysis and recovery of ornithine was similar to methods previously reported (Akers *et al.*, 1972). Optical rotations were determined with a Cary 60 spectrophotometer. Proton magnetic resonance spectra were recorded on Varian A-60 and HR220 spectrometers using 20–40-mg samples in 0.5 ml of D_2O or dimethyl-d_6 sulfoxide (Merck Sharp and Dohme) and tetramethylsilane as internal standard. Mass spectra were obtained with an Associated Electrical Industries MS-12 mass spectrometer at 170° and 70 eV. High-resolution mass determinations were made with a Consolidated Electrodynamics Corporation 21-110 instrument at 90° and 70 eV. The biological activity of HAP was tested using *enb* mutants of *Salmonella typhimurium* (Luckey *et al.*, 1972). A difunctional titrator (Neilands and Cannon, 1955) was used to record equivalents and p*K* values. Ornithine was determined qualitatively (Jirgl, 1965) and quantitatively (Chinard, 1952). Electrophoresis was performed using carbonate for pH 10.0–11.0, borate for pH 8.0–10.0, phosphate for pH 6.0–7.8, pyridinium acetate for pH 4.5–5.5, and HCl as electrolytes (all 0.1 M). Silica gel thin-layer chromatography was performed using the following solvent systems: A, pyridine–acetic acid–water (10:7:3); B, *n*-butyl alcohol–acetic acid–water (4:1:1); C, *tert*-butyl alcohol–2-butanone–water–diethylamine (10:10:5:1), and D, *tert*-butyl alcohol–formic acid–water (14:5:5).

Purification of 1-Hydroxy-3(S)-amino-2-piperidone. To 1 l. of culture supernatant was added 50 ml of a Dowex 50 H^+ slurry (about 10 g of resin). The resin was recovered by filtration, washed with 0.1 N acetic acid, and eluted with 2.0 M pyridinium acetate (pH 5.0). The pyridinium acetate fraction was flash evaporated at 40° to a small volume, acidified to pH 3.0 with glacial acetic acid, and applied to a 1.5 × 40 cm Dowex 50-H^+ column. The column was developed with a linear gradient between 500 ml of 2 N acetic acid and 500 ml of 2 M pyridinium acetate (pH 5.0). The hydroxamate-positive fractions were pooled and flash evaporated at 40° to a small volume. The pyridinium acetate was removed by repeated additions and evaporations of 0.01 N HCl. The

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[1] Abbreviations used are: HAP, 1-hydroxy-3(*S*)-amino-2-piperidone; OHOrn, δ-*N*-hydroxyornithine; AcOHOrn, δ-*N*-acetyl-δ-*N*-hydroxyornithine.

HAP was dissolved in the least possible amount of water and applied to a 3.5 × 110 cm Sephadex G-10 column using water as eluent. The hydroxamate-positive fractions were pooled, flash evaporated, and dried to a powder *in vacuo* over P_2O_5. At this point all attempts at crystallization were unsuccessful; however, the molecular weight by titration was 181 (theoretical 166.5) indicating a purity of 92%. About 50 mg of HAP was recovered per liter of culture.

Separation of Rhodotorulic Acid from 1-Hydroxy-3(S)-amino-2-piperidone or δ-N-Acetyl-δ-N-hydroxyornithine. Dowex 50-H^+ columns (3 cm) in disposable Pasteur pipets were used to separate rhodotorulic acid from HAP or AcOH-Orn in quantities suitable for hydroxamate assays. Normally 0.5–1.0 ml of culture medium acidified to pH 2.0 with glacial acetic acid was applied to a column. Rhodotorulic acid was eluted with 2 or 3 ml of 2.0 N acetic acid while HAP and AcOHOrn were eluted by washing the column with 2 or 3 ml or 2.0 M pyridinium acetate (pH 5.0).

Synthesis of 1-Hydroxy-3(S)-amino-2-piperidone. The synthesis of HAP has been reported previously (Emery, 1966, Isowa *et al.*, 1972). A convenient method, corresponding to the synthesis of 3-acetylamino-1-hydroxy-2-piperidone (Atkin, 1970), is reported here. OHOrn was dried to a glass *in vacuo* over P_2O_5. The ethyl ester of OHOrn was formed by bubbling dry HCl gas through an ethanolic solution (100 ml of absolute ethanol/g of OHOrn) for 2 hr. After stirring overnight 2 equiv of KOH was added. The resulting HAP was purified by ion exchange (see above) and crystallized in 70–75% yield as the hydrochloride salt from ethanol, mp 205–208° (lit. 211–212°, Emery (1966); 208–215°, Isowa *et al.* (1972)).

Results

Titration of the biologically produced HAP revealed a complex buffer zone at pH 7.5–9.5. After HCl hydrolysis pK_a values appeared at pH 2, 5.5, and 9.5, each with a step height exactly half that of the complex buffer zone in the unhydrolyzed material. Oxidation with periodate and titration revealed pK_a values at 2 and 9.5 (1:1 equiv). One equivalent, based on the HCl hydrolysate or assuming two titratable groups in the intact molecule, was 181 g of HAP.

Periodate oxidation produced a product that absorbed sharply at 264 nm, indicating the presence of a parent hydroxylamino compound, and eliminated the ferric ion reaction. The oxidized material gave a typical amino acid color with ninhydrin while the intact compound gave a gray color. Hydrolysis with HCl followed by reduction with tin, or reductive hydrolysis with HI, gave ornithine. The ornithine from the HI hydrolysate was found to be 85% L isomer by comparison with the optical rotation of a commercial sample of L-ornithine.

At pH values lower than 8.8 HAP moved as a cation on electrophoresis. At pH 8.8–8.9 HAP is a neutral molecule and with more alkaline buffers it behaved as an anion. Synthetic HAP, the material of biological origin, and an authentic sample provided by Dr. T. Emery coelectrophoresed, as detected by ninhydrin or iron sprays, at a number of pH values. The three samples also migrated identically on thin-layer chromatography in solvent systems A, B, C, and D and displayed R_F values of 0.72, 0.28, 0.30, and 0.55, respectively.

Hydrolysis with HCl produced a substance which was tetrazolium-positive, iron-negative, and which gave a typical amino acid color with ninhydrin. This substance coelectrophoresed (pH 1.0, 4.5, and 5.5) with authentic OHOrn (see Figure 1 for degradation scheme).

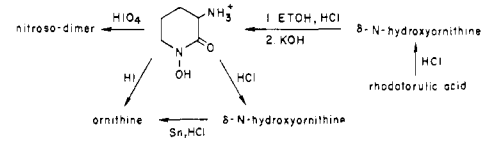

FIGURE 1: Degradation and synthesis of 1-hydroxy-3(*S*)-amino-2-piperidone (HAP).

In the standard hydroxamate assay solution HAP (synthetic or biological) was found to have a maximum absorbance at 490 nm. The molar absorption coefficient at 490 nm varied from 870 to 960 depending on whether the concentration was determined from the weight of dried samples, the amino and hydroxamic acid group equivalents, or the colorimetric determination of ornithine following reduction with HI. The value used for all calculations, 920, represents an average of at least two determinations by each method.

While HAP is stable as a solid, solutions at extreme pH (<1 or >12) decompose within a few minutes as measured by reaction with iron. Solutions near neutral pH were observed to be stable for at least 3 weeks. HAP in the hydroxamate assay solution (pH ~1) has a constant absorbancy for several hours. At pH <6 HAP solutions were observed to gradually hydrolyze to OHOrn (detected by electrophoresis, pH 4.5, tetrazolium spray). Alkaline solutions were shown by electrophoresis (pH 4.5) to gradually acquire at least two ninhydrin-positive, tetrazolium-negative compounds.

An attempt was made to determine the effect of the pH of the culture medium on HAP production. ASKP medium is not buffered above pH 3 and hence 4 g/l. of ammonium acetate was supplied in addition to the other ingredients. The pH of the culture was observed to drop as much as 2 pH units during a 7-day incubation; therefore, little meaningful data were obtained. Generally, cultures of *R. pilimanae* which were adapted to pH 2.8 produced 1–2 mM hydroxamate (total) which is one-fifth to one-tenth that of a pH 6.8 culture. At pH 2.8 usually one-third to two-thirds of the total hydroxamic acid produced is HAP, the remaining being rhodotorulic acid. The production of HAP decreased as the pH was increased, very little is produced at pH 5, and 1 l. of pH 6.8 culture was exposed to Dowex 50 treatment (method same as for HAP purification) before a detectable amount of HAP could be found. Since cell yields at pH 2.8 were about one-third of those at pH 6.8, the capacity of the cell to form OHOrn is relatively constant over this pH range.

The effect of iron on hydroxamate production was determined by assaying for rhodotorulic acid and HAP in duplicate cultures after 10 days of incubation (Figure 2).

OHOrn was added to cultures and culture supernatants under various conditions (see Table I) to determine if the production of HAP was a cellular process. The cultures were assayed after 48 hr and the changes in HAP and rhodotorulic acid concentrations were determined.

For the metabolic tracer experiments involving rhodotorulic acid (Table II) the methods have been reported previously (Akers *et al.*, 1972). Basically, they consist of adding potential ^1H precursors to deuterated cultures and determining the incorporation into the product by proton magnetic resonance.

Cultures adapted to low pH were then adapted to grow in 99.8% D_2O. A deuterated pH 2.8 culture was made 10 mM in OHOrn 3 days after inoculation and the HAP purified after an additional 4 days of growth. The α, β, γ, and δ hydrogen peaks were observed by nuclear magnetic resonance to have areas of

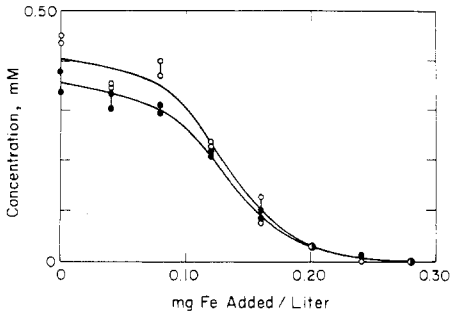

FIGURE 2: The effect of ferric ion concentration on 1-hydroxy-3(*S*)-amino-2-piperidone and rhodotorulic acid formation in low pH medium: (●) rhodotorulic acid; (○) 1-hydroxy-3(*S*)-amino-2-piperidone; (◑) superimposed data points.

1:2:2:2, respectively. Table III lists the chemical shifts and relative peak areas of HAP. Assignments for HAP were made on the basis of relative areas and comparisons made with the assignments for reduced rhodotorulic acid (Llinás, 1971). The HAP samples frequently contained traces (0.1–0.05 equiv) of acetate which appeared as a sharp singlet superimposed on the β hydrogen peak.

The mass spectrum of biologically produced HAP is recorded in Figure 3. The spectrum of synthetic HAP was identical with that of the biological sample; both spectra showed strong molecular ion peaks at *m/e* 130.

TABLE I: Effect of OHOrn on the Formation of HAP.[a]

Culture	OHOrn	Concn Change (mM) HAP	Rhodotorulic Acid
1. Intact culture, pH 2.8, low iron	+	1.083	0.013
	−	0.300	0.016
Whole culture, autoclaved	+	0.443	0.000
	−	0.000	0.003
Culture supernatant	+	0.456	0.000
	−	0.001	0.000
Culture supernatant, autoclaved	+	0.333	0.004
	−	0.001	0.002
pH 2.8 medium, not inoculated	+	0.248	0.002
	−	0.004	0.000
2. Intact culture, pH 2.8, high iron	+	0.986	0.004
	−	0.002	0.006
3. Intact culture, pH 6.8, low iron	+	0.002	0.756
	−	0.001	0.548
Culture supernatant	+	0.002	0.000
	−	0.000	0.002
pH 6.8 medium, not inoculated	+	0.000	0.003
	−	0.001	0.001

[a] Two-day cultures were subcultured and treated as indicated. The OHOrn was then added to half the cultures (final concentration 10 mM) and after 48 hr (24 for culture 3) the changes in HAP and rhodotorulic acid concentrations were determined. Low iron cultures contained no added iron while high iron cultures contained 0.3 mg of Fe/l.

TABLE II: Relative Incorporation of Exogenous Protons into Rhodotorulic Acid.[a]

Exogenous Substrate	Hydrogen Type in Rhodotorulic Acid α	$\beta + \gamma$	δ	CH_3
OHOrn	0.5	1.0	1.0	0.0
HAP	0.0	0.0	0.0	0.1

[a] The relative incorporation into rhodotorulic acid of protons originally present in OHOrn or HAP. The OHOrn figures are relative to the average value obtained for the $\beta + \gamma$ and δ hydrogens. The figures listed are on a per hydrogen basis. The β and γ hydrogens do not appear as separate peaks in the proton magnetic resonance spectrum of rhodotorulic acid. The HAP figures were identical with those obtained when no exogenous substrate was added to a deuterated culture. See Akers *et al.* (1972) for methods of calculation. Exogenous substrates were 10 mM.

HAP was not observed to support the growth of siderochrome-requiring mutants of *S. typhimurium*.

The effect of iron on the formation of rhodotorulic acid from AcOHOrn is recorded in Table IV.

Discussion

The detection of substantial quantities of HAP in *R. pilimanae* cultures grown at pH 2.8 helps confirm the proposed pathway for rhodotorulic acid biosynthesis (Akers *et al.*, 1972). This finding has also enabled examination of the regulation of the process and it allows certain predictions regarding the properties of the enzyme systems involved in the synthesis.

Once HAP was found to contain OHOrn its characterization was facilitated by comparison with synthetic and authentic specimens.

The molar absorbance of HAP is similar to other monohydroxamates but the wavelength of maximum absorption is at a slightly lower wavelength (Akers *et al.*, 1972; Seifter *et al.*, 1960). This may be due to some angular distortion in the hydroxamic acid, since aspergillic acid, a monohydroxamic acid also confined in a ring, was observed to have maximum absorbance at 470 nm in our assay system.

Since all the carbon-bound hydrogens originally present in

TABLE III: Proton Magnetic Resonance of HAP.[a]

Hydrogen	Multiplicity	Biol Material Ppm[b]	Biol Material Rel Area	Synthetic Material Ppm[b]	Synthetic Material Rel Area
α	Quartet	3.29	1.0	3.30	1.0
β	Broad peak	1.84	2.3	1.87	2.2
γ	Broad peak	1.51	2.0	1.50	1.9
δ	Quartet	3.45	1.9	3.45	1.9

[a] Proton magnetic resonance of HAP. Parts per million figures are downfield shifts from tetramethylsilane. [b] Ppm = parts per million.

TABLE IV: Effect of Iron on the Formation of Rhodotorulic Acid from AcOHOrn.[a]

Culture	Concn Change, mM	
	Rhodotorulic Acid	AcOHOrn
pH 6.8 ASKP culture	+4.10	−6.47
pH 6.8 ASKP culture + 2 mg of Fe/l.	+0.04	−0.29

[a] Effect of iron on the formation of rhodotorulic acid from δ-*N*-acetyl-δ-*N*-hydroxyornithine (AcOHOrn). Both cultures were made 10 mM in AcOHOrn on day 2 and the AcOHOrn and rhodotorulic acid concentrations determined on day 4.

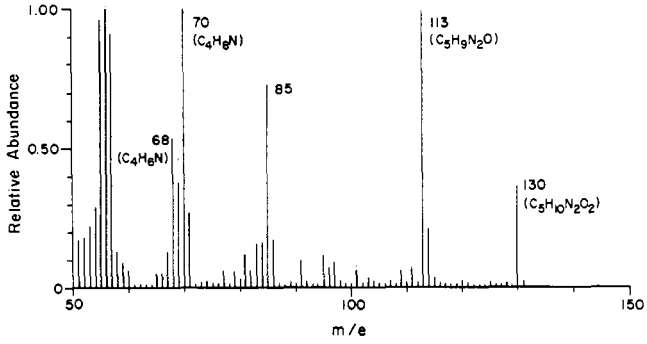

FIGURE 3: The mass spectrum of biologically produced 1-hydroxy-3(*S*)-amino-2-piperidone. The empirical formulas that were determined by high-resolution analysis are also shown.

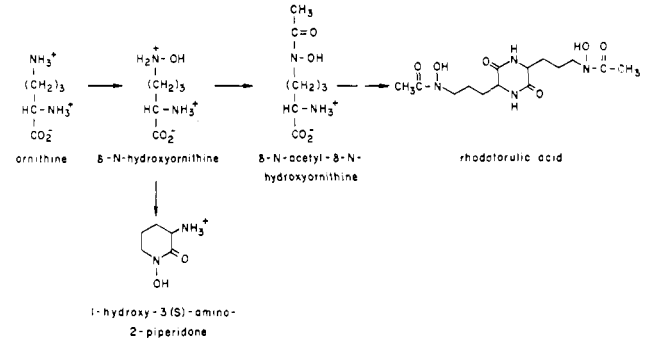

FIGURE 4: Accepted pathway for the biosynthesis of rhodotorulic acid showing the metabolic position of 1-hydroxy-3(*S*)-amino-2-piperidone.

OHOrn reappear in HAP, OHOrn is considered to be a precursor of HAP. Although HAP can form spontaneously from OHOrn, the data in Table I indicate slightly increased cyclization in pH 2.8 medium that contained (or once contained) *R. pilimanae*. Presumably the reactive species is the protonated-acid form of OHOrn. Acylation of the α-amino group on OHOrn type compounds promotes immediate cyclization to *N*-hydroxy lactams (Atkin, 1970; Isowa *et al.*, 1972). As there was no enhancement of rhodotorulic acid production by addition of OHOrn, the latter may not enter *R. pilimanae* in acid conditions. No cyclization of exogenous OHOrn was observed in pH 6.8 cultures. In fact, OHOrn rapidly disappeared from pH 6.8 cultures, most decomposing within 3 days with none detectable by day 6. These observations are consistent with the hypothesis that OHOrn is excreted by *R. pilimanae* (at least in low pH conditions) and spontaneously cyclizes. The cyclization to some extent may be promoted by cofactors produced by the organism. The excretion of OHOrn at low pH may not be a general phenomenon since *Ustilago sphaerogena*, which produces OHOrn (Emery, 1966) as an intermediate in the biosynthesis of ferrichrome, failed to produce HAP when adapted to pH 2.8.

The coordinate repression of the synthesis of rhodotorulic acid and HAP by iron (Figure 2) also supports the conclusion that both substances have a common precursor (OHOrn), the synthesis of which is regulated by the metal ion. For pH 2.8 cultures the concentration of iron that caused half-suppression of maximum rhodotorulic acid synthesis was four times that observed for pH 6.8 cultures (Atkin, 1970).

When [^{1}H]HAP was added to deuterated *R. pilimanae* cultures (pH 6.8) that were rapidly producing rhodotorulic acid, all of the rhodotorulic acid formed came from endogenous precursors. Similarly, Emery (1966) observed no incorporation of [^{14}C]HAP into ferrichrome. When [^{1}H]OHOrn was used as an exogenous substrate the β, γ, and δ hydrogens were incorporated directly into rhodotorulic acid (Table II). The α hydrogen figure is thought to be less than unity because of the involvement of thio ester intermediates in rhodotorulic acid biosynthesis (Akers *et al.*, 1972). Because of the instability of OHOrn at pH 6.8 this does not prove, but only suggests, that OHOrn is a precursor of rhodotorulic acid. However, the hydrogen retention ratios observed indicate that OHOrn was either incorporated directly or reduced to ornithine. When Emery (1966) demonstrated the position of OHOrn in ferrichrome biosynthesis, he observed no formation of ornithine from OHOrn at neutral pH. The data in Table II indicate that HAP is a side product of the rhodotorulic acid pathway (Figure 4).

However, the possibility cannot be excluded that in *R. pilimanae* HAP is formed from the lactam *via* an oxazirane intermediate (Black *et al.*, 1971). This is particularly intriguing when considering the similar enzymatic oxidation of flavacol to neoaspergillic acid (Micetich and MacDonald, 1965) and the occurrence of cyclic ε-*N*-hydroxylysine in mycobactins (Snow, 1970).

The formation of HAP from endogenous precursors was observed to be repressed by the addition of iron to cultures; however, the formation of HAP from exogenous OHOrn was not repressible by iron. This indicates that iron is involved in the repression of OHOrn formation (Figure 4) from ornithine (Akers *et al.*, 1972). Also, as the incorporation of AcOHOrn into rhodotorulic acid is inhibited by iron (Table IV), the enzyme(s) involved in diketopiperazine formation must be repressible by iron. Since two of the three or four enzymatic steps involved in rhodotorulic acid biosynthesis were shown to be repressible by iron, the genes for the entire pathway are probably clustered in an operon.[2]

Acknowledgments

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[2] After this manuscript was submitted Anke and Diekmann (1972) reported that no rhodotorulic acid synthetase could be found in extracts of *Rhodotorula glutinis* grown in the presence of 20 mg of $FeCl_3 \cdot 6H_2O$/l.

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Synthesis and Biological Activity of Some 2′ Derivatives of Adenosine 3′,5′-Cyclic Phosphate†

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ABSTRACT: A series of 2′ derivatives of cAMP were synthesized and their biological properties *in vitro* were investigated by studying their ability to serve as substrates for and inhibitors of cAMP phosphodiesterases, and to activate cAMP-dependent protein kinases. The unblocked 2′-OH in the ribo configuration was found to be necessary for the activation of protein kinase by cAMP. 2′-O-Substituted derivatives and 2′-deoxy-cAMP were relatively good substrates for and inhibitors of cAMP phosphodiesterases while 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate was both a relatively poor substrate and inhibitor. The product of phosphodiesterase action of the 2′-*O*-substituted derivatives was found to be the corresponding 2′-*O*-substituted 5′-nucleotide. N^6-Butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP were studied in the same way. The results indicate that N^6-butyryl-cAMP is the active form of N^6,2′-*O*-dibutyryl-cAMP.

Various derivatives of adenosine 3′,5′-cyclic phosphate, most notably N^6,2′-*O*-dibutyryl-cAMP[1] and several 8-alkylthio-cAMP analogs (Free *et al.*, 1972), have been shown to be more effective than cAMP itself in many whole cell systems (see reviews by Robison *et al.*, 1971, and Drummond and Severson, 1971). Reasons for the low activity of cAMP might include the low permeability of cAMP across cell membranes and its intracellular or extracellular hydrolysis by cAMP phosphodiesterase. It has been proposed that N^6,2′-*O*-dibutyryl-cAMP is both more rapidly transported across cell membranes and less rapidly hydrolyzed by the phosphodiesterase (Posternak *et al.*, 1962; Robison *et al.*, 1971). It has further been suggested that the acylated derivatives of cAMP were deacylated by soluble esterases before becoming active (Posternak *et al.*, 1962; Henion *et al.*, 1967). Exposure of HeLa S3 cells to N^6,2′-*O*-dibutyryl-cAMP led to intracellular levels of N^6-butyryl-cAMP that were 30 times that of basal cAMP levels (Kankel and Hilz, 1972; Kankel *et al.*, 1972). Consistent with these observations, it has been shown that N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP were not hydrolyzed by cAMP phosphodiesterase from beef heart (Moore *et al.*, 1968), liver (Levine and Washington, 1970; Menahan *et al.*, 1969), adipose tissue (Hepp *et al.*, 1969; Blecher *et al.*, 1971), or brain (Drummond and Powell, 1970). Thus, in some systems, at least, N^6-butyryl-cAMP is postulated to be the active metabolite of N^6,2′-*O*-dibutyryl-cAMP (Kankel *et al.*, 1972); however, a comparable understanding of the biologically active form of the 2′ derivatives of cAMP has awaited investigation. In this paper, we wish to report on the *in vitro* enzymatic properties of some representative 2′ derivatives of cAMP in comparison with N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP.

Experimental Section

Synthetic. N^6,2′-*O*-Dibutyryl-cAMP, N^6-butyryl-cAMP, and 2′-*O*-butyryl-cAMP were purchased from Sigma Chemical

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; 2′-*O*-acetyl-cAMP, 2′-*O*-acetyladenosine 3′,5′-cyclic phosphate; 2′-*O*-methyl-cAMP, 2′-*O*-methyladenosine 3′,5′-cyclic phosphate; 2′-*O*-Dnp-cAMP, 2′-*O*-(2,4-dinitrophenyl)adenosine 3′,5′-cyclic phosphate; 2′-*O*-butyryl-cAMP, 2′-*O*-butyryladenosine 3′,5′-cyclic phosphate; N^6-butyryl-cAMP, N^6-butyryladenosine 3′,5′-cyclic phosphate; N^6-2′-*O*-dibutyryl-cAMP, N^6-2′-*O*-dibutyryladenosine 3′,5′-cyclic phosphate; ara-cAMP, 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate; and 2′-deoxy-cAMP, 2′-deoxyadenosine 3′,5′-cyclic phosphate.