Orotidylate Decarboxylase: Insights into the Catalytic Mechanism from Substrate Specificity Studies†

Keith Shostak and Mary Ellen Jones*

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

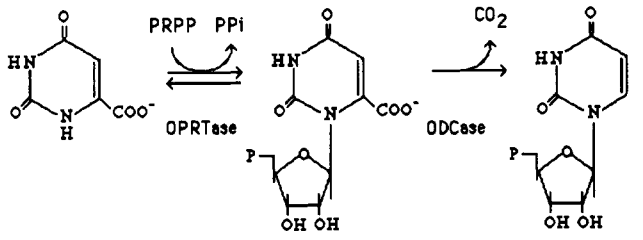
Received July 1, 1992; Revised Manuscript Received August 3, 1992

ABSTRACT: Pyrimidine nucleotides were tested as substrates for pure yeast orotidylate decarboxylase in an attempt to gain insight into the nature of the catalytic mechanism of the enzyme. Substitutions of the 5-position in the pyrimidine ring of the orotidylate substrate resulted in compounds that are either excellent inhibitors or substrates of the enzyme. The 5-bromo- and 5-chloroorotidylates are potent inhibitors while the 5-fluoro derivative is a good substrate with a turnover number 30 times that observed with orotidylate. When carbon 5 of the pyrimidine ring is replaced by nitrogen in 5-azaorotidylate, the resulting compound is unstable in solution with a half-life of 25 min at pH 6. However, studies with freshly generated 5-azaorotidylate show that an enzyme-dependent reaction occurs, presumably decarboxylation. This enzyme reaction follows simple Michaelis–Menten kinetics. Because the 5-aza group is not electrophilic, an enzyme mechanism utilizing a nucleophilic addition of the enzyme at the 5-position is ruled out. We also present studies that are not compatible with a mechanism requiring the formation of a Schiff's base prior to decarboxylation. The enzyme is tolerant of modest substitution at the 4-position, for the 4-keto group can be replaced with a thioketone. However, no catalysis is observed when the same substitution is made at the 2-position. Similarities in the substrate specificity of orotate phosphoribosyltransferase and orotidylate decarboxylase led us to compare the amino acid sequences of the two enzymes; significant (20%) sequence homology was observed. The decarboxylation of orotidylate by orotidylate decarboxylase is unusual because, unlike other decarboxylases, the reaction requires no known cofactors. In addition to the studies above that do not support the formation of an enzyme–substrate addition product, we carried out atomic absorption and chemical tests to see if the enzyme contained tightly bound zinc; none was found. However, spectral studies to explore the interaction between the enzyme and 6-aza-UMP, a potent inhibitor, again rule out a mechanism involving loss of the double bond between positions 5 and 6 but are consistent with a tautomeric shift from the 2-keto, 4-keto form of the free inhibitor to a 2-enol, 4-keto form once the inhibitor is bound to the enzyme.

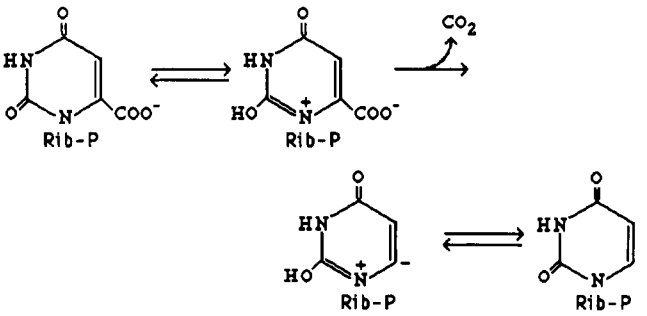
Orotidylate decarboxylase (OMP decarboxylase,[1] EC 4.1.1.23) catalyzes the conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP) in the final step of de novo pyrimidine nucleotide biosynthesis (Scheme I). The previous step in the biosynthesis involves the transfer of a phosphoribosyl group to orotate to form OMP. The reaction is catalyzed by orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10). In this study, OPRTase was used to convert various orotate derivatives to their respective nucleotide analogs in order to study the substrate specificity of OMP decarboxylase.

Two unique catalytic mechanisms have been advanced for the enzymatic decarboxylation of OMP based on model compound studies. The first mechanism involves the formation of a nitrogen ylide intermediate (Scheme II) which acts as an electron sink to aid the breaking of the carbon–carbon bond (Beak & Siegel, 1976). Ylide formation is proposed to occur by the protonation of a ketonic oxygen on the substrate, and no covalent bonds are formed between the enzyme and substrate. The second mechanism utilizes a nucleophilic enzyme residue to form a covalent enzyme–substrate adduct

Scheme I



Scheme II



(Scheme III). In this mechanism, a Michael addition occurs at the 5-position of the substrate before the elimination of the carboxyl group (Silverman & Groziak, 1982). After decarboxylation, the enzyme–substrate bond is broken to release UMP.

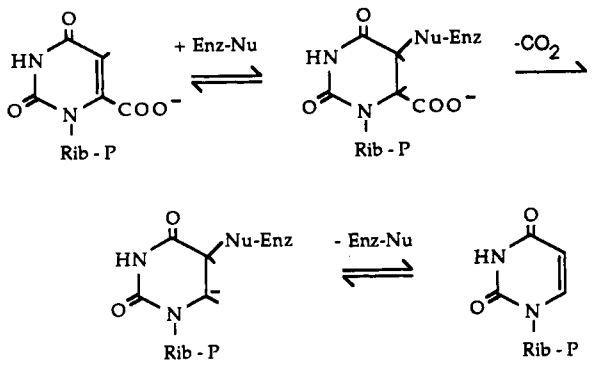
In the ylide or zwitterion mechanism (Scheme II), the enzyme provides an environment in which the basicity of the

† This work was supported by Grant GM 34539 from the National Institutes of Health and NCI Training Grant CA 09156.

[1] Abbreviations: OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; OPRTase, orotate phosphoribosyltransferase; PRPP, 5-phosphoribosyl 1-pyrophosphate; PP_i, inorganic pyrophosphate; Rib-P, ribose 5′-monophosphate; BMP, barbiturate 5′-monophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)-amino]ethanesulfonic acid; FAB, fast atom bombardment.

0006-2960/92/0431-12155$03.00/0

Scheme III



2-oxygen of the substrate is increased so that an active site residue can protonate the keto group. This increase would be facilitated by a hydrophobic active site to cause the necessary pK shifts or the presence of a metal ion to stabilize the zwitterionic transition state. Zinc has been proposed to be bound at the active site from the observation that 6-thiocarboxamide-UMP has more affinity for yeast orotidylate decarboxylase by 4–5 orders of magnitude than 6-carboxamide-UMP (Ondetti et al., 1979). We therefore tested for the presence of enzyme-bound zinc.

With respect to other possible covalent mechanisms, we examined the remote possibility of a Schiff's base mechanism involving the 2-keto group of the substrate analogous to the mechanism of the reaction catalyzed by acetoacetate decarboxylase (Westheimer, 1962). According to this mechanism, a zwitterionic intermediate is formed as in the ylide mechanism, but the Schiff's base serves as an electron sink. A preliminary test for this mechanism is an oxygen exchange between water and the keto group (Hamilton & Westheimer, 1959); we carried out such a study.

Previous studies on the mechanism of OMP decarboxylase have focused on the characteristics of enzyme–inhibitor interactions (Acheson et al., 1990; Levine et al., 1980). Smiley et al. (1991) have observed a large ^{13}C isotope effect in this enzymatic reaction establishing that no covalent step occurs before decarboxylation. The mechanism studies reported here are in agreement with earlier studies but serve now to further characterize this protein. Studies with various nucleotides have defined the structural requirements for binding to the enzyme and subsequent catalysis. We also examined the spectral properties of enzyme-bound inhibitors to see if we could identify the nature of the transition state.

MATERIALS AND METHODS

Materials. 5-Fluoroorotic acid, OMP, UMP, PRPP, yeast OPRTase, and inorganic pyrophosphate phosphatase were purchased from Sigma Chemical Co. 5-Azaorotic acid, 2-thioorotate, and [^{18}O]water (97% enriched) were obtained from Aldrich. Pure OPRTase from *Salmonella typhimurium* was a kind gift from Dr. Charles Grubmeyer of New York University (Bhatia et al., 1990). Pure yeast OMP decarboxylase was prepared from an overproducing yeast strain and purified on Affigel Blue as described by Bell and Jones (1991).

Enzyme Assays. All ODCase assays were performed in 1 mM 2-mercaptoethanol–50 mM potassium phosphate, pH 6.0, at 23 °C in a volume of 0.5 mL. The extinction coefficient used for the conversion of OMP to UMP was 1650 M^{-1} cm^{-1} at 285 nm (Liberman et al., 1955). Apparent k_{cat} and K_m values for alternate substrates in the presence of OMP decarboxylase were determined from absorbance changes upon substrate conversion to product by double-reciprocal plots. Apparent K_i values were determined by double-reciprocal plots using a spectrophotometric assay with 4-thio-OMP as substrate (Shostak et al., 1990). Assays were performed under conditions where the reaction rates were linear with enzyme concentration and time.

General Methods. All spectra and assays were performed on a Milton Roy Model 3000 diode-array spectrophotometer. All synthesized nucleotides were purified before analysis on a semipreparative strong anion exchange HPLC column (Whatman Partisil 10 SAX, 500 × 9.4 mm). The flow rate was 4.0 mL/min with either 100 mM potassium phosphate, pH 3.5, or 0.4 M ammonium bicarbonate as the eluting buffers.

5-Fluoro-OMP. This compound is readily synthesized using yeast OPRTase in the presence of 5-fluoroorotate, PRPP, $MgCl_2$, and inorganic pyrophosphatase (Dahl et al., 1959). The change in extinction coefficient for the conversion of 5-fluoro-OMP to 5-fluoro-UMP at pH 6.0 was determined to be 750 M^{-1} cm^{-1} at 290 nm. The decarboxylation of 5-fluoro-OMP by OMP decarboxylase has been previously demonstrated and the product characterized as 5-fluoro-UMP (Dahl et al., 1959).

4-Thio-OMP. This compound was also synthesized enzymatically, and the decarboxylation products were characterized (Shostak et al., 1990). The extinction coefficient for the conversion of 4-thio-OMP to 4-thio-UMP is 3350 M^{-1} cm^{-1} at 365 nm.

2-Thio-OMP. To a 200-μL solution of 5.2 mM 2-thio-6-carboxyuracil (2-thioorotate) was added 200 μg of OPRTase from *S. typhimurium* in the presence of 25 mM PRPP, 10 units of inorganic pyrophosphatase, and 5 mM $MgCl_2$ in 10 mM Tris–HCl, pH 8.0. The reaction proceeded for 3 h at 30 °C and was terminated by boiling. The spectral changes observed during the enzymatic conversion of 2-thioorotate to 2-thio-OMP are shown in Figure 3. Purification by anion-exchange HPLC yielded a product with a longer retention time than 2-thioorotate, consistent with the conversion of 2-thioorotate to the nucleotide monophosphate.

5-Aza-OMP. This compound is relatively labile in aqueous solution, where the corresponding base, 5-azaorotate, is stable. Because 5-aza-OMP decomposes readily in water, the synthesis was expedited by using large amounts of pure OPRTase from *S. typhimurium* (Bhatia et al., 1990) under conditions described in the text. OMP decarboxylase assays were performed when a steady-state level of 5-aza-OMP was present; i.e., a window of time existed in the synthesis of this compound during which the rate of formation of the nucleotide in the presence of OPRTase was equal to the rate of decomposition in solution. Under the conditions described in the text, steady-state levels of 5-aza-OMP existed anywhere between 1 and 3 min after initiation of the enzymatic synthesis. Decarboxylase activity was assayed during this window of time. The extinction coefficient for the enzymatic conversion of 5-aza-OMP to a decomposition product (Figure 1) was found to be 6550 M^{-1} cm^{-1} at 238 nm.

5-Bromo-OMP. This compound was obtained by a procedure used in the aqueous synthesis of 5-bromo-UMP after Moore and Anderson (1959). Bromine water was slowly added to a solution of OMP in water with stirring until a pale brown color developed, indicating the presence of a slight excess of bromine. As with UMP, the reaction appears to occur immediately upon bromine addition. The mixture was extracted several times with chloroform and the aqueous layer dried. Product was obtained in 75% yield after HPLC using an extinction coefficient in water of 7000 M^{-1} cm^{-1} at 278 nm.

The spectral characteristics of the transformation of OMP to 5-bromo-OMP are similar to those observed with UMP to 5-bromo-UMP (Moore & Anderson, 1959).

5-Chloro-OMP. The procedure was the same as with 5-bromo-OMP synthesis except that chlorine gas was bubbled through the OMP solution. An excess of halogen was avoided here to prevent the formation of a large amount of dichloro derivatives. Product was obtained from the reaction mixture by HPLC purification in 45% yield as judged by an extinction coefficient in water of 6500 M^{-1} cm^{-1} at 280 nm.

Analysis of OMP Decarboxylase for Zinc Content. The solvent of yeast OMP decarboxylase was exchanged in an Amicon Centricon 10 microconcentrator several times with a metal-free 20% glycerol–water solution. The final enzyme concentration was 9 μM. Both the filtrate and the enzyme were analyzed for zinc by two methods. The first method utilized diphenylthiocarbazone (dithizone), as previously described by Song et al. (1976). The second method was atomic absorption spectroscopy performed on an Instrument Laboratories Video 22 atomic absorption spectrophotometer. Samples were aspirated at a rate of 4 mL/min into an acetylene–air flame. All standard curves were prepared in the original glycerol–water solution used in the solvent exchange of the enzyme as described above.

Analysis of UMP Formed from OMP for Oxygen Exchange with [^{18}O]Water. OMP (1 mg, sodium salt) was dissolved in 100 μL of water or 97% enriched [^{18}O]water. A third sample was prepared with UMP (1 mg, sodium salt) dissolved in [^{18}O]water. The pH of these solutions with the nucleotide phosphates as buffers was 7.0. To each sample was added 1 μL of OMP decarboxylase (8 μg), and the reaction was allowed to proceed for 40 min at 23 °C. The samples were dried and resuspended in water for three cycles.

Samples were analyzed for ^{18}O incorporation by negative-ion fast atom bombardment mass spectroscopy. Data were acquired with a VG ZAB-4F instrument. Samples were dissolved in 5 μL of water; 1-μL samples were then added to 1 μL of triethanolamine on the probe tip. The accelerating voltage used was 8 kV with xenon used as the FAB gas at 8 kV.

RESULTS

Spectral Characteristics of 5-Aza-OMP Decomposition. In the presence of 50 μg of pure *S. typhimurium* OPRTase, we reconfirmed that 50 nmol of 5-azaorotic acid (Figure 1, panel A) could be converted to 5-aza-OMP at pH 6.0 (Figure 1, panel B). We observed that this conversion leads to a decrease in the absorption at pH 6.0 of the 5-aza-OMP product at 238 nm while a new absorption band near 215 nm appears (Figure 1, panel B). The spectral change represents a first-order process with a $t_{1/2}$ of 25 min. Two experiments were performed to ensure the decomposition observed was not due to trace orotidylate decarboxylase activity in the OPRTase preparation. First, the half-time of 5-aza-OMP decomposition was shown to be independent of the amount of pure OPRTase in the reaction mixture. Second, the presence of 50 μM 6-aza-UMP, which would inhibit any orotidylate decarboxylase activity, yielded the same half-time of 25 min. The apparent nonenzymatic conversion of 5-aza-OMP to a decomposition product absorbing at 215 nm exhibited a single isosbestic point at 232 nm, suggesting the formation of a single decomposition product. At more basic pH values, the absorption band at 215 nm decayed slowly and no isosbestic point was observed.

When the same experiment was repeated in the presence of OMP decarboxylase (Figure 1, panel C), a more rapid

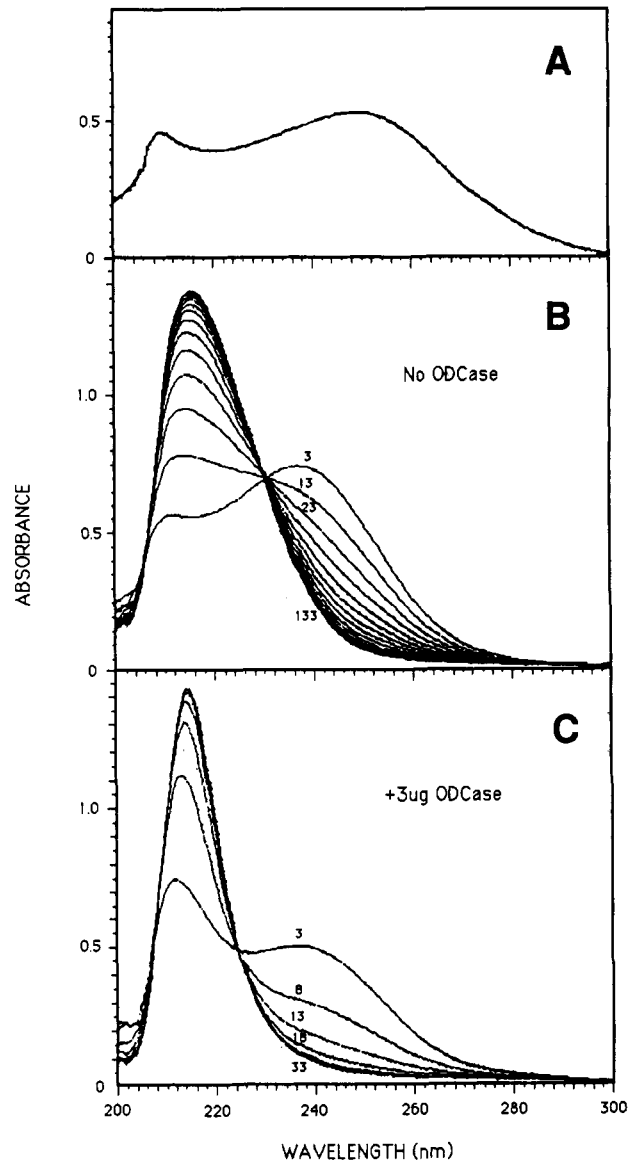


FIGURE 1: Spectra of 5-azaorotate (A) or the decay of 5-aza-OMP with time in the absence (B) or presence (C) of OMP decarboxylase (ODCase). A 0.5-mL mixture of 50 mM potassium phosphate, pH 6.0, 1 mM 2-mercaptoethanol, 50 μg of *Salmonella* OPRTase, 250 μM PRPP, 5 mM $MgCl_2$, and 10 units of inorganic pyrophosphatase was recorded as the spectral baseline. The mixture was made 100 μM in 5-azaorotate and a spectrum immediately recorded (A). In panel B, the reaction was allowed to proceed for 3 min, at which point spectra were taken every 10 min. The times in minutes after the addition of OPRTase are shown above several curves. Panel C was obtained under the same conditions as panel B except that 3 μg of OMP decarboxylase was present in the original mixture and spectra were taken every 5 min.

decay of 5-aza-OMP absorbance at 238 nm was observed concomitant with the rapid appearance of a peak at 215 nm. The isosbestic point for this process is observed at 225 nm rather than 232 nm. In addition to the shifted isosbestic point, the spectrum of the decomposed product at 215 nm exhibits a sharper peak than the one observed in the absence of decarboxylase. The rate of decay of 5-aza-OMP was linearly correlated to OMP decarboxylase concentration where half-times of less than 1 min were achieved at high decarboxylase concentrations. When the absorbance decay at 238 nm in the presence of 1 μg of OMP decarboxylase added was monitored during steady-state 5-aza-OMP formation and decomposition, apparent k_{cat} and K_m values could be obtained for this enzymatic process (Table I). No inhibition of OMP decar-

Table I: Substrate Activity of Selected Orotidylate Analogs

compound	k_{cat} (s^{-1})	app K_m or K_i (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1} \times 10^6$)	$\Delta\epsilon_{(nm)}$[a] ($M^{-1}\ cm^{-1}$)
OMP	19	1.5	12.7	$1650_{(285)}$
5-fluoro-OMP	570	56	10.2	$750_{(290)}$
4-thio-OMP	9	20	0.45	$3350_{(365)}$
5-aza-OMP	5	30	0.17	$6550_{(238)}$
5-chloro-OMP	ND[b]	0.12		
5-bromo-OMP	ND	0.98		
2-thio-OMP	ND	29		

[a] The molar extinction coefficients at the denoted wavelengths were used for assays with the respective compounds as substrates. [b] Not detected.

boxylase activity was observed in the presence of 100 μM decomposition products formed either enzymatically or non-enzymatically at pH 6.0.

OMP Decarboxylase Activity with Potential Substrates. OMP decarboxylase was found to catalyze absorption changes in several OMP analogs that are consistent with the spectral changes that are observed in the decarboxylation of OMP to UMP (Liberman et al., 1955). Apparent k_{cat} and K_m values were obtained from the absorbance changes as analyzed by double-reciprocal plots (Table I). Of all the substrates tested, the natural substrate, OMP, was the most efficient as judged by the ratio of k_{cat} to K_m. 5-Fluoro-OMP was decarboxylated at 30 times the rate of OMP, but with slightly less efficiency due to a high K_m value. 4-Thio-OMP was decarboxylated at about half the rate of OMP with a K_m value more than 1 order of magnitude higher than the natural substrate. 5-Aza-OMP was the poorest of the substrates in terms of efficiency, with significantly altered apparent k_{cat} and K_m values.[2] 5-Chloro- and 5-bromo-OMP do not appear to be substrates for yeast OMP decarboxylase. No spectral changes were observed in these compounds over a 1-h period in the presence of 20 μg of OMP decarboxylase where a catalytic rate less than 10^{-5} s^{-1} could have been detected. Similarly, 2-thio-OMP is not a substrate by these standards. Unlike 2-thio-OMP, 5-chloro- and 5-bromo-OMP are excellent inhibitors of yeast OMP decarboxylase. None of the compounds tested exhibited a time-dependent inactivation of the enzyme.

Analysis of OMP Decarboxylase for Zinc Content. Yeast OMP decarboxylase was analyzed for zinc by two methods. In the dithizone colorimetric method, standard curves were generated from 0 to 0.2 μg/mL zinc. Assuming that one zinc atom binds per subunit, the values assayed should have fallen in the range of 0.027–0.11 μg/mL zinc. The results of triplicate assays in this range of the enzyme fell below 0.01 μg/mL. Similar results were obtained with atomic absorption spectroscopy with theoretical enzyme–zinc concentrations of up to 0.54 μg/mL, suggesting an absence of zinc in OMP decarboxylase.

Product Analysis for Oxygen Exchange with Solvent. To test for solvent oxygen incorporation into the UMP product, OMP decarboxylation was carried out in [^{18}O]water. The resulting product was analyzed by FAB negative-ion mass spectroscopy. Controls of UMP incubated in [^{18}O]water in the absence of enzyme and UMP generated from OMP in [^{16}O]water catalyzed by the enzyme showed m/z peaks at

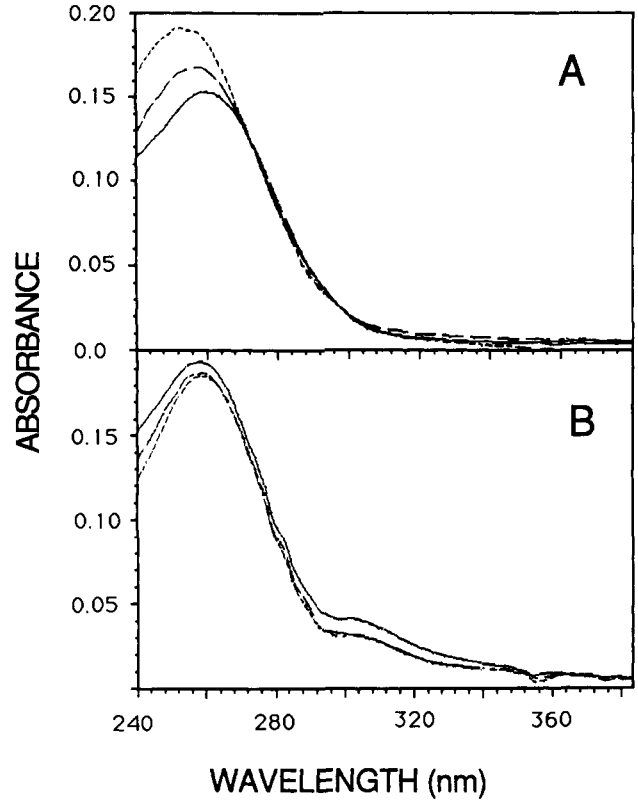


FIGURE 2: Difference spectra of 25 μM 6-aza-UMP in the absence (A) or presence (B) of 36 μM OMP decarboxylase at pH 5.5 (solid line), 7.0 (long dashes), and 8.5 (short dashes). Samples were read in a microcuvette with volumes of 40 μL. Reaction mixtures contained 50 mM sodium MES, potassium phosphate, or Tris–HCl at pH 5.5, 7.0, or 8.5, respectively. The buffer and/or enzyme absorbances were subtracted from each sample by taking a baseline with all components added except 6-aza-UMP. The resulting spectra were taken after the addition of a 1-μL aliquot of 6-aza-UMP.

367, 345, and 323 corresponding to disodium, monosodium, and free-base anions of [^{16}O]UMP, respectively. The spectra of UMP generated from the decarboxylation of OMP in [^{18}O]-water were identical to those seen with [^{16}O]UMP (data not shown). Therefore, it appears that no net incorporation of oxygen from the solvent to the product occurs during catalysis.

Spectral Studies of Enzyme-Bound Inhibitors. 6-Aza-UMP, a potent inhibitor of OMP decarboxylase, exhibits a pK_a value of 7.0 (Handschumacher, 1960). The inhibition constant is pH-dependent, suggesting that the active site binds only the anionic species (Levine et al., 1980). This compound, like most pyrimidines, exhibits spectral changes upon titration of the N-3 proton (Figure 2, panel A). By obtaining difference spectra, where enzyme absorbance is subtracted from the absorbance of 6-aza-UMP plus enzyme, one may observe the spectral properties of the inhibitor bound at the active site. At pH values through the pK_a of 6-aza-UMP, no significant spectral changes were observed between the samples with enzyme present (Figure 2, panel B). The pH-insensitivity of the 6-aza-UMP spectra suggests that the pyrimidine ring is shielded from the solvent and bound to the active site. The addition of enzyme does change the spectra above 280 nm so that one observes a shoulder on the main peak absorbing at approximately 305 nm. As a control, the same experiment was repeated using lysozyme instead of OMP decarboxylase; no shoulder was observed, suggesting that the observed shoulder is not due to artifacts in subtraction of the protein spectra but arises as a consequence of inhibitor binding to the active site of OMP decarboxylase. A similar shoulder is observed in the spectrum of 2-thioorotate but disappears when

[2] 5-Azaorotate compounds form a complex with magnesium ion, a required cofactor for these experiments, which may have altered the values observed for 5-aza-OMP as substrate in Table I. The presence of excess Mg^{2+} has been shown to inhibit the binding of 5-azaorotate to yeast OPRTase, and magnesium binding to 5-aza-OMP might have an inhibitory effect on OMP decarboxylation of 5-aza-OMP (Ashton et al., 1989).

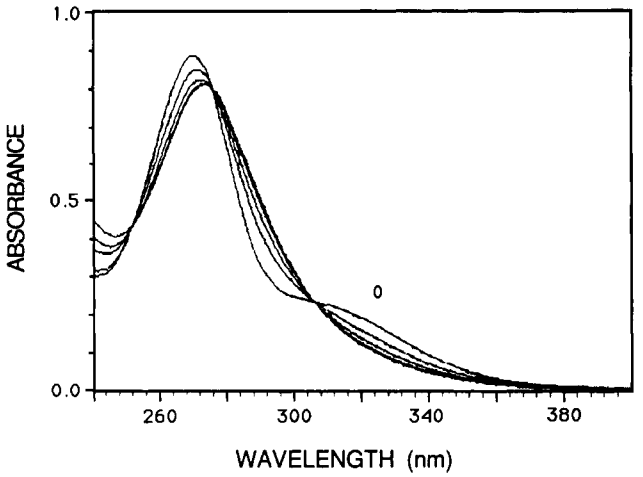


FIGURE 3: Spectra of the conversion of 2-thioorotic acid to 2-thio-OMP by OPRTase. A 500-μL solution containing 20 mM sodium BES, pH 7.0, 5 mM $MgCl_2$, 1 unit of inorganic pyrophosphatase, 300 μM PRPP, and 10 units of yeast OPRTase was taken as the baseline; 73 μM 2-thioorotate was then added and a spectrum taken as denoted by time zero in the figure. Spectra were taken every 10 min. Controls run in the absence of OPRTase gave 2-thioorotate spectra that were unchanged over 2 h.

this compound is enzymatically converted to the nucleotide by OPRTase (Figure 3). No defined shoulder is observed using BMP as inhibitor in the presence of OMP decarboxylase. The implications of this shoulder are discussed below.

DISCUSSION

Significance of 5-Aza-OMP Transformations. The 5-aza-pyrimidines are known to be unstable compounds in solution (Handschumacher, 1963). It is also known from model studies that unsubstituted 5-azapyrimidine bases are much more stable than their N-1 substituted derivatives (Cihak et al., 1963; Cihak et al., 1964, Pithova et al., 1965). The pyrimidine rings of 5-aza compounds spontaneously open between the 5- and 6-positions to yield biuret derivatives. It is not surprising in these studies that 5-aza-OMP decomposes to a compound with an absorbance peak at 215 nm consistent with a biuret derivative (Figure 1). The short half-time of 25 min for the ring-opening decomposition is inconsistent with early in vivo studies on 5-azanucleotides (Cihak et al., 1963, 1964) but agrees with the observations reported by Ashton et al. (1989) on the conversion of 5-azaorotate to 5-aza-OMP and the subsequent instability of the nucleotide.

What remains to be resolved is the nature of the decomposition of 5-aza-OMP in the absence or presence of OMP decarboxylase. Due to the small quantities of product available and the subsequent difficulties in purification, we were unable to analyze the products for structure determination.

Figure 1 clearly shows that differences exist in the spectra of 5-aza-OMP decomposition products as well as in the rates of formation of these products when they are generated in the absence or presence of OMP decarboxylase. A possible explanation is that, in the absence of OMP decarboxylase, 5-aza-OMP is able to undergo spontaneous decomposition to 1-carboxy-2-phosphoribosylbiuret. In the presence of OMP decarboxylase, 5-aza-OMP is decarboxylated to 5-aza-UMP which immediately decomposes to 2-phosphoribosylbiuret (Cihak et al., 1963, 1964; Pithova et al., 1965).

The decarboxylation of 5-aza-OMP by the yeast enzyme is significant in terms of the two mechanisms discussed earlier. The aza moiety is nonelectrophilic so it is unlikely that this compound can undergo a nucleophilic attack as proposed in the Michael addition mechanism (Scheme III). The covalent mechanism is also inconsistent with the large ^{13}C isotope effects observed (Smiley et al., 1991) and evidence that the 5-carbon of barbiturate 5'-monophosphate, a transition-state analog for OMP decarboxylase, does not undergo sp_3 hybridization upon binding to the enzyme (Levine et al., 1980; Acheson et al., 1990). Because 5-aza-OMP is a substrate for OMP decarboxylase, its decarboxylation provides positive evidence against the Michael addition mechanism. This result is consistent with the ylide mechanism (Scheme II).

Steric Effects on Binding and Catalysis. All of the substrates tested exhibited K_m values 1–2 orders of magnitude higher than that of OMP. Substitution of the 5-carbon with an aza group in 5-aza-OMP represents the smallest size replacement in this study. The nitrogen is slightly smaller than the carbon and does not possess a proton. Fluoro replacement for a proton is usually a conservative substitution because the fluoro group has approximately the same radius and hydrophobicity as the hydrogen atom. However, the effects on K_m from this substitution are dramatic. In contrast, 5-chloro- and 5-bromo-OMP do not appear to be decarboxylated yet they bind very well to the enzyme. Thus, it would appear that OMP decarboxylase can tolerate a variety of substitutions at C-5 of the pyrimidine ring that allow effective binding.

Replacement of the 4-keto group with a thioketone to yield 4-thio-OMP gave a product which also exhibited relatively good efficiency in catalysis. However, the same substitution at the 2-position resulted in a compound, 2-thio-OMP, which was neither a substrate nor a good inhibitor. Levine et al. (1980) have suggested that the 2-position may be essential for efficient substrate binding, and these groups could be involved in zwitterion formation (Scheme II).

Electronic Effects on Binding and Catalysis. The 30-fold increase in k_{cat} obtained with 5-fluoro-OMP relative to OMP as substrate is probably an electronic effect in view of the sterically conservative nature of the substitution. The increased rate of decarboxylation could arise from stabilization of a carbanionic transition state by the electronegative fluoro group. The 5-aza substrate is also an electron-withdrawing group; however, a slower catalytic rate relative to OMP was observed. Special considerations exist with 5-aza-OMP such as more dramatic steric alterations from this substitution, the chemical instability of the compound, and the possibility of formation of a complex with magnesium which had to be added for the formation of 5-aza-OMP by OPRTase.

Significance of the 6-Aza-UMP Spectral Shoulder. Reaction conditions were selected such that essentially all of the 6-aza-UMP in the reaction mixture would be bound to OMP decarboxylase at each pH value studied. Because the spectra of the bound inhibitor were essentially pH-insensitive, unlike those of the free inhibitor (Figure 2), we conclude that all of the inhibitor is bound. The common absorbance near 260 nm resembles that of the free anion.

Several conclusions can be drawn from this experiment. First, because the UV absorption was maintained in the bound inhibitor, it seem safe to assume that no covalent interaction exists between the enzyme and inhibitor which resulted in a loss of the double bond between positions 5 and 6. Saturation of this bond would result in a loss of all absorption above 240 nm at neutral or acidic pH values due to a loss of conjugation, as is observed with dihydropyrimidines (Batt et al., 1954). Therefore, if 6-aza-UMP is a true transition-state analog, there is no covalent attachment between enzyme and substrate at C-5 in the transition state. This result confirms earlier

```
Decarboxylase   1 MSKATYKERAATHPSPVAAKLFNIMHEKQTNLCASLDVRTTKELLELVE   49
                                                  : : |:    |::||| :|
Transferase     1 ·····························MPIMLE·DYQKNFLELAIE   18

Decarboxylase  50 ALGPKICLLKTHVD····ILTDFSMEGTVKPLKALSAKYNFLLFE·DRKF   94
                  :   ::  :|        :: :: :  | | |  |   | :::::  | ||
Transferase    19 CQALRFGSFKLKSGRESPYFFNLGLFNTGKLLSNLATAYAIAIIQSDLKF   68

Decarboxylase  95 ADI·GNTVKLQYSAGV··YRIAEWADITN·············AHGVVGPG  128
                    | |  :|    | :  :::||:                  ||  |
Transferase    69 DVIFGPAYKGIPLAAIVCVKLAEIGGSKFQNIQYAFNRKEAKDHGEGGII  117

Decarboxylase 129 IVSGLKQ····AAEEVTKEPRGLLMLAEL··SCKGSLSTGEYTKGTVDIA  172
                  : | | :    ::::|   :  :  ::|:  |:|| :    :     :::
Transferase   118 VGSALENKRILIIDDVMTAGTAINEAFEIISSAKGQVVGSIIALDRQEVV  168

Decarboxylase 173 KSDKDFVIGFIAQRDMGGRDEGYDWLIMTPGVGLDDKGDALGQQYRTVDD  222
                  :                                 |||  :   |  :
Transferase   169 ST·····························DDKEGLSATQTVSKKY  186

Decarboxylase 223 VVSTGSDIIIVGRGLFAKGRDAKVEGERYRKAGWEAYLRRCGQQN      267
                    :  | : ::    :: ||    |     |  :| ||  :|
Transferase   187 GIPVLSIVSLIHIITYLEGRITAEE·····KSKIEQYLQTYGASA      226
```

FIGURE 4: *Protein sequence comparison between yeast orotidylate decarboxylase (Mizukami & Hishinuma, 1988) and OPRTase (deMonitgny et al., 1989). The protein sequences were derived from nucleotide sequences of the URA3 and URA5 genes, respectively. Periods in the amino acid sequence indicate gaps created to obtain maximum sequence alignment. Identical residues between the two sequences are shown in bold type connected by a line. Functionally similar residues are indicated by a colon (Amuro et al., 1985). Sequence alignment was aided by the Gap program from the Genetics Computer Group at the University of Wisconsin.*

experiments in which there was no evidence of adduct formation at the 5-position of BMP (Acheson et al., 1990). Therefore, the Michael addition mechanism (Silverman & Groziak, 1982) is not viable as an OMP decarboxylase mechanism.

Second, the appearance of an absorption shoulder of enzyme-bound 6-aza-UMP may yield information as to the tautomeric state of the bound inhibitor. A similar band is observed in the spectrum of 2-thioorotate at acidic and neutral pH values. However, that band disappears once 2-thioorotate is converted to the nucleotide by OPRTase (Figure 3). The shoulder may disappear because of an enol to keto shift at the 2-position as 2-thioorotate is converted to the nucleotide (Psoda et al., 1974; Psoda & Shugar, 1979; Igarashi-Yamamoto et al., 1981). The stabilization of a 2-enol form of enzyme-bound 6-aza-UMP is entirely consistent with the zwitterionic mechanism (Scheme I) and may account for the absorption shoulder seen in the bound inhibitor. Although the orientation of 6-aza-UMP in the active site is reversed from that proposed by Levine et al. (1980), it is consistent with data in the accompanying paper on studies of site-directed mutants of OMP decarboxylase (Smiley & Jones, 1992). The observation that BMP does not give rise to the same phenomenon when bound to the active site is not surprising. We have observed that neither BMP nor its pyrimidine base exhibits wavelength shifts in response to changes in ionization state [Stimson (1949) and Levine et al. (1990); confirmed by Acheson et al. (1990)].

Probability of a Covalent Decarboxylation Mechanism. The studies in this paper and others (Levine et al., 1980; Acheson et al., 1990; Shostak & Jones, 1991; Smiley et al., 1991) argue against a covalent mechanism involving either position 5 or 6 of the pyrimidine ring. By performing the decarboxylation reaction in [^{18}O]water and noting that the UMP product contains no ^{18}O, we can argue against Schiff's base mechanism that would involve obligatory oxygen exchange between bulk solvent water and carbon 2 (or carbon 4) of the substrate. It should be noted that the water released when a Schiff's base is formed in some enzyme reactions does not exchange with the solvent. The present studies clearly demonstrate that the enzyme decarboxylates OMP wihout the help of zinc. The high affinity of 6-thiocarboxamide for the active site of OMP decarboxylase (Ondetti et al., 1979) may be eventually explained by the ease of enolization of thioketones resulting in a negative charge at the 6-position rather than the high affinity of zinc–sulfur complexes. An enzyme–substrate bond at the 1- or 3-position seems out of the question since these imines are nucleophilic in the pyrimidine ring. These observations, the spectral studies, and the inability of 2-thio-OMP to act as a substrate of OMP decarboxylase all reconfirm that the zwitterionic mechanism is valid for this enzyme (Scheme II).

Activity of OPRTase with Orotate Derivatives. An interesting correlation was found between the specific activity of OPRTase with orotate analogs as substrates and OMP decarboxylase with the corresponding OMP analogs. In 50 mM potassium phosphate, pH 6.0, 5 mM $MgCl_2$, 300 μM PRPP, 1 unit of inorganic pyrophosphatase, and 200 μM pyrimidine substrate, the relative specific activity (moles of product per minute per milligram of protein) of orotate analogs with yeast OPRTase was in decreasing order, as follows: 5-fluoroorotate > orotate > 5-azaorotate > 4-thio-6-carboxyuracil > 2-thio-6-carboxyuracil. 5-Chloroorotate and 5-bromoorotate did not appear to be substrates for yeast OPRTase nor were the corresponding nucleotides substrates for yeast OMP decarboxylase. The relative rates of phosphoribosyl transfer by OPRTase correlate well with the relative rates of OMP decarboxylase activity with modified orotidylates where 5-fluoro-OMP > OMP > 4-thio-OMP > 5-aza-OMP. 2-Thio-

OMP was the only OPRTase substrate tested that did not appear to be an OMP decarboxylase substrate.

These observations led us to look for similarities between the protein sequences of OPRTase and OMP decarboxylase. A structural relationship between the two enzyme sequences was sought by comparison of yeast OPRTase (deMonitgny et al., 1989) and OMP decarboxylase (Mizukami & Hishinuma, 1988) protein sequences. The results of this comparison (Figure 4) yield a 20% homology and 43% similarity (Devereux et al., 1984) in similar residues between one of the two monofunctional yeast OPRTases and the sole monofunctional yeast OMP decarboxylase. This result suggests that the enzymes may share an evolutionary relationship. In mammals, OPRTase and OMP decarboxylase exist on a single polypeptide chain of a bifunctional protein, UMP synthase. In UMP synthase, OPRTase occupies the amino terminal domain while OMP decarboxylase occupies the carboxy terminal domain (Ohmstede et al., 1986). Comparison of the amino terminal OPRTase domain with the carboxy terminal OMP decarboxylase domain in the human bifunctional protein sequence reported by Suttle et al. (1988) shows a 20% homology and 47% similarity between the two domains.

All phosphoribosyltransferases, including OPRTase, contain a sequence that is highly conserved. For yeast OPRTase, this sequence starts with Gly_{119} and ends with Thr_{138}. The single invariant amino acid in this sequence is an aspartate residue (Asp_{132} of the yeast sequence). This aspartate is always preceded by a glutamate or another aspartate where these two residues are surrounded by hydrophobic amino acids. The acidic and hydrophobic residues are conserved in the suggested alignment (Figure 4).

In OMP decarboxylase sequences, the most highly conserved region runs from Phe_{86} through Thr_{100} of the yeast enzyme. The invariant amino acids in all known OMP decarboxylases are equivalent to Asp_{91}, Lys_{93}, Asp_{95}, Ile_{97}, and Thr_{100} of the yeast protein. Both the decarboxylase and the transferase share a high homology in this region with three of the invariant amino acid residues (equivalent to yeast OMP decarboxylase Asp_{91}, Lys_{93}, and Ile_{97} residues) and the number of charged residues being essentially conserved: Lys_{93} of OMP decarboxylase is an essential catalytic residue which probably donates a proton to the oxygen atom of C-2 of the substrate to form the zwitterion (Smiley & Jones, 1992). It will be interesting to see if this region surrounds the pyrimidine base in OPRTase.

ACKNOWLEDGMENT

We thank Mohit Bhatia and Dr. Charles Grubmeyer of New York University for sending us pure *Salmonella* OPRTase, Dr. Kenneth Tomer of the NIEHS for running the FAB mass spectroscopy samples, Drs. Doug Lee and Jan Chlebowski of the Medical College of Virginia for the atomic absorption analysis, and Drs. Richard Wolfenden, Jeffrey Smiley, Willis Person, and Michael Lea for discussions concerning this work.

REFERENCES

Acheson, S. A., Bell, J. B., Jones, M. E., & Wolfenden, R. (1990) *Biochemistry 29*, 3198–3202.

Amuro, N., Paluh, J. L., & Zalkin, H. (1985) *J. Biol. Chem. 260*, 14844–14849.

Ashton, R. W., Strauss, R. S., Chung, S. H., & Sloan, D. L. (1989) *Arch. Biochem. Biophys. 272*, 421–432.

Batt, R. D., Martin, K. J., Ploesser, J. T., & Murray, J. (1954) *J. Am. Chem. Soc. 76*, 3663–3665.

Beak, P., & Siegel, B. (1976) *J. Am. Chem. Soc. 98*, 3601–3606.

Bhatia, M. B., Vinitsky, A., & Grubmeyer, C. (1990) *Biochemistry 29*, 10480–10487.

Brody, R. S., & Westheimer, F. H. (1978) *J. Biol. Chem. 254*, 4238–4244.

Cihak, A., Skoda, J., & Sorm, F. (1963) *Collect. Czech. Chem. Commun. 28*, 3297–3303.

Cihak, A., Skoda, J., & Sorm, F. (1964) *Collect. Czech. Chem. Commun. 29*, 300–308.

Dahl, J. L., Way, J. L., & Parks, R. E. (1959) *J. Biol. Chem. 234*, 2998–3002.

deMonitgny, J., Belarbi, A., Hubert, J. C., & Lacroute, F. (1989) *MGG, Mol. Gen. Genet. 215*, 455–462.

Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.

Hamilton, G., & Westheimer, F. H. (1959) *J. Am. Chem. Soc. 81*, 6332.

Handschumacher, R. E. (1963) *Cancer Res. 23*, 634–639.

Igarashi-Yamamoto, N., Tajiri, A., Hatano, S., Shibuya, T., Ueda, T. (1981) *Biochim. Biophys. Acta 656*, 1.

Levine, H. L., Brody, R. S., & Westheimer, F. H. (1980) *Biochemistry 19*, 4993–4999.

Lieberman, I., Kornberg, A., & Simms, E. S. (1955) *J. Biol. Chem. 215*, 403–415.

Lue, N. F., Chasman, D. I., Buchman, A. R., & Kornberg, R. D. (1987) *Mol. Cell. Biol. 7*, 3446–3451.

Mizukami, M., & Hishinuma, F. (1988) *Agric. Biol. Chem. 52*, 3067–3071.

Moore, A. M., & Anderson, S. M. (1959) *Can. J. Chem. 37*, 590–598.

Ohmstede, C. A., Langdon, S. D., Chae, C. B., Jones, M. E. (1986) *J. Biol. Chem. 261*, 4276–4282.

Ondetti, M. A., Cushman, D. W., Sabo, E. F., & Hong, S. C. (1979) *Drug Action and Design: Mechanism-Based Enzyme Inhibitors*, pp 285–287, Elsevier North Holland, Inc., New York.

Pithova, P., Piskala, A., Pitha, J., & Sorm, F. (1965) *Collect. Czech. Chem. Commun. 30*, 90–98.

Psoda, A., Kazimierczuk, Z., & Shugar, D. J. (1974) *J. Am. Chem. Soc. 96*, 6832.

Psoda, A., & Shugar, D. J. (1979) *Acta Biochim. Pol. 26*, 55.

Shostak, K., Christopherson, R. I., & Jones, M. E. (1990) *Anal. Biochem. 191*, 365–369.

Silverman, R. B., & Groziak, M. P. (1982) *J. Am. Chem. Soc. 104*, 6434–6439.

Smiley, J. A., Paneth, P., O'Leary, M. H., Bell, J. B., & Jones, M. E. (1991) *Biochemistry 30*, 6216–6223.

Smiley, J. A., & Jones, M. E. (1992) *Biochemistry* (following paper in this issue).

Song, M. K., Adham, N. F., & Rinderknecht, H. (1976) *Am. J. Clin. Pathol. 65*, 229–233.

Stimson, M. M. (1949) *J. Am. Chem. Soc. 71*, 1470–1474.

Westheimer, F. H. (1962) *Proc. Chem. Soc., London* 253.

Suttle, D. P., Bugg, B. Y., Winkler, J. K., & Kanalas, J. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1754–1758.

Registry No. OMP, 2149-82-8; 5-fluoro-OMP, 70629-37-7; 4-thio-OMP, 132418-78-1; 5-aza-OMP, 15963-84-5; 5-chloro-OMP, 144182-31-0; 5-bromo-OMP, 144182-32-1; 2-thio-OMP, 144182-33-2; OPRTase, 9030-25-5; 6-aza-UMP, 2018-19-1; Zn, 7440-66-6; OMP decarboxylase, 9024-62-8; 2-thioorotate, 144182-34-3; bromine, 7726-95-6; chlorine, 7782-50-5; 5-azaorotic acid, 144182-35-4; 2-thioorotic acid, 6953-78-2.