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Synthesis of Pyrimidin-2-one Deoxyribosides and their Ability to Support the Growth of the Deoxyriboside-Requiring Organism *Lactobacillus acidophilus* R26

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In the past, several methods for reducing pyrimidine nucleosides and nucleotides have been described. Levene & LaForge (1912) reduced uridine and cytidine with hydrogen by using palladium as catalyst. Burke (1955) made use of sodium and ethanol in liquid ammonia. Also, hydrogen with rhodium on alumina (Cohn & Doherty, 1956; Green & Cohen, 1957) was shown to reduce specifically the double bond in the 5,6-position of pyrimidines, their nucleosides and nucleotides.

In this Laboratory it was found (Laland & Roth, 1956; Haavaldsen, Laland, McKinley-McKee & Roth, 1959) that sodium amalgam in water reduced pyrimidine nucleosides and nucleotides as shown by the disappearance of their light absorption at 260 mμ. From the reduction mixture, the sugar or the sugar phosphate respectively could be isolated after mild acid hydrolysis.

We have examined some of the products produced when thymidine is reduced with sodium amalgam in dilute acetic acid. Our attention was drawn to a substance which exhibited a violet fluorescence when exposed to ultraviolet light, which had a maximum light absorption in 0·1N-hydrochloric acid at 324 mμ and no absorption at 260 mμ, and which supported the growth of the deoxyriboside-requiring strain *Lactobacillus acidophilus* R26 Orla Jensen. The substance is most likely 1-(2-deoxy-β-D-*erythro*pentofuranosyl)-5-methylpyrimidin-2-one (IIC) (referred to as 5-methylpyrimidin-2-one deoxyriboside below). By treating uracil in a similar manner, a substance was isolated which proved to be identical with the pyrimidin-2-one (IIA) prepared from 2-aminopyrimidine (Brown, 1950). Similarly, when thymine and deoxyuridine were treated with sodium amalgam in dilute acetic acid, 5-methylpyrimidin-2-one (IIB) and pyrimidin-2-one deoxyriboside (IID) respectively were isolated.

The efficiency of the growth-promoting property of 5-methylpyrimidin-2-one deoxyriboside towards *Lactobacillus acidophilus* has been determined and its possible incorporation into DNA examined.

A preliminary account of some of this work has already been given (Helgeland, Laland & Serck-Hanssen, 1963).

MATERIALS AND METHODS

Melting points are uncorrected.

Nucleosides and pyrimidines. Thymidine and deoxyuridine were purchased from Mann Research Laboratories Inc., New York 6, N.Y., U.S.A., thymine from L. Light and Co. Ltd., Colnbrook, Bucks., uracil from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and 5,6-dihydrothymidine from Sigma Chemical Co., St Louis, Mo., U.S.A. Pyrimidin-2-one was a gift from Professor D. J. Brown, The Australian National University, Canberra.

Tritiated thymidine. [^{3}H]Thymidine, labelled in the methyl group, was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The specific activity was 9·64 c/m^{3}mole.

Micro-organism. Lactobacillus acidophilus R26 Orla Jensen was a gift from Professor E. Hoff-Jørgensen, Universitetets Biokemiske Institutt, Juliane Maries vej 30, Copenhagen, Denmark, and was maintained as described by Hoff-Jørgensen (1957).

Microbiological assay. The inoculum was prepared in the following manner. A portion (2·5 ml.) of basal medium (Hoff-Jørgensen, 1957) was mixed in a centrifuge tube with 2·5 ml. of a solution containing 0·1 μmole of thymidine, autoclaved for 10 min. at 120° and inoculated from the stock culture. After 24 hr. at 37° the bacteria were centrifuged down, washed with 10 ml. of sterile 0·9% NaCl and again centrifuged. The washed bacteria were suspended in 10 ml. of sterile 0·9% NaCl and the suspension was used as inoculum.

For bioautography, 80 ml. of basal medium was added to 80 ml. of water and 3·2 g. of Difco Bacto-Agar, the mixture autoclaved at 120° for 10 min. and cooled to 45°, 5 ml. of inoculum added and the mixture poured into a trough. The paper strip to be tested was applied to the agar for 15 min. and the agar incubated for 18 hr. at 37°. Since addition to the medium of 2,3,5-triphenyltetrazolium chloride completely inhibited growth, this compound could not be used to facilitate the detection of growth zones.

For growth curves, 1 ml. of basal medium and 1 ml. of an aqueous solution of the material to be tested were mixed in tubes (1 cm. diam. × 7·5 cm. long). The tubes were autoclaved at 120° for 10 min., inoculated with 0·05 ml. of inoculum and incubated without shaking at 37°. At intervals the tubes were read at 650 mμ in a Coleman Junior spectrophotometer.

Thymidine phosphorylase. This enzyme was prepared from horse liver by the method of Friedkin & Roberts (1954).

Paper chromatography. For preparative purposes descending chromatography on Whatman no. 3MM paper

was used, otherwise Whatman no. 1. The solvent systems used were butan-1-ol–ethanol (96%, v/v)–aq. NH_3 (sp.gr. 0·88)–water (4:1:2:1, by vol.) and propan-1-ol–aq. NH_3 (sp.gr. 0·88) (3:2, v/v). Chromatograms were photographed in the ultraviolet light by using a Chromatolite lamp (Hanovia Lamps, Slough, Bucks.). Deoxyribose-containing substances were detected by the diphenylamine method (Buchanan, Dekker & Long, 1950).

5,6-Dihydropyrimidines and deoxyribosides were detected by spraying with 0·5N-NaOH. After 30 min. at room temperature the chromatogram was sprayed with the *p*-dimethylaminobenzaldehyde reagent (Fink, McGaughey, Cline & Fink, 1956).

Elementary analysis. These were done by Dr Ing. A. Schoeller, Mikroanalytisches Laboratorium, Kronach/Ofr., Germany.

Infrared-absorption spectra. These were measured in a Perkin-Elmer spectrophotometer model 21 by Miss E. Augdahl, Department of Chemistry, University of Oslo. The compounds were pressed in potassium bromide disks.

Measurement of radioactivity. Radioactivity was measured at infinite thinness with a Tracerlab flow counter.

Ultraviolet-absorption spectra. These were measured in 0·1N-HCl, water and 0·1N-NaOH by using a Beckman DU spectrophotometer.

Spectrophotometric determination of dissociation constants. The pK_a values were determined by a spectrophotometric method (Shugar & Fox, 1952). The following solutions were used: 0·1N-HCl (pH 1), 0·01N-HCl (pH 2), 0·05M-glycine–HCl buffer (pH 2·2–5·0), 0·05M-citrate buffer (pH 5·0–6·2), 0·1M-phosphate buffer (pH 6·2–8·0), 0·05M-glycine–NaOH buffer (pH 8·6–10·6), 0·01N-NaOH (pH 12) and 0·1N-NaOH (pH 13).

Reduction of pyrimidines and pyrimidine deoxyribosides. An aqueous solution of 0·2N-acetic acid containing 4 mg. of pyrimidine or nucleoside/ml. was used. If the total volume of the solution was less than 100 ml., 125 mg. of 3% sodium amalgam/ml. of solution was used. With larger portions, the yield of pyrimidin-2-one was increased if 1% sodium amalgam was used (400 mg./ml.). The amalgam was added in small portions with stirring.

Preparation of 5[^{3}H]-methylpyrimidin-2-one deoxyriboside. A portion (4·05 mg.) of [^{3}H]thymidine was dissolved in 1 ml. of 0·2N-acetic acid and treated with 125 mg. of 3% sodium amalgam. The desired product was isolated from the reaction mixture by paper chromatography on Whatman no. 1 paper in the butan-1-ol–ethanol–aq. NH_3–water solvent followed by paper electrophoresis on Whatman no. 1 paper in 0·1N-NaOH–borate buffer, pH 10. The product was finally purified by running it twice on a paper chromatogram in the above solvent system.

RESULTS

Examination of reduction mixture from thymidine. On paper chromatography in butan-1-ol–ethanol–aq. ammonia (sp.gr. 0·88)–water the fastest-moving substance (R_F 0·62) exhibited a violet fluorescence in ultraviolet light, gave a positive diphenylamine reaction and is the 5-methylpyrimidin-2-one deoxyriboside. A slower-moving band (R_F 0·58) gave positive diphenylamine and *p*-dimethylaminobenzaldehyde reactions and was most likely 5,6-dihydrothymidine. In addition, there were three unidentified slower (but close-moving), fluorescent, diphenylamine-positive and ultraviolet-absorbing substances. Bioautography of a chromatogram revealed the presence of only two growth-supporting zones, namely 5-methylpyrimidin-2-one deoxyriboside and a zone consisting of the three close-moving substances.

Reduction mixtures from thymine, uracil or deoxyuridine. The chromatograms showed a pattern similar to that described for thymidine. In each case a pyrimidin-2-one was present, and it moved ahead of the corresponding 5,6-dihydro compound which again moved ahead of three unidentified substances. The 5-methylpyrimidin-2-one obtained from thymine exhibited a violet fluorescence in ultraviolet light, whereas pyrimidin-2-one and its deoxyriboside did not.

5-Methylpyrimidin-2-one deoxyriboside. A solution of reduced thymidine (from 200 mg. of thymidine) was passed through a column (2 cm. diam. × 24 cm. long) of Dowex 1 (X4) previously washed with 0·005M-potassium tetraborate (Khym & Zill, 1952) and the column subsequently eluted with water. The combined water eluate was concentrated in vacuum and chromatographed in the butan-1-ol–ethanol–aq. ammonia (sp.gr. 0·88)–water solvent. The fluorescent band of 5-methylpyrimidin-2-one deoxyriboside was eluted with water, concentrated to dryness in vacuum, and the product (6 mg.) crystallized from ethanol as white plates. It had m.p. 140–155° but sintered at 104°; the light-absorption maxima at pH 1, 6, and 13 were at 324, 314 and 321 mμ respectively (ϵ 8200, 6100 and 7100 respectively) (Found: C, 52·3; H, 6·2; N, 12·3. Calc. for $C_{10}H_{13}N_2O_4$: C, 53·1; H, 6·2; N, 12·4%).

5-Methylpyrimidin-2-one. A solution of reduced thymine (from 1·5 g. of thymine) was passed through a column (3·2 diam. × 60 cm. long) of Amberlite IR-120 (H^+ form). The column was first eluted with N-hydrochloric acid and subsequently with 2N-hydrochloric acid which eluted the 5-methylpyrimidin-2-one. The solution was concentrated to dryness in vacuum and chromatographed in the butan-1-ol–ethanol–aq. ammonia (sp.gr. 0·88)–water solvent. The fluorescent band of 5-methylpyrimidin-2-one was eluted with water, concentrated to dryness and the material (44 mg.) crystallized from ethyl acetate as long needles. It had m.p. 210°, and the light-absorption maxima at pH 1, 6 and 13 were at 322, 310 and 303 mμ respectively (ϵ 5700, 4800 and 4500 respectively) (Found: C, 54·5; H, 5·6; N, 25·4. Calc. for $C_5H_7ON_2$ C, 54·6; H, 5·5; N, 25·5%).

Pyrimidin-2-one. This substance was isolated from a solution of reduced uracil (1·5 g.) in a

Table 1. *Absorption maxima at different* pH *values of pyrimidin-2-one and derivatives*

	$\lambda_{max.}$ (mμ)				
	Pyrimidin-2-one				
pH	(*a*)*	(*b*)†	Pyrimidin-2-one 2′-deoxyriboside	5-Methyl-pyrimidin-2-one	5-Methylpyr-imidin-2-one 2′-deoxyriboside
0	309	309	311	322	324
6	298	298	303	310	314
13	292	292	315	303	321

* Quoted from Brown, Hoerger & Mason (1955). † Prepared from uracil.

manner identical with that described above for 5-methylpyrimidin-2-one. The material obtained (44 mg.) crystallized from ethyl acetate in long needles. The light-absorption maxima at pH 1, 6 and 13 were at 309, 298 and 292 mμ respectively.

Pyrimidin-2-one deoxyriboside. A solution of reduced uracil deoxyriboside was concentrated in vacuum and chromatographed on paper in the butan-1-ol–ethanol–aq. ammonia (sp.gr. 0·88)–water solvent. The pyrimidin-2-one deoxyriboside was eluted and rechromatographed in the same solvent system. This substance was required for its ultraviolet-absorption spectra only and no attempt was made to obtain it in crystalline form.

Physical and chemical properties of pyrimidin-2-one and derivatives. The material prepared from uracil by the sodium amalgam method had an infrared-absorption spectrum (see Fig. 1*a* and 1*b*) identical with that of pyrimidin-2-one prepared from 2-aminopyrimidine (Brown, 1950). The two substances had identical ultraviolet-absorption maxima (see Table 1) at pH 0, 6 and 13. The dissociation constants of the substances were also similar (see Table 2). Neither substance was fluorescent in ultraviolet light.

The infrared-absorption spectra of 5-methylpyrimidin-2-one and of 5-methylpyrimidin-2-one deoxyriboside are shown in Fig. 1(*c*) and 1(*d*).

The ultraviolet-absorption spectra of 5-methylpyrimidin-2-one deoxyriboside, 5-methylpyrimidin-2-one and pyrimidin-2-one deoxyriboside respectively are shown in Figs. 2, 3 and 4. Characteristically they all have ultraviolet-absorption maxima above 300 mμ and no absorption at about 260 mμ. The exact locations of the absorption maxima at different pH values are recorded in Table 1.

The spectra of 5-methylpyrimidin-2-one deoxyriboside and pyrimidin-2-one deoxyriboside in 0·1 N-sodium hydroxide are not recorded in Figs. 2 and 4 since a rapid irreversible increase in light absorption takes place. This increase is followed by a decrease, and after 10 days at room temperature in 0·1 N-sodium hydroxide the original maxima had disappeared and new maxima at lower wavelengths appeared. For 5-methylpyrimidin-2-one deoxyriboside, the new maximum is located at 273 mμ, and for pyrimidin-2-one deoxyriboside at 267 mμ.

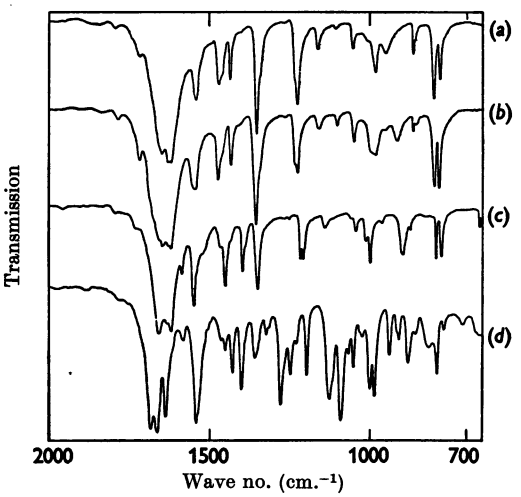


Fig. 1. Infrared-absorption spectra of (*a*) pyrimidin-2-one made from 2-aminopyrimidine, (*b*) pyrimidin-2-one made from uracil, (*c*) 5-methylpyrimidin-2-one, and (*d*) 5-methylpyrimidin-2-one deoxyriboside.

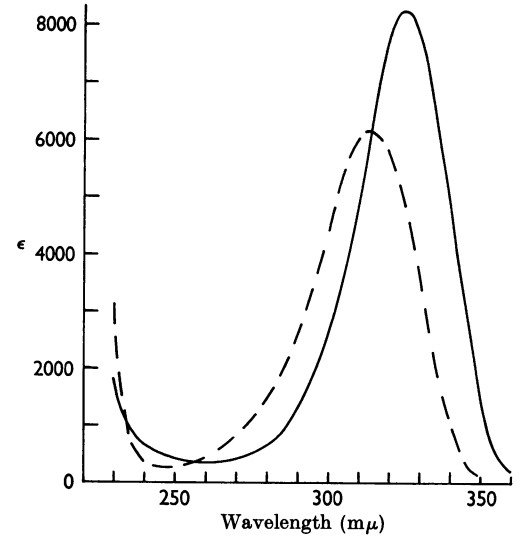


Fig. 2. Absorption spectra of 5-methylpyrimidin-2-one deoxyriboside in 0·1 N-HCl (——) and water (- - - -).

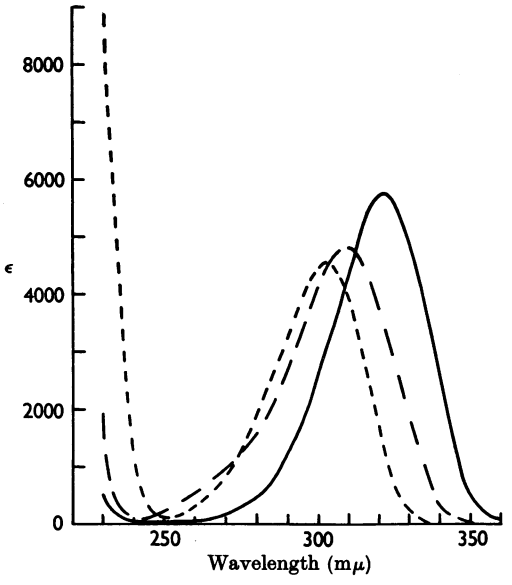


Fig. 3. Absorption spectra of 5-methylpyrimidin-2-one in 0·1 N-HCl (——), water (– – – –) and 0·1 N-NaOH (- - - - -).

A comparison of the dissociation constants of the various pyrimidin-2-one derivatives described in the present paper are given in Table 2. They all have a dissociation constant at approx. pH 2, and the dissociation constant at approx. pH 9 is lacking in the nucleosides. The nucleosides appear to have a group with a dissociation constant at about pH 12 (probably due to the sugar; Fox & Shugar, 1952), since a significant displacement of the absorption maxima to larger wavelengths is observed when changing pH from 11 to 12. The irreversible changes of the nucleosides which take place in alkali made it difficult to assign a definite value for this dissociation constant.

Ability of 5-methylpyrimidin-2-one deoxyriboside to support the growth of Lactobacillus acidophilus. The growth curves for four different concentrations of 5-methylpyrimidin-2-one deoxyriboside and those of corresponding amounts of thymidine are shown in Fig. 5. It is apparent that 5-methylpyrimidin-2-one deoxyriboside supports the growth of the organism. As calculated from the turbidity of the cultures after 21 hr. of growth in the concentration range 0–12·5 μM, 5-methylpyrimidin-2-one

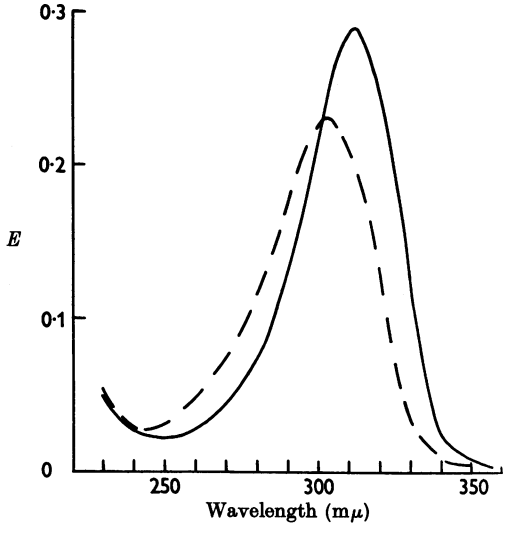


Fig. 4. Absorption spectra of pyrimidin-2-one deoxyriboside in 0·1 N-HCl (——) and water (– – – –).

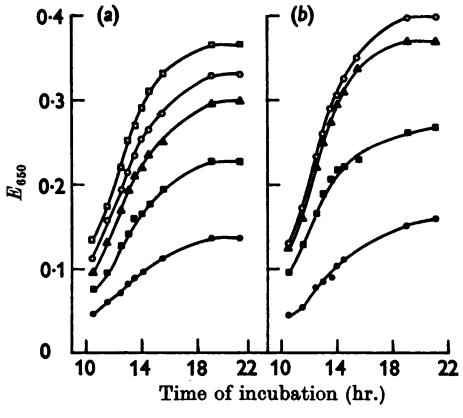


Fig. 5. Growth curves of *L. acidophilus* in the presence of 5-methylpyrimidin-2-one 2′-deoxyriboside (*a*) and thymidine (*b*) respectively. In both cases the following concentrations (μM) were used: ●, 0; ■, 2·5; ▲, 7·5; ○, 12·5; □, 25. With thymidine, the curves obtained with the two highest concentrations were identical and thus only the curve for 12·5 μM is recorded.

Table 2. *Dissociation constants of pyrimidin-2-one and derivatives*

	Pyrimidin-2-one		Pyrimidin-2-one 2′-deoxyriboside	5-Methyl pyrimidin-2-one	5-Methylpyrimidin-2-one 2′-deoxyriboside
	(*a*)*	(*b*)†			
pK_{a_1}	2·24	2·3	2·1	2·4	2·3
pK_{a_2}	9·17	9·2	Approx. 12	9·3	Approx. 12

* Quoted from Brown *et al.* (1955).

† Prepared from uracil.

deoxyriboside had on an average 85% of the activity of thymidine. However, 25 μM-5-methylpyrimidin-2-one deoxyriboside does not give as good a growth as does 12·5 μM-thymidine, which gives maximal growth. Hence, such high concentrations of 5-methylpyrimidin-2-one deoxyriboside are inhibitory.

5,6-Dihydrothymidine when tested in a similar manner did not support the growth of the organism.

Possible incorporation of 5-methylpyrimidin-2-one deoxyriboside into deoxyribonucleic acid in Lactobacillus acidophilus. A portion (6·1 μmoles) of 5[^{3}H]-methylpyrimidin-2-one deoxyriboside having $4{\cdot}5 \times 10^6$ counts/min./μmole was dissolved in 2·6 ml. of 70% (v/v) ethanol and kept for 24 hr. The solution was then added aseptically to 1 l. of autoclaved medium (500 ml. of basal medium and 500 ml. of water) to give a final concentration of 6·1 μM. The culture was incubated for 15½ hr. at 37°, and the cells were harvested, washed four times with 250 ml. of 0·9% sodium chloride each time and disintegrated in a Hughes (1951)bacterial press. The broken cells were extracted three times with 20 ml. of 0·6N-perchloric acid. Then 3·1 mg. of DNA was isolated from the cell residue by the procedure of Schneider (1957). It was dissolved in 2 ml. of water and 0·1 ml. portions were plated and counted in duplicate. The samples were not different from that of background and hence no incorporation of 5[^{3}H]-methylpyrimidin-2-one had taken place.

5-Methylpyrimidin-2-one deoxyriboside as substrate for horse-liver thymidine phosphorylase. The incubation mixture contained 5-methylpyrimidin-2-one deoxyriboside (1·4 mg.), enzyme (1 mg.) in 0·5 ml. of 0·05M-phosphate buffer, pH 7·4. As a control, thymidine (1·0 mg.) and enzyme (0·5 mg.) in 0·5 ml. of 0·05M-phosphate buffer, pH 7·4, was used. Both mixtures were incubated for 15 hr. at 37°. Acetone (10 ml.) was added to precipitate the enzyme. The filtrates were concentrated *in vacuo* to dryness and dissolved in 1 ml. of water, and 0·1 ml. was put on paper and run together with deoxyribose 1-phosphate in the propan-1-ol–aq. ammonia (sp.gr. 0·88) system and the chromatogram sprayed with the diphenylamine reagent. No deoxyribose 1-phosphate was detectable in the 5-methylpyrimidin-2-one deoxyriboside incubation mixture whereas the thymidine incubation mixture contained deoxyribose 1-phosphate.

DISCUSSION

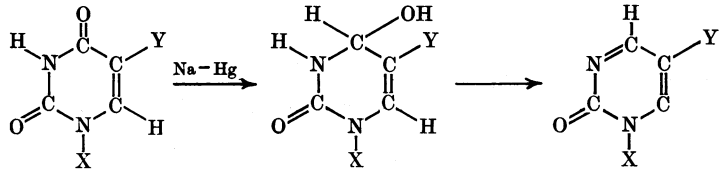
Treatment of uracil (IA), thymine (IB) and their deoxyribosides (IC, ID) with sodium amalgam in aqueous acetic acid has been shown to yield among other products the corresponding pyrimidin-2-ones (IIA, IIB, IIC, IID respectively). The reaction mechanism shown in Scheme 1 is suggested.

It has previously been shown (Marshall & Walker, 1951; Brown & Short, 1953) that pyrimidin-2-one exists in neutral solution mainly as the amide. The great similarity between the light-absorption spectrum of pyrimidin-2-one deoxyriboside and pyrimidin-2-one (Table 1) in neutral solution confirms the structure of the latter.

The 5-substituted pyrimidin-2-one and the corresponding deoxyriboside exhibit in neutral solution a bathochromic shift in the absorption maximum of 11–13 mμ, as compared with the unsubstituted pyrimidin-2-one. Substitution of a methyl group in the 5-position in uracil to give thymine produces a bathochromic shift of 3–5 mμ in the absorption maximum. It is therefore not unreasonable to assume that the bathochromic shift in the absorption maxima in the 5-substituted pyrimidin-2-one is due to the 5-substitution.

It is only the 5-substituted pyrimidin-2-ones that exhibit fluorescence in ultraviolet light.

5-Methylpyrimidin-2-one and its deoxyriboside have almost identical spectra in 0·1N-hydrochloric acid. This is also the case with pyrimidin-2-one and its deoxyriboside. This finding indicates a similar



(I) (II)

(IA) and (IIA): X = H; Y = H
(IB) and (IIB): X = H; Y = CH_3
(IC) and (IIC): X = 2-deoxyribosyl; Y = CH_3
(ID) and (IID): X = 2-deoxyribosyl; Y = H

Scheme 1.

structure for the cations. Formation of the cations is due to protonization of the imide nitrogen in the 3-position in the ring. In alkali, however, the pyrimidin-2-ones and the corresponding deoxyribosides have very different spectra. 5-Methylpyrimidin-2-one or pyrimidin-2-one have an acid character owing to the dissociation of the $-N_{(1)}H-CO-$ group, but this cannot occur in the deoxyribosides because of replacement of hydrogen by deoxyribosyl.

The glycosidic linkage in the pyrimidin-2-one deoxyriboside and the 5-methyl derivative is labile (gives the diphenylamine reaction) to acid. The stability of this linkage to acid in deoxyuridine and thymidine has been explained by the aromatic character of the ring, due to resonance in the molecule (Daly, Allfrey & Mirsky, 1949). The reason for the acid lability of the pyrimidin-2-one deoxyribosides, however, is probably due to the protonation of the $N_{(3)}$ atom (Dekker, 1960).

5-Methylpyrimidin-2-one deoxyriboside supports the growth of the deoxyriboside-requiring organism *L. acidophilus* R 26, but is not incorporated as such into DNA. Thus the enzyme trans-*N*-deoxyribosylase which is present in this organism can use the substance as substrate. This is in accordance with the low specificity of this enzyme (Roush & Betz, 1958). Other enzymes, such as thymidine phosphorylase involved in deoxyriboside metabolism, are more specific and cannot use 5-methylpyrimidin-2-one deoxyriboside as a substrate.

SUMMARY

1. Pyrimidin-2-one, 5-methylpyrimidin-2-one and the corresponding 2′-deoxyribosides have been isolated after reduction of uracil, thymine and their 2′-deoxyribosides respectively with sodium amalgam in aqueous acetic acid.

2. The pyrimidin-2-ones described have ultraviolet-absorption maxima in the region 292–324 mμ, the location of the maximum being pH-dependent. They all exhibit a dissociation constant at approx. pH 2 due to the protonization of the $N_{(3)}$ of the ring. Pyrimidin-2-one and 5-methylpyrimidin-2-one have a dissociation constant at approx. pH 9 due to the dissociation of the group $-C_{(2)}O-N_{(1)}H-$. As expected, the 2′-deoxyribosides show no dissociation in this region. The 2′-deoxyribosides of pyrimidin-2-one and 5-methylpyrimidin-2-one give the diphenylamine reaction and possess therefore an acid-labile glycosidic linkage.

3. The 5-methylpyrimidin-2-one 2′-deoxyriboside supports the growth of the deoxyriboside-requiring organism *Lactobacillus acidophilus* R 26 Orla Jensen. Its activity as a growth factor is about 85% of that of thymidine. By using 5[^{3}H]-methylpyrimidin-2-one 2′-deoxyriboside it was shown that the 5-methylpyrimidin-2-one ring was not incorporated into DNA.

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