crystals. These were extracted with 5 ml. of water. Evaporation of the extract left 0.327 g. (16%) of crystals, m.p. 239–241° or m.p. 237–239° after crystallization from dimethylformamide. This material gave an infrared spectrum identical with that of 2,3′-anhydro-1-(*cis*-3,*trans*-4-dihydroxycyclopentyl)-thymine (XII).

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

Synthesis and Proof of Structure of the Cyclopentane Isostere of Thymidine. Steric Control in the Prins Condensation of Formaldehyde with a Cyclopentenylthymine[1]

BY K. C. MURDOCK AND ROBERT B. ANGIER

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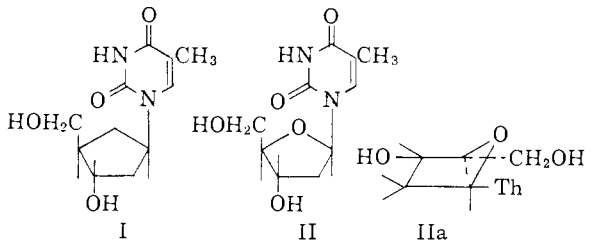
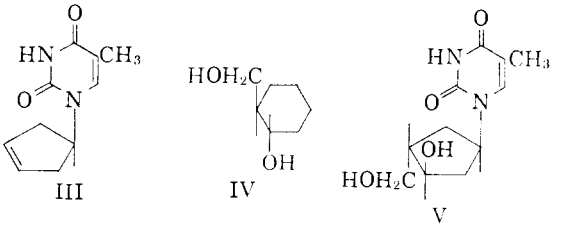
1-(3-Cyclopenten-1-yl)-thymine (III) was converted stereospecifically to the cyclopentane isostere I of thymidine. A convenient reaction sequence developed for this transformation included: (a) sulfuric acid-catalyzed condensation with paraformaldehyde in acetic acid, (b) reaction with acetic anhydride and sulfuric acid for the acetolysis of cyclic formals and (c) methanolysis of acetate groups. The isolation of intermediates was not required. The relative configurations at the three asymmetric centers of the diol I were established by two sequences of reactions leading to definitive intramolecular ring closures. The resulting cyclized products IX, X and XIV are anhydronucleoside analogs. It is postulated that the sterospecificity in the synthesis of I results from the formation of a non-classical carbonium ion intermediate such as XVb in which formaldehyde is bound to the thyminyl group. The cyclopentane isostere XVIII of thymidylic acid was synthesized.

Antimetabolites or possible metabolic substitutes for thymidine are of special interest because the enzyme systems required for the incorporation of thymidine into deoxyribonucleic acid seem to be uniquely associated with the capacity for rapid cell proliferation.[2a] We hoped to prepare the cyclopentane isostere I of thymidine(II) as a likely tool for cancer chemotherapy,[2b] for the induction of mutations,[3] or for the study of nucleic acid metabolism.[4] The steric relationships in I and II should be very similar. A comparison of



I II IIa

results from X-ray, molecular polarizability and proton magnetic resonance studies of nucleosides and other compounds shows the expected similarities in bond lengths and angles in tetrahydrofuran-[5] and cyclopentane[6] ring systems. In both systems there are favored conformations in which four of the ring atoms are approximately coplanar while a fifth atom is puckered about 0.5 Å. out of the plane of the other four. From proton magnetic resonance spectroscopic data it has been concluded that thymidine has considerable conformational rigidity, with the oxygen atom of the tetrahydrofuran ring puckered up as indicated in conformation IIa.[5b] From an inspection of molecular models an analogous conformation would also be predicted as the most stable form for the cyclopentane isostere I, since it should minimize non-bonded interactions between the substituents on the cyclopentane ring. Syntheses leading to 1-(3-cyclopenten-1-yl)-thymine (III) are reported separately.[7] A stereospecific approach to the problem of the three



III IV V

asymmetric centers in the diol I *via* an epoxide derivative of the olefin III was theoretically attractive but experimentally impractical.[8] Another possible approach to the diol I was to apply the Prins reaction to the olefin III. This reaction, the acid-catalyzed condensation of olefins with formaldehyde, has been used occasionally for the synthesis of 1,3-diols. Reaction in sulfuric acid has given 1,3-diols in fair to good yields, predominantly in

(1) Presented at the 141st Meeting of the American Chemical Society, Washington, D. C., March, 1962.

(2) (a) J. N. Davidson in "Biological Structure and Function," T. W. Goodwin and O. Lindberg, eds., Academic Press, Inc., New York, N. Y., 1961, p. 95; (b) A. D. Welch, *Cancer Res.*, **21**, 1475 (1961); S. Kit, *ibid.*, **20**, 1121 (1960); D. F. Gamble, H. W. Bond and A. Burger in "Medicinal Chemistry," 2nd ed., A. Burger, ed., Interscience Publishers, Inc., New York, N. Y., 1960, p. 1077; C. P. Rhoads, *Acta Unio Intern. contra Cancrum*, **16**, 522 (1960).

(3) E. Freese, *Proc. Natl. Acad. Sci. U. S.*, **45**, 622 (1959); R. M. Litman and A. B. Pardee, *Biochim. Biophys. Acta*, **42**, 131 (1960); Z. Lorkiewicz and W. Szybalski, *Biochem. Biophys. Res. Comm.*, **2**, 413 (1960).

(4) R. E. Handschumacher and A. D. Welch in "The Nucleic Acids," Vol. 3, E. Chargaff and J. N. Davidson, eds., Academic Press, Inc., New York, N. Y., 1960, p. 453.

(5) (a) E. Alver and S. Furberg, *Acta Chem. Scand.*, **13**, 910 (1959); M. Spencer, *Acta Cryst.*, **12**, 59 (1959); (b) R. U. Lemieux, *Can. J. Chem.*, **39**, 116 (1961); C. D. Jardetzky, *Fed. Proc. Abstr.*, **20**, 355 (1961); "Abstr. 5th Internat. Congress Biochem.," Moscow, 1961, p. 70; *J. Am. Chem. Soc.*, **83**, 2919 (1961); **84**, 62 (1962); **82**, 229 (1960).

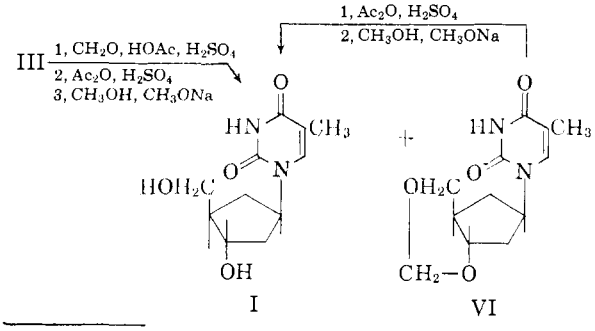
(6) F. V. Brutcher, Jr., T. Roberts, S. J. Barr and N. Pearson, *ibid.*, **81**, 4915 (1959); R. A. Raphael in "The Chemistry of Carbon Compounds," Vol. IIA, E. H. Rodd, ed., Elsevier Press, New York, N. Y., 1953, p. 76; R. J. W. Le Fèvre and C. G. Le Fèvre, *Chemistry & Industry*, 54 (1956); M. Löw, *Tetrahedron Letters*, No. **1**, 3 (1960); A. Almenningen, O. Bastiansen and P. N. Skancke, *Acta Chem. Scand.*, **15**, 711 (1961).

(7) K. C. Murdock and R. B. Angier, *J. Org. Chem.*, **26**, 3317 (1962).

(8) K. C. Murdock and R. B. Angier, *J. Am. Chem. Soc.*, **84**, 3718 (1962).

the form of their cyclic formals (*m*-dioxanes).[9] These formals are remarkably resistant to hydrolysis, but have been cleaved smoothly by acetolysis with acetic anhydride; methanolysis of acetate groups then gave the free diols.[10] We found that the olefin III in sulfuric acid did not react with formaldehyde, but instead underwent an intriguing intramolecular cyclization, reported separately.[8] The Prins reaction in acetic acid containing only a catalytic amount of sulfuric acid characteristically gives complex mixtures of products (*cf.* ref. 11), but at least in some cases the reaction is stereospecific. Thus Blomquist and Wolinsky,[12] and Smissman and Mode[13] recently reinvestigated the product mixture from cyclohexene and proved that the diacetate and formal components were derivatives of a diol IV in which the hydroxyl- and hydroxymethyl substituents were exclusively *trans*. Analogous stereochemistry was also reported for the reaction with a rigid cyclohexene derivative, *trans*-Δ^2-octalin.[14] These findings augured well for the conversion of the cyclopentenylthymine III to the desired *trans*-diol I. But it was expected that formaldehyde would also attack concurrently and preferentially from the lower, less hindered side of the cyclopentene ring of III to give a larger amount of another *trans*-diol, V.

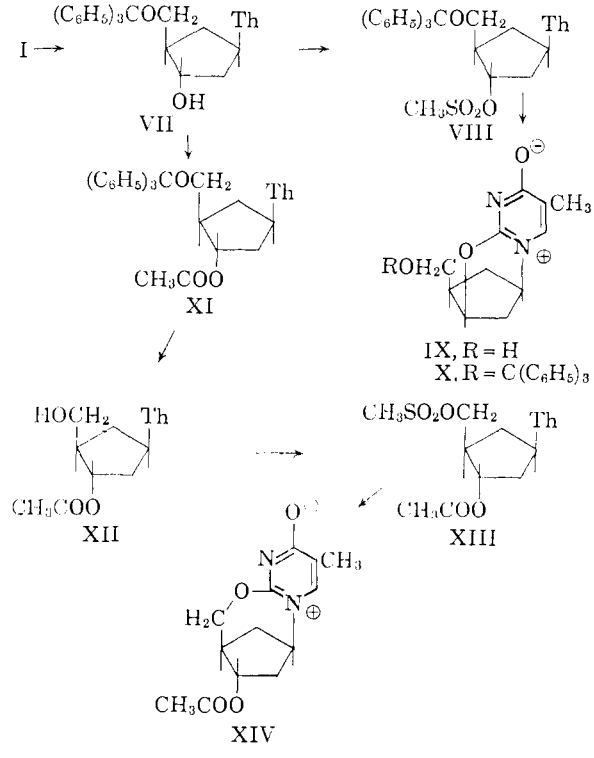
Actually, the results were surprisingly satisfactory. A three-step sequence was carried out without attempting to purify the intermediates. Condensation with formaldehyde in acetic acid containing sulfuric acid (26 mole % relative to the olefin III) was followed by acetolysis[10] with acetic anhydride in the presence of much less sulfuric acid (2 mole %), to cleave formals. Subsequent removal of acetate groups with sodium methoxide in methanol gave a mixture which was resolved[15,16] by partition chromatography.[15] The compounds isolated included starting olefin (III, 17%) and two substances with compositions corresponding to the desired diol (I, 9%) and its formal (VI, 17%). The structure of the formal VI was established by conversion to the diol I, using another acetolysis–methanolysis sequence in which an increased amount of sulfuric acid was present during the acetolysis.



The sequence from the olefin III was substantially improved by simply allowing all of the sulfuric acid from the first step to remain during the acetolysis. After subsequent methanolysis neither the starting olefin nor the formal was detected in the reaction mixture. The diol I was isolated in 30% yield without recourse to partition chromatography. An interesting sulfonate salt was obtained as a by-product (see below).

As would be expected, the pK_a of diol I (9.9) is very close to that of thymidine (II, pK_a 9.8).[17] The ultraviolet absorption maximum appears at a somewhat higher wave length (273 *vs.* 267 mμ,[17] in 0.1 *N* HCl).

The stereochemistry of the diol I was established with intramolecular cyclization reactions similar to those used by Michelson and Todd in their proof of the configuration of the glycosidic center in thymidine.[18] The primary hydroxyl group was blocked selectively with trityl chloride in pyridine, forming the trityl ether VII in 71% yield. This



trityl ether VII and methanesulfonyl chloride afforded a methanesulfonate (VIII). In a subsequent cleavage of the trityl ether linkage of VIII with 80% acetic acid during six minutes at 100°, the reaction mixture became strongly acidic and a cyclized product IX was obtained in which both the trityl and methanesulfonate groups were

(9) E. Arundale and L. A. Mikeska, *Chem. Revs.*, **51**, 505 (1952); M. G. J. Beets, *Rec. trav. chim.*, **70**, 20 (1951); M. G. J. Beets and E. A. Drucker, *ibid.*, **72**, 247 (1953).

(10) A. Heslinga, *ibid.*, **78**, 473 (1959); C. C. Price and I. V. Krishnamurti, *J. Am. Chem. Soc.*, **72**, 5335 (1950).

(11) J. Colonge and J. Crabalona, *Bull. soc. chim. France*, 1505 (1959); 98, 102 (1960).

(12) A. T. Blomquist and J. Wolinsky, *J. Am. Chem. Soc.*, **79**, 6025 (1957).

(13) E. E. Smissman and R. A. Mode, *ibid.*, **79**, 3447 (1957).

(14) E. E. Smissman and D. T. Witiak, *J. Org. Chem.*, **25**, 471 (1960).

(15) For partition chromatographic work we are indebted to Mr. C. Pidacks and his group; for their general procedure see ref. 16.

(16) H. M. Kissman, C. Pidacks and B. R. Baker, *J. Am. Chem. Soc.*, **77**, 18 (1955).

(17) J. J. Fox and D. Shugar, *Biochim. Biophys. Acta*, **9**, 369 (1952).

(18) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955).

gone. When some triethylamine was included in the reaction mixture to neutralize the methanesulfonic acid formed, the same product IX was isolated along with a comparable amount of a derivative X of IX in which the trityl group was retained. The characteristic ultraviolet absorption maxima of both IX and X (near 232 and 256 mμ), their distinctive infrared spectra and the solubility of IX in water all show that they are indeed cyclization products, closely related to the anhydronucleosides and analogous zwitterions.[8]

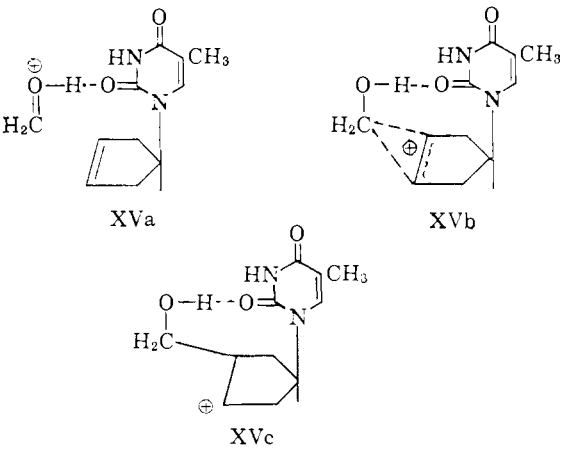
Since the cyclization reaction leading to the anhydro compounds IX and X would involve a nucleophilic backside attack on the carbon atom bearing the methanesulfonate group, it may be concluded that this group and the parent secondary hydroxyl function were *trans* with respect to the thymine residue.

The notably facile cyclization of the methanesulfonate VIII provides a clear-cut example of a phenomenon already commented on in general by Goodman, *et al.*,[19] and also encountered by us,[8] namely, the high reactivity of cyclopentane derivatives in nucleophilic displacement reactions, as compared with the lower reactivity of derivatives of tetrahydrofuran. Thus, with the same reaction conditions that were applied to VIII, Michelson and Todd apparently observed no cyclization of the tetrahydrofuran counterpart (3′-methanesulfonyl-5′-tritylthymidine) of VIII; the product was the expected 3′-methanesulfonylthymidine, obtained in good yield.[18] (In their structural studies methanesulfonate functions were exchanged for iodo groups with sodium iodide in acetone. Cyclizations to anhydro derivatives were then accomplished in acetonitrile containing silver acetate and traces of an amine.[18,20])

A second reaction sequence began with conversion of the hydroxy trityl ether VII to an acetate XI. Removal of the trityl blocking group gave an acetoxy alcohol XII which was then converted to an acetoxy methanesulfonate XIII. After some exploratory work (described in the Experimental section) it was found that the most clean-cut cyclization of XIII was accomplished at 100° in dimethylformamide. The acid binder included in the mixture was a hindered amine, diisopropylmethylamine,[8,21] which apparently was completely resistant to the alkylating action of XIII. Reaction was complete after four hours and no by-products were detected. The product was the acetylated anhydro compound XIV as evidenced by analyses, infrared and ultraviolet absorption spectra, and high solubility in water. After an examination of molecular models, it may be concluded from this cyclization that the hydroxymethyl and thyminyl residues of the diol I are *cis*. These cyclization sequences thus establish that the stereochemistry of I is analogous with that of thymidine (II) at all three asymmetric carbon atoms.

A corollary conclusion from the above structure proof is that the acid-catalyzed reaction of the substituted cyclopentene III with formaldehyde gives derivatives of a diol with *trans*-hydroxyl and hydroxymethyl groups, in agreement with the earlier results with cyclohexene systems.[12-14 cf. 22]

It was surprising that in the synthesis of the diol II, neither paper chromatography nor partition chromatography revealed any trace of the expected isomer V of this diol. Apparently the thyminyl residue of the olefin III exercised a strong steric control which directed attacking formaldehyde molecules to the upper side of the cyclopentene ring. Such control could result from the formation of proton-linked intermediates such as XVa and XVb. Subsequent, back-side attack of the



symmetrical, delocalized carbonium ion XVb by acetic acid or formaldehyde would lead stereospecifically to acetate or formal VI intermediates convertible only to *trans*-diol (racemic I). A classical carbonium ion intermediate such as XVc seems unlikely, since it would be expected to lead to a mixture of *cis*- and *trans*-diols.[22]

The previously mentioned sulfonate salt encountered during the isolation of I was an anhydro compound, probably XVI. The presence of the anhydro linkage was apparent from the infrared and ultraviolet absorption spectra. This salt was converted to its conjugate acid by passing it through a column packed with a polysulfonic acid ion-exchange resin. A zwitterionic structure XVII for the acid[23] was indicated by its notably low acid-

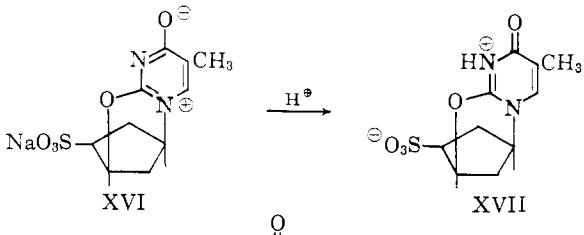
(19) L. Goodman, A. Benitez and B. R. Baker, *J. Am. Chem. Soc.*, **80**, 1680 (1958).

(20) There is some uncertainty about the stereochemistry of Michelson and Todd's "3′-deoxy-3′-iodothymidine" since the exchange of iodo- and methane-sulfonate groups normally occurs with inversion. However, it has been shown that a toluenesulfonate derivative of uridine is converted to an iodo compound with an over-all retention of configuration resulting from two successive inversions. The first gave an anhydro intermediate which then suffered a second inversion with ring opening when attacked by iodide ion (D. M. Brown, D. B. Parihar and A. Todd, *J. Chem. Soc.*, 4242 (1958)).

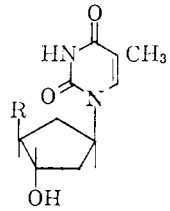
(21) E. L. Carpenter, U. S. Patent 2,453,062, June 4, 1946; S. Hünig and M. Kiessel, *Chem. Ber.*, **91**, 380 (1958).

(22) Several cycloalkenes with arenesulfonate substituents undergo intramolecular alkylation at the double bond; the stereospecificity of accompanying addition of solvent at the other end of the double bond seems to require reaction intermediates which are non-classical, three-membered carbonium ions analogous to XVb. For a summary of these investigations see S. Winstein and P. Carter, *J. Am. Chem. Soc.*, **83**, 4485 (1961). Contrastingly, in the Prins reaction with an arylethylene derivative (anethole) the reaction was not stereospecific and was interpreted in terms of a classical carbonium ion intermediate (P. S. Portoghese and E. E. Smissman, *J. Org. Chem.*, **27**, 719 (1962)).

(23) A hydrochloride salt analogous to XVII was described in the preceding paper (ref. 8).



XVI XVII



XVIII, R = $CH_2OPO_3H_2$
XIX, R = COOH

ity (pK_a 2.8) and by infrared absorption maxima characteristic of $-\overset{|}{\underset{|}{N}}H^{\oplus}$ and $-SO_3^{\ominus}$ groups.[24,25]

A preparation of the cyclopentane isostere XVIII of thymidylic acid used the hydroxy acetate XII as a suitably blocked reactant. Phosphorylation of the hydroxyl group with polyphosphoric acid[26] was followed by acidic hydrolysis of acetate and pyrophosphate linkages and purification of the product by ion-exchange chromatography. In the analysis of the product XVIII it was found that the phosphate ester linkage was much more resistant to acidic hydrolysis than in the nucleotides,[27] although hydrolysis was complete after seven hours in 60% perchloric acid at 149°.

A catalytic oxidation of the diol I with molecular oxygen was done according to a procedure developed for the selective oxidation of the primary hydroxyl groups of sugar derivatives.[28] The products isolated were the hydroxy carboxylic acid XIX and thymine.

Biological Testing.[29]—The thymidine isostere I, the corresponding carboxylic acid XIX and the various cyclopentylthymines described in the preceding paper[8] were tested for their ability to supplement or antagonize the growth-promoting action of thymidine for the bacterium *Pediococcus cerevisiae* ATCC 8081.[30,31] All were inactive. No activities were shown against a wide assortment of bacteria and fungi. The diol I and the most closely related alcohols and diols were also tested against three mouse tumors[32] with negative results.[31a]

Experimental[33]

Melting points are corrected. Evaporations were conducted under reduced pressure. Solids were pressed with potassium bromide for infrared spectral determinations. Paper chromatograms were run by the descending method using Whatman No. 1 paper. Compounds on the developed chromatograms were detected as dark spots when viewed under an ultraviolet lamp with a filter giving maximum transmission at 254 mμ. With the one-phase system butanone–water, 9:1, R_f-values were most reproducible when chromatograms were allowed to develop for just one hour. Other solvent systems used for paper chromatography were butanol–5 *N* acetic acid, 7:3, and 0.5% sodium carbonate.

1-(*trans*-3-Hydroxy-*cis*-4-hydroxymethylcyclopentyl)-thymine (I) and its Formal VI.—A solution of 1.921 g. (10 mmoles) of 1-(3-cyclopenten-1-yl)-thymine (III)[7] and 0.258 g. of 96.5% sulfuric acid in 15 ml. of acetic acid was agitated at *ca.* 60° with 0.420 g. (1.27 mmoles) of "approximately 91%" paraformaldehyde until the latter had dissolved. The stoppered reaction flask was kept for 17 hr. in an oven at 69°, cooled, and the contents were then neutralized by the addition of 2.5 ml. of 2 *N* sodium hydroxide solution. The mixture was evaporated, the residual gum was extracted with 30 ml. of chloroform, the extract was evaporated to dryness, then evaporated with another 30 ml. of chloroform to remove residual acetic acid. To a solution of the residual gum in 15 ml. of acetic anhydride was added 0.020 g. of 96.5% sulfuric acid. The reaction flask was stoppered, heated for 2.5 hr. in an oven at 69°, evaporated to dryness at *ca.* 1 mm., then taken to dryness twice more with 20-ml. portions of methanol. A solution of the residue in 20 ml. of methanol was heated under reflux for 1.0 hr. with 12.0 ml. of *N* sodium methoxide[34a] in methanol, cooled, acidified to *p*H 5 with concentrated hydrochloric acid, then evaporated to dryness. The residue was heated with 20 ml. of 1-propanol and the solution filtered. The concentrated filtrate deposited 0.211 g. of a crystalline solid, m.p. 188–197° (fraction A). Evaporation of the mother liquor, agitation of the residue with 30 ml. of methylene chloride and separation of the solid by filtration gave fraction B, 1.113 g., m.p. 167–184.° Evaporation of the methylene chloride mother liquor left 1.041 g. of a glass, fraction C. Fractions A, B and C were each subjected to partition chromatography,[15] using the system ethyl acetate–heptane–water (5:5:2). The content of the eluate was monitored with the aid of a spectrophotometer set to

(24) B. Witkop, J. B. Patrick and H. M. Kissman, *Chem. Ber.*, **85**, 949 (1952).

(25) A. Simon and H. Kriegsman, *ibid.*, **89**, 1718 (1956); D. E. Freeman and A. N. Hambly, *Austral. J. Chem.*, **10**, 229 (1957); H. Gerding and J. W. Maarsen, *Rec. trav. chim.*, **7**, 374 (1958).

(26) R. H. Hall and H. G. Khorana, *J. Am. Chem. Soc.*, **77**, 1871 (1955); A. M. Michelson, *J. Chem. Soc.*, 1957 (1958).

(27) A. Marshak and H. J. Vogel, *J. Biol. Chem.*, **189**, 597 (1951).

(28) K. Heyns and H. Paulsen, *Angew. Chem.*, **69**, 600 (1957); C. B. Reese, K. Schofield, R. Shapiro and A. Todd, *Proc. Chem. Soc.*, 290 (1960); K. Heyns and J. Lenz, *Chem. Ber.*, **94**, 348 (1961).

(29) We would like to thank Mr. A. C. Dornbush and Miss Alice Craig for microbiological testing results and Dr. A. Vogel and his group for anti-tumor testing.

(30) A. E. Bolinder and W. G. Kurz, *Acta Chem. Scand.*, **13**, 2160 (1959); N. Grossowicz and F. Mandelbaum, *Science*, **133**, 1773 (1961).

(31) This organism was selected over other candidates because thymine, a possible metabolite from these compounds, does not support its growth (H. E. Sauberlich, *Arch. Biochem.*, **24**, 224 (1949)). In a plate-cup assay with 5-bromo-2′-deoxyuridine a zone of inhibition was surrounded by a zone of growth augmentation. This behavior is in accord with published work with other organisms and mammalian cell cultures in which this compound variously antagonizes or replaces thymidine.[4] Thus *P. cerevisiae* appears to be a suitable organism for the testing of thymidine analogs.

(31a) NOTE ADDED IN PROOF.—Preliminary experiments by Dr. T. R. Breitman have shown that relatively high concentrations of the thymidine isostere I inhibit the incorporation of radioactive thymidine into deoxyribonucleic acid of a thymine-requiring mutant (15 T-, U-) of *Escherichia coli*. Addition of I to an enzyme system from regenerating rat liver inhibited both thymidine phosphorylation and incorporation into deoxyribonucleic acid.

(32) E. H. Dearborn, *Acta Unio Intern. contra Cancrum (Suppl.)*, **15**, 76 (1959).

(33) We thank Mr. L. Brancone, Mr. W. Fulmor, and their groups for elemental and spectroscopic analyses.

(34) (a) A lesser amount of sodium methoxide (procedure from footnote 26 of ref. 16) was inadequate for complete removal of acetate groups. (b) With a comparable propanol extract from another run paper chromatograms revealed only a single spot, running parallel with that from purified diol I, thus contraindicating the presence of isomeric diol V. The three solvent systems mentioned at the beginning of the Experimental section gave R_f values of 0.30, 0.55 and 0.82, respectively. The propanol extract was evaporated and the residue subjected to partition chromatography[15] with the system 1-butanol–ethyl acetate–water (1:1:1), monitoring the eluate continuously with a recording ultraviolet spectrophotometer. A spectral peak recorded at the end of the first hold-back-volume was compact and symmetrical, indicating that it was due to a single product. Evaporation of the corresponding eluate fraction and agitation of the residue with 2-methoxyethanol gave colorless crystals (yield 30%), m.p. 209–211° or m.p. 210–212° after admixture with the analytical sample of diol I.

measure the absorption of ultraviolet light at 270 mμ. From fractions B and C there were eluted in the first hold-back-volumes 0.023 g. and 0.296 g. (17% recovery) of crystals, m.p. 165–168°, obtained by evaporating the eluates corresponding to the ultraviolet absorption peaks, then washing the residues with benzene. This material did not depress the m.p. (168–169°) of the starting olefin III and gave an infrared spectrum identical with that of III. From fractions A, B and C there were eluted in the fourth hold-back-volume 0.048 g. + 0.067 g. + 0.197 g. (12%) of the formal VI, m.p. 229–230°, raised to m.p. 230–231° by recrystallization from methylene chloride–toluene. The infrared absorption bands of lowest wave length (at 3.11 and 3.25 μ) were very similar to those in the starting olefin III in position and relative intensity, thus contraindicating the presence of a hydroxyl group; $\lambda_{max}^{CH_3OH}$ 272 mμ (ε 8,400).

Anal. Calcd. for $C_{12}H_{16}O_4N_2$: C, 57.13; H, 6.39; N, 11.11. Found: C, 56.99; H, 6.68; N, 10.95.

After the normal elution of fractions A and B was completed, the columns were eluted with methanol. The methanol was evaporated and the residues crystallized from 2-methoxyethanol to give 0.095 g. + 0.126 g. (9%) of the diol I, m.p. 210–212°, raised to m.p. 212–213° by recrystallization from 2-methoxyethanol: $\lambda_{max}^{0.1\ N\ HCl}$ 273 mμ (ε 10,000), minimum 238 mμ (ε 2,800); $\lambda_{max}^{0.1\ N\ NaOH}$ 272 mμ (ε 7,600), minimum 248 mμ (ε 4,000); pK_9 9.9 (determined spectrophotometrically). The product gave a sharply defined infrared absorption spectrum.

Anal. Calcd. for $C_{11}H_{16}O_4N_2$: C, 54.99; H, 6.71; N, 11.66. Found: C, 55.01; H, 6.94; N, 11.62.

A solution of 0.100 g. of the formal VI and 13 mg. of 96.5% sulfuric acid in 0.75 ml. of acetic anhydride was kept in an oven at 70° for 3 hr. Removal of acetic anhydride, methanolysis with sodium methoxide and the subsequent work-up were as in the following experiment. The product was identical with the above diol I (m.p., mixture m.p., infrared spectrum and paper chromatography).

1-[*trans*-3-Hydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (I) and the Sodium Salt XVI of 2,3′-Anhydro-1-(*cis*-3-hydroxy-4-sulfocyclopentyl)-thymine.—To a solution of 9.61 g. (0.05 mole) of 1-(3-cyclopenten-1-yl)-thymine (III)[7] and 1.29 g. (0.0125 mole) of 96.5% sulfuric acid in 80 ml. of acetic acid was added 2.100 g. (0.07 mole) of "⩾ 95%" paraformaldehyde. The reaction flask was stoppered and heated in an oven at 70° for 17 hr., shaking it occasionally until the last of the paraformaldehyde had dissolved. The solution was evaporated to a thick sirup at 35–40°, dissolved in 75 ml. of acetic anhydride and heated at 70° for 2.5 hr. This solution was evaporated to a thick sirup, then evaporated twice more with 25-ml. portions of methanol, leaving a residue almost free of the odor of acetic acid. This was dissolved in 25 ml. of methanol, a solution of 1.75 g. (0.076 g.-atom) of sodium in 75 ml. of methanol was added and the resulting solution (pH 13–14) heated under reflux for 1 hr. The cooled mixture was adjusted to *p*H 5.6 by the addition of 4.4 ml. of concentrated hydrochloric acid. Evaporation to dryness left a residue which was agitated with 100 ml. of hot 1-propanol.[34b] Not all of the gum dissolved. The extract was allowed to stand 18 hr., when more of a partly crystalline gum had separated. These gums were saved. The supernatant liquor was evaporated to give a residue which became partly crystalline. This was macerated to a smooth paste over the steam-bath with 10 ml. of 1-propanol, then allowed to cool and stand overnight. The solid was collected by filtration, washed with 1-propanol, with 2-methoxyethanol and with ether; 4.18 g., m.p. 205–210°. This solid slowly crystallized from 50 ml. of 2-methoxyethanol, finally at 5°, to give 2.94 g. of product, m.p. 211–213°, or m.p. 212–213° after admixture with diol I from the preceding experiment. The concentrated mother liquor afforded another 0.66 g. (total 30%) of product, m.p. 211–212°.

The above gums crystallized from dimethylformamide (10 ml.) and then from methanol–pyridine to give 0.46 g. of the sodium sulfonate XVI, m.p. 215–218°, or m.p. 214–221° after drying *in vacuo* at 117° for 24 hr.; $\lambda_{max}^{CH_3OH}$ 232 and 253 mμ (ε 8,200 and 8,000). This material was very soluble in water. The sharp infrared absorption peaks at 6.00, 6.17 and 6.55 μ are characteristic of anhydro compounds while those at 8.4 (broad), 9.34 and 9.52 μ are in agreement with those reported for sodium sulfonates.[25] Paper chromatography with 1-butanol–5 *N* acetic acid, 7:3, revealed only one spot (R_f 0.07); the dimethylformamide mother liquor apparently contained both XVI and a considerable amount of a second compound of similarly high polarity (R_f 0.10), a compound with an orange fluorescence (R_f 0.00) and diol I (R_f 0.59). The spot representing the latter compound ran exactly parallel with a purified sample of diol I. No other spots were seen.

Anal. Calcd. for $C_{10}H_{11}O_6N_2SNa$: C, 40.81; H, 3.77; N, 9.52. Found: C, 41.36; H, 3.94; N, 9.63; ash, 20.56.

2,3′-Anhydro-1-(*cis*-3-hydroxy-4-sulfocyclopentyl)-thymine (XVII).—A solution of 0.150 g. of the sodium sulfonate XVI in 2 ml. of aqueous methanol was added to a column 6 mm. in diameter which was slurry-packed with 1.0 g. of well-washed Dowex-50 polysulfonic acid resin. Elution with aqueous methanol was continued until the eluate was no longer acidic. The eluate was concentrated to *ca.* 0.5 ml. over the steam-bath. More methanol was added and the concentration was repeated. A crystalline product gradually separated; 0.091 g. (66%), m.p. 180–183° dec. Vacuum drying during 15 hr. at 100° was accompanied by "chalking" of the crystals, suggesting loss of solvent. The dried material (m.p. 174–177° dec.) had darkened slightly; $\lambda_{max}^{CH_3OH}$ 229 and 258 mμ (ε 6,000 and 7,100). The pattern of infrared absorption peaks at 5.97, 6.23, 8.3 (broad), 9.40 and 9.58 μ was as in the precursor sodium salt. The strong peaks at 6.55 and 6.71 μ were gone and new peaks appeared at 3.76 (broad), 4.24, 4.97 (–$\overset{|}{\underset{|}{N}}H^+$)[24] and 5.82 μ (⟩$\overset{|}{\underset{|}{N}}H^+$).[24] The peak at 2.88 μ was also much stronger.

Anal. Calcd. for $C_{10}H_{12}O_6N_2S$ (272.3): C, 44.10; H, 4.44; N, 10.29; S, 11.78. Found: C, 42.88; H, 4.99; N, 10.23; S, 12.44, 12.24; neut. equiv., 277; pK_a, 2.8.

1-[*trans*-3-Hydroxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (VII).—A solution of 0.480 g. (2.0 mmoles) of vacuum-dried 1-[*trans*-3-hydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (I) and 0.572 g. (2.05 mmoles) of trityl chloride in 14 ml. of dry pyridine was allowed to stand 9 days at *ca.* 25°, then poured very gradually into 100 ml. of ice-water. The resulting mixture was set aside at 5° for 2 days during which time the gummy product became crystalline. The solid was collected, washed with water and with methanol, then crystallized from methanol; 0.697 g. (71%), m.p. 171–173°. A recrystallized sample, m.p. 169–171°, was dried over phosphorus pentoxide for 16 hr. at 80° and ⩽ 1 mm.; $\lambda_{max}^{CH_3OH}$ 270 mμ (ε 10,700).

Anal. Calcd. for $C_{30}H_{30}O_4N_2 \cdot 0.25\ H_2O$: C, 73.97; H, 6.31; N, 5.75. Found: C, 74.19; H, 6.36; N, 6.01.

1-[*trans*-3-Methanesulfonyloxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (VIII).—To an ice-cold solution of 0.500 g. of vacuum-dried 1-[*trans*-3-hydroxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (VII) in 5 ml. of dry pyridine was added 0.25 ml. of redistilled methanesulfonyl chloride. The solution was kept at 0° for 15 hr., 0.10 ml. of water was added, the mixture kept several hours at 0°, then poured into 50 ml. of ice-water. Filtration and washing with water gave 0.709 g. of product, m.p. 146–157° dec. at a heating rate of 6°/min. This melting point was markedly dependent on the rate of heating (*e.g.*, m.p. 136–139° at 2°/min.). Material recrystallized three times from ethanol melted as before, but gave a slightly sharper infrared absorption spectrum in which weak maxima at 3.55, 7.07 and 10.1 μ had been eliminated. Maxima at 7.35, 7.48, 8.49 and 8.55 μ agree with those reported for sulfonate esters.[35] The analytical sample was dried over phosphorus pentoxide for 10 hr. at 60° and ⩽ 1 mm.

Anal. Calcd. for $C_{31}H_{32}O_6N_2S \cdot 0.25H_2O$: C, 65.88; H, 5.80; N, 4.96. Found: C, 65.99; H, 6.15; N, 5.22.

2,3′-Anhydro-1-[*cis*-3-hydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (IX) and 2,3′-Anhydro-1-[*cis*-3-hydroxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (X).—A solution of 0.670 g. of 1-[*trans*-3-methanesulfonyloxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (VIII) in 4 ml. of acetic acid, 1 ml. of water and 0.300 g. of triethylamine was heated at 100° for 10 min. The resulting solution was evaporated to dryness at ⩽ 50°, finally at ⩽ 1 mm. A solution of the residual sirup in 6 ml. of acetone gradually (3

(35) R. D. Guthrie and H. Spedding, *J. Chem. Soc.*, 953 (1960).

days) deposited 0.248 g. of a solid. After washing with methylene chloride there remained 0.049 g. of a crystalline solid, m.p. 201–208°. Paper chromatograms with three solvent systems each showed only one spot. Recrystallization from 1-propanol gave 0.030 g. of the hydroxymethyl product IX, m.p. 214–218°, raised to m.p. 219–221° by drying over phosphorus pentoxide for 7.5 hr. at 115° and $\leq$ 1 mm.; $\lambda_{max}^{CH_3OH}$ 232 and 256 mμ (ε 8,900 and 7,600); λ_{max}^{KBr} 2.90 (OH), 6.01, 6.25, 6.39 and 6.56 μ. This material was very soluble in water.

Anal. Calcd. for $C_{11}H_{14}O_3N_2 \cdot 0.25H_2O$: C, 58.27; H, 6.45; N, 12.36. Found: C, 58.25; H, 6.74; N, 11.84.

The methylene chloride washes from the crude hydroxymethyl compound were evaporated to dryness. The solid residue was washed with water and with acetone, then crystallized from ethanol to give 0.082 g. of the trityloxymethyl product X, m.p. 260–262°; $\lambda_{max}^{CH_3OH}$ 232 (shoulder) and 255 mμ (ε 19,100 and 9,400); λ_{max}^{KBr} 2.90 (H_2O?), 6.01, 6.1 (shoulder), 6.18 and 6.56 μ.

Anal. Calcd. for $C_{30}H_{28}O_3N_2 \cdot 0.25H_2O$: C, 76.82; H, 6.12; N, 5.98. Found: C, 76.86; H, 6.25; N, 6.25.

In an attempt to dehydrate this material it was recrystallized from ethanol and dried over phosphorus pentoxide for 9 hr. at 192° and $\leq$ 1 mm. without significantly changing the melting point (261–263°) or the analytical results (C, 76.78; H, 6.30; N, 6.30).

1-[*trans*-3-Acetoxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (XI).—A solution of 0.333 g. of 1-[*trans*-3-hydroxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (VII) in 3.5 ml. of dry pyridine and 0.8 ml. of acetic anhydride was allowed to stand for 22 hr., then added dropwise with stirring to 50 ml. of ice-water. The resulting solid was collected and washed with water; 0.380 g., m.p. 120–127°, resolidified, remelted 197–200°. Material recrystallized from ethanol melted at 119–124°, gradually resolidified, then remelted at 198–200°. The analytical sample was dried over phosphorus pentoxide for 16 hr. at 80° and 1 mm.

Anal. Calcd. for $C_{32}H_{32}O_5N_2 \cdot H_2O$: C, 70.83; H, 6.32; N, 5.16; H_2O, 3.32. Found: C, 71.04; H, 6.83; N, 5.50; H_2O (Karl Fischer), 1.34.

1-[*trans*-3-Acetoxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (XII).—A solution of 0.327 g. of 1-[*trans*-3-acetoxy-*cis*-4-(trityloxymethyl)-cyclopentyl]-thymine (XI) in 5 ml. of 80% acetic acid was heated under reflux for 10 min., cooled, and diluted with 25 ml. of ice-water. Trityl alcohol was removed by filtration. The filtrate was evaporated and the residual oil crystallized from 1-propanol to give 0.116 g. (68%) of product, m.p. 193–196°, raised to m.p. 195–197° by recrystallization from 1-propanol; $\lambda_{max}^{CH_3OH}$ 272 mμ (ε 10,200).

Anal. Calcd. for $C_{13}H_{18}O_5N_2$: C, 55.31; H, 6.43; N, 9.92. Found: C, 55.36; H, 6.66; N, 9.99.

1-[*trans*-3-Acetoxy-*cis*-4-(methanesulfonyloxymethyl)-cyclopentyl]-thymine (XIII).—A solution of 0.085 g. of vacuum-dried 1-[*trans*-3-acetoxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (XII) in 1.5 ml. of dry pyridine was chilled with an ice-bath during the dropwise addition of 0.25 ml. of redistilled methanesulfonyl chloride. After 16 hr. at 0° there was added dropwise with swirling and cooling 0.1 ml. of water. After another 1.5 hr. at 0° the mixture was added gradually to 10 ml. of ice-water. The resulting solution was allowed to stand at 0° for several hours. The colorless crystals which had separated were collected and washed with water; 0.052 g., m.p. 142–144°.

Anal. Calcd. for $C_{14}H_{20}O_7N_2S$: C, 46.66; H, 5.59. Found: C, 46.47; H, 5.98.

The mother liquor was extracted with 3 × 10 ml. of chloroform, the extracts were evaporated, the residual gum agitated with 1 ml. of water, then allowed to stand 2 hr. at 0°. The solid was collected by filtration and washed with water; 0.049 g. (total yield 95%), m.p. 139–141°.

2,1″-Anhydro-1-[*trans*-3-acetoxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (XIV).—A solution of 0.039 g. of 1-[*trans*-3-acetoxy-*cis*-4-(methanesulfonyloxymethyl)-cyclopentyl]-thymine (XIII) and 0.034 g. of methyldiisopropylamine[8] in 1.0 ml. of dry dimethylformamide was kept in an oven at 100° for 4 hr., cooled, then evaporated to dryness. The solid residue was washed by slurrying in butanone, leaving 0.014 g. of crystalline product, m.p. 206–208°; $\lambda_{max}^{CH_3OH}$ 232 and 253 mμ (ε 8,300; 7,000); λ_{max}^{KBr} 5.73 (–OC-OCH_3), 6.00, 6.14 and 6.52 μ. This material was very soluble in water.

Anal. Calcd. for $C_{13}H_{16}O_4N_2$: C, 59.08; H, 6.10; N, 10.60. Found: C, 58.90; H, 6.47; N, 10.52.

Conditions for the cyclization of the methanesulfonate XIII were first explored using sub-milligram amounts in sealed lengths of melting point capillary tubing. Reactions were followed by paper chromatography, using butanone–water (9:1) as the developing solvent. In 80% acetic acid with added triethylamine there was no cyclization after 5 hours at room temperature. At 100° there was a rapid formation of cyclized product which then underwent a decomposition (probably a hydrolytic cleavage of the anhydro linkage; *cf.* ref. 8) which was complete after 1 hour. Checks at 1, 2, 4 and 8 minutes revealed that starting material was gone after 8 minutes. The maximum amount of cyclized product (R_f 0.34) appeared to be present at 4 minutes, although starting material (R_f 0.92) and the decomposition product (R_f 0.74) were also present.

trans-2-Hydroxy-*cis*-4-(thymin-1-yl)-cyclopentylmethyl Phosphate (XVIII).[36]—A small amount of radioactive phosphate was included in the reaction mixture. This facilitated the identification and purification of the phosphorylated product. First 0.0282 g. (0.1 mmole) of 1-[*trans*-3-acetoxy-*cis*-4-(hydroxymethyl)-thymine (XII) and then 0.109 g. of phosphorus pentoxide were dissolved in 0.139 g. of 85% phosphoric acid containing 10 μc. of $H_3P^{32}O_4$. The solution was heated for 2.0 hr. in an oven kept at 59°, 1.0 ml. of water was added, the resulting solution was heated under reflux on a steam-bath for 0.5 hr., then cooled. Inorganic phosphate was precipitated by the addition of 2.35 ml. of 4 *N* lithium hydroxide. The mixture (pH $\geq$ 14) was heated on the steam-bath for 30 min. to saponify any remaining acetate groups, and then was allowed to stand overnight at 5°. Lithium phosphate was removed by centrifugation at 5°, washing with 4 × 1 ml. of cold, 0.02 *N* lithium hydroxide. The combined supernatant fractions were diluted to a volume of 50 ml., then chromatographed on a 1 × 10-cm. column of Dowex-1-chloride anion exchange resin. A linear gradient elution was used, adding 1 l. of 0.5 *N* ammonium bicarbonate to 1 l. of water and taking 10-ml. eluate cuts. Aliquots from each cut were assayed for radioactivity and for ultraviolet absorption at 272 mμ. For the calculation of yields it was assumed that products in neutral or acidic solutions all had the same ultraviolet absorption properties as 1-[*trans*-3-hydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (I), *i.e.*, λ_{max} 272 mμ (ε 10,000). A sharp eluate peak centered at cut 17 (53% yield) had no radioactivity. Evaporation of cuts 16–18 and recrystallization of the residue from water gave a product (m.p. 209–211°) with an infrared spectrum identical with that of 1-[*trans*-3-hydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]-thymine (I).[37] A second peak centered at cut 33 (40% yield) had ultraviolet absorption tailing out to cut 50 so that it partially overlapped two non-chromophoric radioactivity peaks centered at cuts 45 and 50. (The latter phosphorus peak was about twice the size of the former and presumably represented inorganic orthophosphate.) A small, radioactive peak centered at cut 61 (4%) was not investigated except to note that the ratio of phosphorus to chromophore was the same as it was in the first part of the second peak. In the second peak the ratio of chromophore to radioactivity was constant from cuts 30 to 42, with symmetry of the peak up to cut 37. Cuts 30–37 (purified product, 25% yield) were pooled: $\lambda_{max}^{0.1\ N\ HCl}$ 272 mμ, with an apparent ratio of chromophoric pyrimidine to phosphorus of 0.93/1, based on a colorimetric phosphate assay.[38] For this assay the phosphate ester linkage was first cleaved by evaporating an aliquot to dryness and then heating the residue at 149° for 7 hr. in 60% perchloric acid. With milder conditions known to be adequate for the complete hydrolysis of nucleoside phosphate esters (60% perchloric acid, 100°, 1 hr.),[37] the apparent phosphorus content was only 13% as great. Paper chromatography of the puri-

(36) This experiment was done in collaboration with Dr. T. R. Breitman.

(37) It seems most likely that this diol (I) was formed indirectly, *i.e.*, by cyclization of a pyrophosphate intermediate to give an anhydro compound such as XIV. Subsequent saponification would give I. For a precedent see E. R. Walwick, W. K. Roberts and C. A. Dekker, *Proc. Chem. Soc.*, 84 (1959).

(38) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

fied product with the system isobutyric acid–water–concentrated ammonia (66:33:1)[39] showed only one spot, with R_f 0.50, *vs.* R_f's of 0.32 for 5′-thymidylic acid, 0.79 for the thymidine isostere I, and 0.69 for thymidine.

1-(*cis*-4-Carboxy-*trans*-3-hydroxycyclopentyl)-thymine (**XIX**).—A solution (*p*H 9) of 0.361 g. (1.5 mmoles) of 1-(*trans*-3-hydroxy-*cis*-4-hydroxymethyl)-thymine (I), 0.318 g. (3 mmoles) of sodium carbonate and 0.758 g. (9 mmoles) of sodium bicarbonate in 15 ml. of water was sealed with platinum catalyst (pre-reduced, from 0.038 g. of platinum oxide) and oxygen at 35 p.s.i. into a 183-ml., stainless steel bomb. This was rocked and heated at 85–90° for 24 hours, cooled, opened, charged with oxygen as before, sealed and rocked at 88–92° for another 47 hours. The cooled mixture was acidified and de-ionized by the careful, portionwise addition of 4.0 g. of Dowex-50 polysulfonic acid resin, and then filtered. The dark filtrate and washes were evaporated to a volume of *ca.* 5 ml., adjusted to *p*H 7 with concentrated aqueous ammonia, allowed to stand for 2 hours, then filtered from 0.048 g. of a solid which had separated, m.p. 317–319°. This material gave an infrared spectrum identical with that of thymine. The filtrate was adjusted to *p*H 8.5–9.0 with more ammonia, then subjected to chromatography[40] on a 1.5 × 27-cm. column of Dowex-1 formate. Eluate peaks were located qualitatively by evaporating a droplet from each cut (10–12 ml.) on filter paper; thymine derivatives at concentrations down to about 0.3 μmole/ml. were detected as dark spots when viewed under an ultraviolet lamp with a filter giving maximum transmission at 254 mμ. Quantitative assessments of peak homogeneities were made after appropriate dilutions, using a Beckman DU ultraviolet spectrophotometer, then plotting cut numbers *vs.* optical densities on graph paper. Water (cuts 1–15) eluted a peak centered at cut 7 with a shoulder at cut 10. Evaporation of cuts 6–8 left a glass which crystallized overnight. It was then washed with ethanol, leaving 0.011 g. (3% recovery) of crystals, m.p. 210–212°. These gave an infrared spectrum identical with that of the starting diol I. Dilute formic acid (0.05 *N*, cuts 16–29) gave a peak centered at cut 24. Evaporation of cuts 22–26 left 0.021 g. of a solid, m.p. 315–318° dec., which gave an infrared spectrum identical with that of thymine. Stronger formic acid (0.55 *N*, cuts 30–46) gave a peak centered at cut 40. Cuts 39–45 were evaporated and residual formic acid was removed by evaporation to dryness with 3 × 1 ml. of water. The residue was heated with 6 drops of water, allowed to stand and then collected by filtration; yield 0.018 g. (5%), m.p. 250–254°; $\lambda_{max}^{H_2O}$ 273 mμ (ε 9,000), min. 238 mμ (ε 1,680); λ_{max}^{KBr} 2.90 (–OH), 3.19 sh (–NH), 3.9, 8.20 and 8.30 (–COOH). When the potassium bromide disk was ground with the calculated amount of 0.01 *N* methanolic potassium hydroxide, then dried and repressed, it gave a spectrum in which the 3.9, 8.20 and 8.30 bands were gone and a new band at 6.35 μ (–COOK) was present.

Anal. Calcd. for $C_{11}H_{14}O_5N_2$: C, 51.96; H, 5.55; N, 11.02. Found: C, 51.42; H, 4.95; N, 11.02.

In a similar experiment with a heating period of 24 hours, the yield of hydroxy acid XIX was 1.2%, and 67% of the starting diol I was recovered.

(39) R. Zetterström and M. Ljunggren, *Acta Chem. Scand.*, **5**, 291 (1951).

(40) In an earlier run this ion exchange chromatography was done by a gradient elution method patterned after a procedure of H. Busch, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **196**, 717 (1952). The concentrations of formic acid in peak eluate fractions were determined by potentiometric titration. These concentrations were then used in the modified chromatography which is described.

COMMUNICATIONS TO THE EDITOR

THE MECHANISM OF ESTROGEN BIOSYNTHESIS

Sir:

One of the more important biochemical problems related to steroid metabolism concerns the biosynthetic sequence and mechanism involved in the transformation of androgens to estrogens. It has been shown that 19-hydroxyandrost-4-ene-3,17-dione and 19-oxoandrost-4-ene-3,17-dione are excellent substrates for conversion to estrogens using human placental microsome preparations and are considered to be on the pathway going from androst-4-ene-3,17-dione to estrogen.[1] It also has been found that the 19-hydroxy compound forms both formaldehyde and formic acid in its conversion to estrogen while the 19-oxo structure yields mostly formic acid.[2] In attempting to fit these facts into a mechanism for estrogen formation, information concerning the stereochemistry of the hydrogen eliminated at C-1 was necessary. This communication is concerned with this point.

Two samples of androst-4-ene-3,17-dione specifically labeled with tritium at C-1 were prepared. The distribution of tritium in compound I was 25% 1α and 75% 1β and in compound II 93% 1α and 7% 1β. The proof for the distribution of tritium is outlined in the preceding publication.[3] These two compounds were then incubated with a placental aromatizing system.

Experimental.—Two sets of incubations were carried out with human placental microsomes supplemented with a reduced triphosphopyridine-nucleotide (TPNH) regenerating system as described earlier.[1] In the first experiment, the incubations, run with a total of 16.7 mg. of I, were extracted with methylene chloride. The combined crude extracts were partitioned between 50% aqueous methanol (mobile upper phase) and carbon tetrachloride (lower phase) in a Craig countercurrent distribution apparatus (99 transfers) and estrone was obtained from the appropriate tubes.[4] In analyzing for radioactivity, sample weights were determined by direct weighing of crystals (method A) and by spectrophotometric analyses (method B) using the absorption peak at 242 mμ for androst-4-ene-3,17-dione and the Kober reaction chromogen[5] for estrone. In experiment 2, the incubations containing a total of 200 μg. of II were extracted with methylene chlo-

(1) T. Morato, M. Hayano, R. I. Dorfman and L. R. Axelrod, *Biochem. Biophys. Research Commun.*, **6**, 334 (1961).

(2) R. I. Dorfman, C. Gual, T. Morato, M. Hayano and M. Gut, Abstracts, International Congress on Hormonal Steroids, Milano, Italy, May, 1962, p. 270.

(3) H. J. Brodie, M. Hayano and M. Gut, *J. Am. Chem. Soc.*, **84**, 3766 (1962).

(4) This method of isolating pure estrone from tissue incubations of androstenedione has been well documented (B. Baggett, L. L. Engel, K. Savard and R. I. Dorfman, *J. Biol. Chem.*, **221**, 931 (1956); K. Ryan, *ibid.*, **234**, 268 (1959); see also ref. 7).

(5) J. B. Brown, *J. Endocrinol.*, **8**, 196 (1952).