SPECTROPHOTOMETRIC STUDIES OF NUCLEIC ACID DERIVATIVES AND RELATED COMPOUNDS AS A FUNCTION OF pH

IV. ON THE STRUCTURE OF OROTIDINE. A STUDY OF N-METHYLATED OROTIC ACIDS*, **

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INTRODUCTION

It has been amply demonstrated[1,2,3] that of the free pyrimidines orotic acid occupies a rather unique position in being the sole effective precursor of nucleic acid and nucleotide pyrimidines in certain microorganisms and animal tissues. Recently[4,5], *in vitro* studies have shed light upon the metabolic pathway of this conversion. These studies indicate that orotic acid and 5-phosphoribosylpyrophosphate react in the presence of orotidine-5'-phosphate phosphorylase to give orotidine-5'-phosphate (O5P), the latter being decarboxylated enzymically to uridine-5'-phosphate (U5P). The enzymic conversion of orotic acid to uridine nucleotides has also been demonstrated by HURLBERT AND REICHARD[6] using particle-free extracts from a number of animal and avian tissues.

The structure of O5P is listed by LIEBERMAN, KORNBERG AND SIMMS[5] as a 5-phosphoribofuranosyl derivative of orotic acid in which the sugar moiety is linked at the N^1 position of the pyrimidine ring. This reasonable assumption of structure is based primarily upon the transformation of O5P in the presence of a relatively-purified enzymic preparation of U5P[5]. This enzymic reaction, of itself, does not unequivocally establish the structure of O5P. For example, the point of attachment of the ribose phosphate residue to the pyrimidine ring might be at N^3 or at O^2 and yet with the proper crude enzyme preparation a rearrangement of the sugar radical might be effected, resulting in the formation of U5P.

The similarity of the spectrum of O5P at pH 7.0 to that of orotidine (peak at 266 mμ, 280/260 = 6.5 at pH 7.0) as reported by LIEBERMAN *et al.*[5] and the fact that O5P may also be synthesized by a malt phosphatase by phosphate transfer of phenyl phosphate to orotidine[5] shows that O5P is indeed a phosphoryl derivative of orotidine. The structure of orotidine, however, is by no means certain. MICHELSON *et al.*[7], who isolated this compound from the mycelia of a *Neurospora* mutant showed that, unlike

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** For part III see reference 10.

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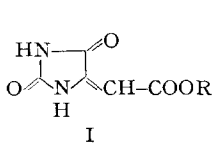
other naturally-occurring pyrimidine nucleosides, orotidine was easily hydrolyzed by acid to orotic acid and ribose. While this lability to mineral acid may be due to the presence of the 6-carboxy function, the possibility (as pointed out by MICHELSON *et al.*) of the existence of a glycoside linkage at other positions of the pyrimidine ring must also be considered. In addition, the lactol ring structure of orotidine was not characterized. Since derivatives of orotic acid (*i.e.* O5P) are probably on the main pathway of pyrimidine biosynthesis, an investigation into the structure of orotidine (and thereby O5P) was undertaken.

It was shown in previous studies[8,9] that the ultraviolet absorption spectra of 1-methyluracil as a function of pH was similar to that for uridine (pH 1.0 to 12.0) but markedly different from that of 3-methyluracil. It was suggested[8] that a study of the spectra of 1-and 3-methylorotic acids as a function of pH would provide information useful for the positional allocation of the sugar rest to the pyrimidine in orotidine. In order to identify the position of attachment by this spectrophotometric method it is necessary, (a) to have structurally-authenticated N-methyl orotic acids, and (b) to demonstrate that the spectral behavior of these N-methylated orotic acids bears the same relationship to orotic acid as do the N-methylated uracils to uracil. Finally, if orotidine (assuming the above-mentioned conditions are met) does, indeed, bear the glycosyl radical at position 1 of the pyrimidine, then the spectrum of orotidine (curves up to pH 12.0) should be similar to that of 1-methylorotic acid. In the high alkaline region (pH 12–14), the nucleoside should exhibit a new dissociation spectrally, as does uridine, owing to the effect of ionization of the sugar moiety at these pH values upon the chromophore of the pyrimidine ring[8,10].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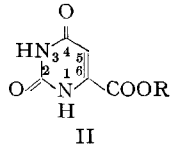
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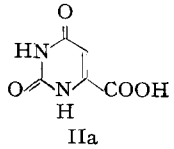
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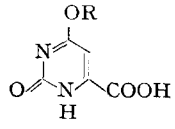
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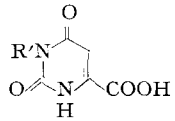
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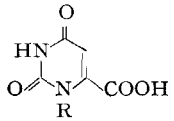
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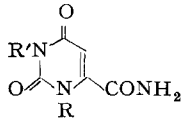
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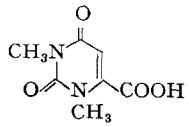
VII

RESULTS AND DISCUSSION

Characterization of methylated orotic acids

As shown in recent years by MITCHELL AND NYC[11], the condensation reaction of urea with ethyloxalacetate described by MÜLLER[12] gives a 5-[carbethoxymethylidene] hydantoin (I), rather than the ethyl ester of orotic acid (II, R = Et) as had been proposed previously[12,13,14]. Treatment of I with hot aqueous alkali results in the simultaneous rearrangement and saponification of I to uracil-6-carboxylic acid (II a). The studies of MITCHELL AND NYC thus adequately explain the inability of BACHSTEZ[13] to obtain I by the treatment of orotic acid with methanol and hydrogen chloride.

BACHSTEZ concluded that the reaction of orotic acid with alcoholic hydrogen chloride gave "O-Alkylverbindungen", presumably 4-alkoxy derivatives (III), and reported melting points for

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the methylated and ethylated derivatives of 298–300° and 324–327° respectively. WHEELER[14], on the other hand, found that treatment of uracil-6-carboxylic acid with methanolic hydrogen chloride gave a product of melting point 230° which he described as uracil-6-methylcarboxylate (II) (see Table I).

BISCARO AND BELLONI[15], who first isolated orotic acid from milk, found that treatment of the silver salt of IIa with methyl or ethyl iodide gave *"etere orotico"* with melting points of 248–250° and 200° respectively. BACHSTEZ repeated their work in this regard and obtained methyl and ethyl derivatives with essentially similar melting points (see Table I). By virtue of the dissimilarity of the melting points of the compounds obtained from the reaction with alkyl halides from those obtained by the reaction of orotic acid with alcoholic hydrogen chloride, BACHSTEZ[13] concluded that the procedure of the Italian investigators[15] gave rise to *"N-Alkylverbindungen"* (either IV or V). As "true" carboxy esters of orotic acid (II), BACHSTEZ AND WHEELER list the products from the reaction of urea with esters of oxalacetic acid which, as mentioned above, have since been shown to be hydantoin esters (I) by MITCHELL AND NYC.

Finally, BACHSTEZ treated a series of N-substituted ureas with ethyloxalacetate and obtained, after saponification with hot aqueous alkali, a new series of mono-substituted orotic acids which differed in melting points from all the previously described derivatives. Since compounds of this latter series gave higher melting points than the corresponding derivatives obtained from the reaction of silver orotate with alkyl halide, he concluded (by analogy with BEHREND AND DIETRICH'S[16] alkylation studies with 6-methyluracil) that the condensation of substituted ureas with esters of oxalacetic acid gave rise, after subsequent saponification, to N^3-substituted orotic acids (IV). This led him to the further conclusion that the products obtained from the reaction of silver orotate with alkyl halides were N^1-substituted derivatives (V).

TABLE I

A. TREATMENT OF OROTIC ACID WITH ALCOHOLS AND HYDROGEN CHLORIDE

Alcohol used	*Melting points of products obtained*		
	WHEELER[14]	BACHSTEZ[13]	*This work*
Methanol	230*	298–300	243–245
Ethanol	—	324	210–211

B. TREATMENT OF SILVER OROTATE WITH ALKYL HALIDES

Alkyl halide used	*Melting points of products obtained*		
	BISCARO AND BELLONI[15]	BACHSTEZ[13]	*This work*
CH_3I	248–250	250	243–245
C_2H_5I	200	211–213	210–211

* Uncorrected melting point. All others are corrected.

Thus, of the four series of substituted orotic acids, namely, the "O-Alkylverbindungen" (III), the N^3 (IV), the N^1 (V), and the "COOH-Gruppe alkylierte" derivatives (II), the latter series has been characterized as hydantoin esters[11]. The remaining three series remain to be proved.

With regard to the "O-Alkylverbindungen" of BACHSTEZ, we find that treatment of orotic acid with methanolic or ethanolic hydrogen chloride gives rise to crystalline compounds of melting points of 243–245° and 210° respectively rather than the reported 298° and 324° (see Table I). The spectra of these products, though generally similar to that for orotic acid[9], differ by the absence of spectral shifts in the region of pH 1–7, attributable to the absence of a free carboxyl group in the 6 positions (see spectrum and discussion of 1,3-dimethylorotic acid below). Treatment of these compounds with dilute, warm alkali followed by acidification with mineral acid re-

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generates orotic acid. It is therefore concluded that these substances are true carboxyl esters of orotic acid (II), as might be expected, rather than ethers.

With regard to the "N-Alkylverbindungen" reported as N^1-alkylated orotic acids[13], we find that the products obtained by the action of methyl or ethyl iodide upon silver orotate are identical with those obtained above from the reaction of orotic acid and alcoholic hydrogen chloride as determined by melting points, mixed-melting points, and spectral behavior. It is obvious, therefore, that both these procedures give rise to true esters of orotic acid.

For characterization of the fourth series, the "N^3-Alkylverbindungen", a methylorotic acid was prepared by the condensation of N-methylurea with ethyloxalacetate and saponified in warm alkali according to BACHSTEZ. Attempts to nitrate and decarboxylate this compound (presumably IV) to the known, monomethylated 5-nitrouracil with fuming nitric acid and sulfuric acid failed to give identifiable products. It was found, however, that simply by heating IV at 300° it was decarboxylated quantitatively to the well-characterized 3-methyluracil. Thus BACHSTEZ'S assignment of the alkyl substituent to N^3 of the pyrimidine ring in this series is correct.

It has been shown by BEHREND AND STRUVE[17] that oxidation of 6-methyluracil with potassium ferricyanide in the presence of ammonia yields uracil-6-carboxamide (VI, R, R′ = H). Adaptation of their procedure to the oxidation of 1,6-dimethyluracil resulted in an unambiguous synthesis of VI (R = CH_3, R′ = H). Treatment of this compound with warm, aqueous KOH for several hours and acidification of the solution with mineral acid gave crystalline 1-methylorotic acid (V). V, when treated with fuming nitric acid and sulfuric acid, was simultaneously nitrated and decarboxylated to yield 1-methyl-5-nitrouracil, showing quite conclusively that V is indeed the hitherto-unreported, true 1-methyl orotic acid.

Similarly, oxidation of 1,3,6-trimethyluracil with ammoniacal solution of potassium ferricyanide resulted in the isolation of 1,3-dimethyluracil-6-carboxamide (VI) as evidenced by elemental analyses and absorption spectrum (the spectrum of VI is unaltered between pH values 0 to 13). Treatment of VI with warm, aqueous potassium hydroxide gave only meager yields of the salt of 1,3-dimethylorotic acid (VII). VI was recovered unchanged when treated with sodium nitrite in 50% hydrochloric acid.

It has been reported[13] that dimethyl sulfate fails to react with orotic acid. In order to develop a simpler synthesis of VII, the action of dimethyl sulfate in aqueous alkali upon orotic acid and its monomethylated derivatives was investigated. It was found that contrary to previous reports, orotic acid may be methylated by this reagent to 3-methylorotic acid (IV). 3-Methylorotic acid, when treated with dimethyl sulfate in aqueous alkali, afforded only meager yields of VII with a goodly portion of IV recovered unchanged. 1-Methylorotic acid, however, may be methylated by this process to VII in good yields*.

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* From an examination of molecular models it is evident that the 6-carboxyl function of orotic acid creates a considerable amount of steric hindrance at the N^1-position of the pyrimidine ring and this fact would explain the difficulty to methylate orotic acid or its 3-methyl derivative at N^1. This steric consideration is of importance where synthetic routes to the nucleoside, orotidine, are contemplated. For, while a mercuri derivative of thymine may be N^1-glycosylated to give a nucleoside[18], application of this mercuri procedure to the N^1-ribosylation of a mercuri derivative of orotic acid (or its ester) may be difficult if not unlikely.

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Spectral comparison of N-methylated orotic acids.

From the foregoing discussion, the first condition necessary for the elucidation of the structure of orotidine, namely, the synthesis of *authentic* N-methyl derivatives of orotic acid, is satisfied. In previous studies, the spectra of nucleic acid derivatives and related compounds in aqueous solutions of varying pH values gave a composite picture of each of these compounds in terms of their degrees of dissociation and ionization constants[8,9,10,18,20]. It was shown that the spectrum of orotic acid exhibits three equilibria, one in acidic media ($pK = \sim 2$) and the remaining two in the alkaline region (pK values $= 9.45$ and > 13)[9]. The spectral shifts in the alkaline region, by analogy with the spectrum of uracil[9], should be due to ionization and concomitant enolization involving the 1:2 and the 3:4 positions of the heterocyclic ring. In the acid region the spectral shifts should refer to ionization of the 6-carboxy function.

Proof of these spectral assignments to orotic acid can be derived from an examination of the spectrum of 1,3-dimethylorotic acid (VII, see Fig. 1), a compound in which only the 6-carboxyl group can dissociate. The spectrum of this compound accordingly shows only one equilibrium pattern in acid solutions which is similar to the spectral pattern of orotic acid in the same pH range. With 1.3-dimethyluracil-6-carboxamide this equilibrium is absent, that is, its spectrum is unaltered between pH 0 to 13.

Mono-N-methyl derivatives of orotic acid should show two equilibria each, one in acid solutions attributable to the ionization of the carboxyl group and the other in alkaline media due to the dissociation of either the 1 or 3 hydrogen atom. The spectra of 1- and 3-methylorotic acid (Fig. 2 and 3 respectively) conform to these requirements, namely, both compounds exhibit two equilibria.

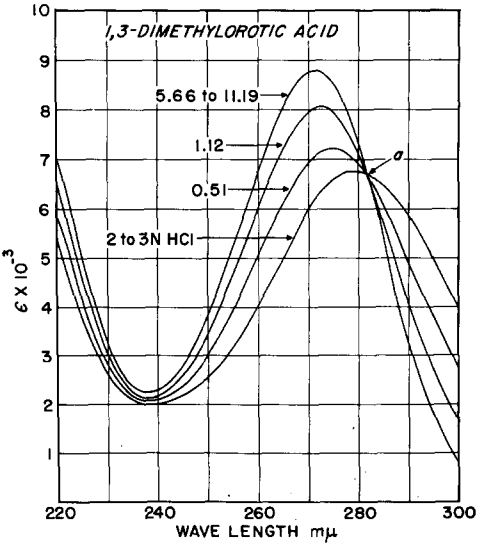

Fig. 1. 1,3-Dimethylorotic acid at pH or normality values indicated. $pKa_{(COOH)} = 0.8$.

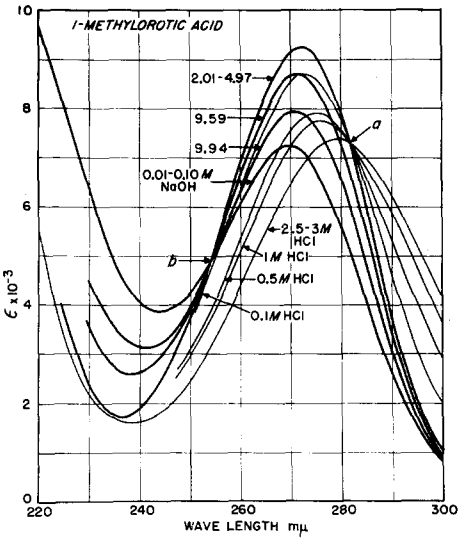

Fig. 2. 1-Methylorotic acid at pH or molarity values indicated. Curves passing through isosbestic point *a* refer to ionization of the carboxyl group, $pKa_1 = 0.7$. Curves through isosbestic *b* denote ionization of the 3-hydrogen atom, $pKa_2 = 9.82$.

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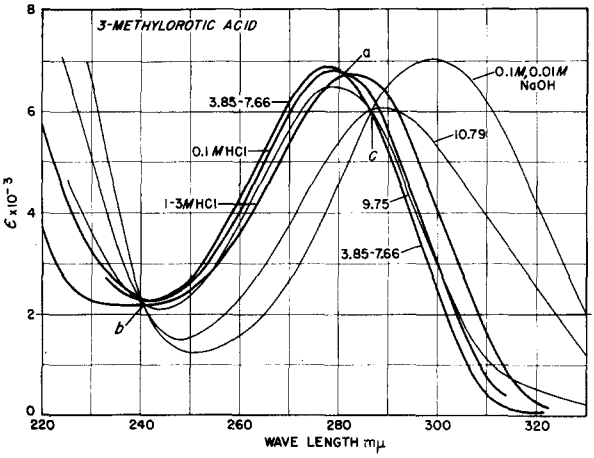
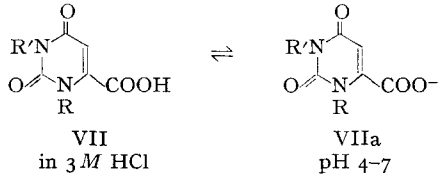


Fig. 3. 3-Methylorotic acid at pH or molarity values indicated. Isosbestic point *a* refers to the COOH dissociation, $pKa_1 < 1.0$. Curves through isosbestic point *b* and *c* denote ionization of the 1-hydrogen atom, $pKa_2 = 10.52$.

In the acid region (*i.e.*, 3 *M* HCl to pH 5) the spectra of orotic acid and of 1- and 3-methylorotic acid are essentially similar to the spectrum of 1,3 dimethylorotic acid. This similarity is based upon, (a) the decrease in extinction and bathochromic displacement of the maximum as the pH of the medium is lowered, and (b) the occurrence of a single isosbestic point for this equilibrium pattern to the right of the maximum at 282–283 mμ. By virtue of the fixed dicarbonyl structure at positions 2 and 4 in 1,3-dimethylorotic acid, the structure of orotic acid and of its mono-N-methylated derivatives in this pH range is:



VII
in 3 *M* HCl

VIIa
pH 4–7

A comparison of the curves of orotic acid and its methylated derivatives at pH 7 (structures VIIa) shows that apart from essentially-additive effects due to N^1 and/or N^3 methylation, a general similarity exists (see Fig. 4). A similar picture holds for the corresponding uracil derivatives[9].

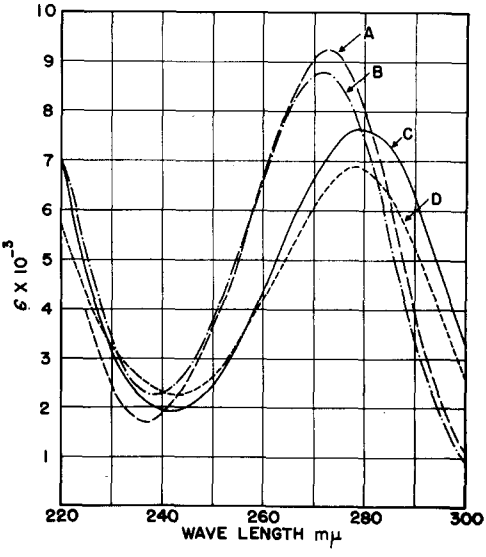


Fig. 4. 1-Methylorotic acid (A), 1,3-dimethylorotic acid (B), orotic acid[9] (C), and 3-methylorotic acid (D) all in aqueous solutions at pH 5–7.

In the alkaline region, however, the spectra of 1- and 3-methylorotic acid differ markedly from each other in a manner similar to the difference noted previously with 1- and 3-methyluracil. As with 1-methyluracil, the shifts in the spectrum of 1-methylorotic acid above pH 5 (all curves passing through isosbestic point *b*) are marked by decrease in extinction accompanied by a slight shift of the maximum toward the shorter wavelengths. A similar pattern is given by 1-methyluracil-6-carboxamide except that the carboxyl dissociation is, of course, absent (see Fig. 5).

It is evident that orotic acid (VIIa, R,R' = H, $pK = \sim 2$) exists at physiological pH in the carboxylate ion form and perhaps this can be correlated with its activity as

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a precursor. From Fig. 6, a similar structural analogy holds for orotidine and is most probably true for O5P as well.

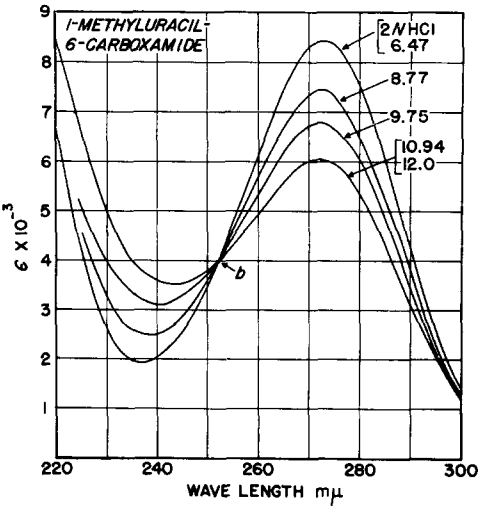

Fig. 5. 1-Methyluracil-6-carboxamide at pH values indicated. $pKa = 9.10$ for ionization at N^3.

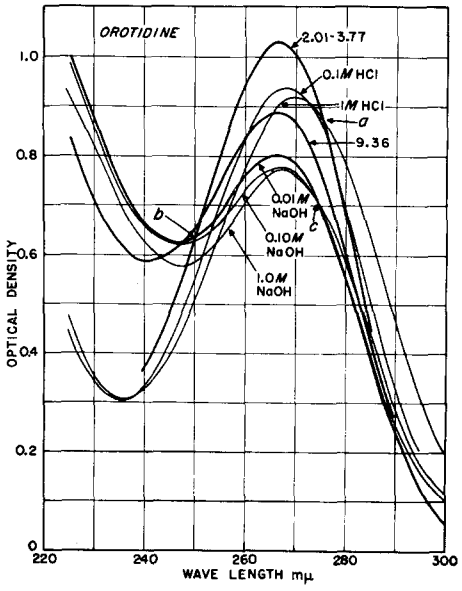

Fig. 6. Orotidine at pH or molarity values indicated. $pKa_{(COOH)} < 2$. Curves through isosbestic point *b* denote ionization at N^3, $pKa_2 \sim 9$. Curves through isosbestic point *c* refer to ionization of the sugar moiety, $pKa_3 \sim 13$.

The spectrum of 3-methylorotic acid, on the other hand, shows a pattern (Fig. 3, all curves passing through isosbestic point *b*) in which, with increasing pH of the medium, the maximum undergoes a decrease in extinction and a bathochromic shift accompanied by the appearance of a new maximum at about 300 mμ. An essentially similar pattern is seen with 3-methyluracil, the first ionic equilibrium of uracil, the second dissociation of cytosine, and the second dissociation of orotic acid, and seems to be characteristic for the dissociation of the hydrogen atom at position 1 of 2,4-pyrimidinediones. It is concluded, therefore, that above pH 7 the order of dissociation of orotic acid is similar to that of uracil[9], that is, involving ionization of the 1 and 3 hydrogen atoms respectively. It is concluded further that in this range the spectral behavior of N-methylated orotic acids bear the same relationship to orotic acid as do the mono-N-methylated uracils to uracil.

The structure of orotidine

A cursory examination of the spectrum of orotidine (Fig. 6) shows its similarity to the spectrum of 1-methylorotic acid (Fig. 2) in the acid region (curves passing through isosbestic points *a*) and in the alkaline region up to pH 12 (isosbestic *b*). Above pH 12 orotidine exhibits a new equilibrium denoted by the curves passing through isosbestic point *c* which is absent in the spectrum of 1-methylorotic acid. An analogous situation exists between 1-methyluracil and uridine[8], between 1-methylcytosine and cytidine[8], between 1-methylthymine* and 1-β-D-ribofuranosylthymine[18]. Indeed, this equilib-

* J. J. Fox and N. Yung, unpublished observations.

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rium in the high alkaline range has been found in all natural or synthetic pyrimidine nucleosides which have been investigated thus far[8,10]. It is therefore concluded that the sugar moiety of orotidine is located at position 1 of the pyrimidine ring. By virtue of the similarity of the spectrum of orotidine and O5P and the fact that O5P may be synthesized by a malt phosphatase by phosphate transfer of phenyl phosphate to orotidine[5], it is also concluded that the sugar rest is similarly located in O5P.

When treated with sodium metaperiodate, barium orotidinate consumed one mole of periodate per mole of nucleoside without the liberation of formic acid in accord with a furanose structure. The structure of orotidine is therefore 1-D-ribofuranosyl-uracil-6-carboxylic acid.

Stability of orotidine

It has been stated that, unlike uridine or cytidine, orotidine is readily hydrolyzed by dilute mineral acid with cleavage (0.5 *N* sulphuric acid at 100°) to orotic acid and ribose[7]. We find that orotidine is stable toward 1.0 *N* HCl at room temperature and may be kept in 3 *N* HCl overnight without any significant alteration of its absorption spectrum. This stability of orotidine may be of value in the quest for improved methods of its isolation from biological sources.

EXPERIMENTAL*

Uracil-6-methylcarboxylate (*II*, $R = CH_3$)

Method A. Orotic acid (5 g) is added to one liter of methanol saturated with hydrogen chloride and the mixture warmed overnight on a steam bath. The warm reaction mixture is filtered from some unchanged orotic acid. Upon cooling, the filtrate deposits needles, 2.5 g, m.p. 243–245° (corr.) to an oil. WHEELER[14] listed 230° to an oil.

Method B. Uracil-6-methylcarboxylate was prepared according to BISCARO AND BELLONI[15] and BACHSTEZ[13] from the silver salt of orotic acid and methyl iodide, m.p. 243–245° (corr. to an oil). BACHSTEZ reported 250° (corr.), BISCARO AND BELLONI gave 248–250° (corr.).

A mixed melting point of the products obtained from methods A and B above gave no depression. Their ultraviolet absorption spectra were identical: at pH 0–3.85, maximum at 285 mμ, minimum at 240 mμ; at pH 9.75, maximum at 317.5 mμ, minimum at 247.5 mμ. Above pH 10 their spectrum shifts to that for orotic acid[9].

Saponification of uracil-6-methylcarboxylate. 1.0 g of II ($R = CH_3$) obtained from either method A or B is added to 25 ml of 1 *N* sodium hydroxide and warmed for 3 to 5 minutes. Upon acidification of the solution with 5 ml of concentrated hydrochloric acid, orotic acid (0.8 g) precipitates, m.p. 345–346° (undepressed by admixture with orotic acid).

Uracil-6-ethylcarboxylate (*II*, $R = C_2H_5$)

Method A. Orotic acid (10 g) in 1 l of ethanol saturated with hydrogen chloride is treated in a manner similar to the above-mentioned methyl analog. 4 g of product are collected, m.p. 199–204°. One recrystallization from ethanol gives pure material, m.p. 210–211° (corr.).

Method B. When prepared according to method B using ethyl iodide, a product is obtained, m.p. 204–205°. One recrystallization from ethanol raised the melting point one degree, (corr.) 210–211°. Admixture of the products obtained from both methods gives no depression.

This ester is apparently quite labile and may be converted to orotic acid simply by boiling in water. Thus heating a sample of II ($R = C_2H_5$) in hot water for one hour and chromatographing the solution on Whatman No. 1 paper along with starting ester and orotic acid in butanol–water (86:14) resulted in two ultraviolet-absorbing spots, a fast-moving spot of R_F similar to that for II ($R = C_2H_5$) and a new component near the origin corresponding to orotic acid.

As with the methyl ester, short-time saponification of ethyl orotate with 1 *N* NaOH followed by acidification with mineral acid regenerates orotic acid.

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* Melting points, unless otherwise specified, are uncorrected. Analyses by the Schwarzkopf Microanalytical Laboratory and by Dr. J. F. ALICINO.

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1-Methyluracil-6-carboxamide (VI, $R' = H$, $R = CH_3$)

1,6-Dimethyluracil (5.0 g) is dissolved in 200 ml of water containing 50 ml of concentrated ammonium hydroxide. Potassium ferricyanide (78 g) is added and the flask heated at 50–70° for 7 hours. After the addition of another 25 ml of NH_4OH, the flask is cooled and filtered from potassium ferrocyanide. The filtrate is concentrated on a water bath until it is free from ammonia and the neutral solution adjusted to a volume of *ca.* 150 ml and cooled. Colorless, glistening platelets precipitate, 2.0 g, m.p. 325–328° (effervescence). An additional 0.25 g to 0.5 g may be obtained from the mother liquor. Recrystallization from water gives pure material, m.p. 327° dec. (corr.).

Anal. Calcd. for $C_6H_7O_3N_3$: C, 42.61; H, 4.14; N, 24.84.
Found: C, 42.80; H, 4.10; N, 25.08.

1-Methylorotic acid (V, $R' = CH_3$)

1-Methyluracil-6-carboxamide (8.8 g) is heated on a steam bath with 500 ml of 0.2 *N* KOH during which time the volume is kept constant by repeated additions of water. When the evolution of ammonia ceases, the basic solution is filtered from some debris and strongly acidified with concentrated HCl to yield 7.5 g (85%) of product, m.p. 245–248°. Two recrystallizations from water give hexagonal rhombs, m.p. 273–275° (with effervescence and re-solidification).

Anal. Calcd. for $C_6H_6O_4N_2$: C, 42.36; H, 3.55; N, 16.47.
Found: C, 42.56; H, 3.40; N, 16.28.

Nitration of 1-methylorotic acid—Synthesis of 1-methyl-5-nitrouracil

1-Methylorotic acid (1.0 g) is added to 20 ml of fuming nitric acid ($d = 1.5$) and warmed at 65° in an open dish until a syrup remains. When foaming stops, 5 ml of water is added after which the syrup solidifies to a white granular precipitate. Cooling and filtration of the mixture yields 0.6 g (60%), m.p. 255–259°. One recrystallization from hot water gives clusters of prisms, m.p. 258–260°. A mixed melting point with authentic 1-methyl-5-nitrouracil[19] is undepressed.

Decarboxylation of 3-methylorotic acid. Synthesis of 3-methyluracil

3-Methylorotic acid (0.5 g) was prepared according to MÜLLER [2] and BACHSTEZ [3] and heated to 310° in a test tube for about 10 minutes until the evolution of gas ceased. The contents are taken up in ethanol, decolorized with charcoal, and the filtrate concentrated to approximately 2 ml. Upon cooling, a solid crystallizes which upon filtration and recrystallization from methanol gives pure compound, m.p. 174–176° (undepressed by admixture with authentic 3-methyluracil). The ultraviolet spectrum at several pH values is identical with that reported for 3-methyluracil[9].

1,3-Dimethyluracil-6-carboxamide

1,3,6-Trimethyluracil (2.7 g) and 25 ml of concentrated ammonium hydroxide are added to a flask containing 50 ml of water and 31 g of potassium ferricyanide and heated at 50–70° for 7 hours. An additional 25 ml of concentrated ammonium hydroxide is added to the reaction mixture. Upon cooling and filtration from potassium ferrocyanide, the green filtrate is taken down to dryness on a steam bath *in vacuo* and the residue extracted several times with 50 ml portions of hot ethanol. The combined alcoholic extracts are concentrated to 25 ml and cooled. Filtration yields 1.5 g, m.p. 208–212°. Recrystallization from ethanol three times gives clusters of long prisms, m.p. 239° eff. (corr.).

Anal. Calcd. for $C_7H_9O_3N_3$: C, 45.90; H, 4.94; N, 22.94.
Found: C, 46.16; H, 4.88; N, 23.04.

The ultraviolet absorption spectrum of this compound is unaltered between pH values 0 to 13. Maximum at 272 mμ, minimum at 240 mμ.

1,3-Dimethylorotic acid

1-Methylorotic acid (0.85 g) is dissolved in 10 ml of 1 *N* sodium hydroxide and 1 ml of dimethyl sulfate is added to the solution. Upon warming on a steam bath a homogeneous solution is obtained, whereupon another 5 ml of base is added bringing the pH of the solution to approximately 8.5. After 25 minutes on the steam bath the volume is taken down *in vacuo* to *ca.* 1 ml and 2 drops of concentrated hydrochloric acid are added. A white precipitate forms immediately. After the addition of another ml of water the cold mixture is filtered and washed with cold 0.1 *N* hydrochloric acid to yield 0.25 g, m.p. 268° (dec.) of product (presumably the sodium salt). Another 0.2 g are obtained from the mother liquor. The combined precipitates are recrystallized by solution into 1.5 ml of water and adding an equal volume of concentrated hydrochloric acid. Upon cooling,

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needle clusters precipitated. A further recrystallization from *ca.* 25% HCl gives clusters of fine needles, m.p. 149–151° (corr.).

Anal. Calcd. for $C_7H_8O_4N_2$: C, 45.65; H, 4.37; N, 15.22.
Found: C, 45.40; H, 4.38; N, 15.03.

Conversion to 1,3-dimethyluracil

Several mg of 1,3-dimethylorotic acid are placed into a small test tube and heated in a bath for 15 minutes at 220°. The sample melts with effervescence and browning and solidifies upon cooling. The residue is dissolved in 0.5 ml of ethanol, treated with charcoal, and filtered. Upon addition of ether to the filtrate and cooling, crystallization occurs on the sides of the container, m.p. 88–93°. Chromatography on Whatman #1 paper along with starting material and 1,3-dimethyluracil in butanol-water (86:14) showed two components. The major component of high R_F value corresponded to that for 1,3-dimethyluracil while the slow-moving component (faint) corresponded to that for starting material. Elution of the major component and spectral determination showed that it was identical with 1,3-dimethyluracil, maximum at 266 mμ, minimum at 234 mμ, with no spectral shifts between pH values 0 to 12.

Methylation of orotic acid (a synthesis of 3-methylorotic acid)

Orotic acid (0.04 mole) is dissolved in 100 ml water containing 0.24 mole of sodium hydroxide and the solution treated with 0.15 mole of dimethyl sulfate. After 30 minutes of warming on a steam bath the solution becomes homogeneous. At the end of one hour the reaction is cooled, filtered from a small amount of insoluble material, and treated with concentrated hydrochloric acid. A solid white mass precipitates which is collected and extracted several times with hot ethanol to separate it from unchanged orotic acid. The combined alcoholic extracts are cooled after which 3-methylorotic acid separates, m.p. 306–311°. Admixture of this material with 3-methylorotic acid prepared according to MÜLLER[12] or BACHSTEZ[13] gives no depression. The absorption spectrum of this product is identical with that for 3-methylorotic acid (see Fig. 3).

3-Methylorotic acid itself, when treated with dimethyl sulfate and alkali as described with orotic acid, is recovered unaltered.

Spectrophotometric studies

Measurements were made with a Beckman Model DU spectrophotometer using techniques and buffers previously employed[10,20]. HCl (1 *N*) was taken as pH 0, 0.01 *N* NaOH as pH 12, 0.10 *N* NaOH as pH 13 and 1.00 *N* NaOH as pH 14. The apparent *pKa* values were determined spectrophotometrically by procedures previously described[9,20]. The values listed on the curves in the figures refer to pH unless specified otherwise.

A 3 mg sample of barium orotidinate (60% pure) was obtained from Dr. I. SLOTNICK of Roswell Park Memorial Institute, Buffalo, for which the authors are indeed grateful. Paper chromatography in propanol–water (3:1) showed it to be free from orotic acid, and it gave only one ultraviolet-absorbing spot. Its absorption spectrum as function of pH (Fig. 6) indicates minor impurities as shown by the lack of sharpness of the isosbestic points §.

Metaperiodate oxidation studies

Solutions of compounds ranging in concentration between 0.001 and 0.002 mM per ml were treated with excess sodium metaperiodate and titrated iodometrically according to the usual

TABLE II

METAPERIODATE OXIDATION STUDIES

Compounds	*Moles IO_4 consumed per mole*				*Acidity titrated**
	within 3 min	*1 h*	*4 h*	*24 h*	
Uridine	0.92	—	1.00	1.00	none
1-Methylorotic acid	0	0	—	0	1.0
Barium orotidinate**	0.45	0.9	0.9	0.9	none

* Equivalents per mole of compound.

** Concentration of an aqueous solution was determined from the molar absorbancy value taken from the spectrum reported by MICHELSON *et al.*[7]. The acidity titrated with 1-methylorotic acid is obviously due to the *free* carboxyl function which is absent in the barium salt of orotidine.

§ For a discussion of the significance of isosbestic points see reference[8], p. 370, and reference [21].

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procedure[22,23]. The acidity produced was determined according to JACKSON AND HUDSON[24]. Uridine and 1-methylorotic acid were run along with the barium salt of orotidine as controls (see Table II).

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SUMMARY

In order to provide a firm basis for the determination of the position of attachment of the sugar moiety to the pyrimidine ring in orotidine, a series of N-methylated orotic acid derivatives have been synthesized and their structure proved by conversion to substances of known structure. The ultraviolet absorption spectra of these N-methylorotic acid derivatives as a function of pH were determined and their "apparent" dissociation constants were measured spectrally.

It was shown that these derivatives bear the same spectral relationship to orotic acid as do the N-methylated uracils to uracil. Information relative to the structure of these compounds in aqueous solutions was deduced therefrom.

The spectrum of orotidine as a function of pH was presented and shown to be very similar to that for 1-methylorotic acid. The sugar residue of orotidine (and thereby of orotidine-5'-phosphate) is therefore linked at the #1 position of the pyrimidine ring.

Metaperiodate oxidation studies have shown that orotidine is a furanoside. The structure of orotidine is, therefore, 1-D-ribofuranosyluracil-6-carboxylic acid.

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