32. *Nucleotides. Part XLVIII.*[1] *The Reaction of Hydroxylamine with Cytosine and Related Compounds.*

By D. M. Brown and P. Schell.

Hydroxylamine reacts with the nucleic acid-derived pyrimidine bases and nucleosides. The corresponding purine derivatives are unaffected by the reagent. Cytosine and related compounds react fastest at about pH 6·5 and the structures of the products have been elucidated. The reaction depends on addition of hydroxylamine to the 4,5-double bond and replacement, by hydroxylamine, of the amino-group. Methyl and hydroxymethyl substituents at position 5 reduce the reaction rate very markedly. The products are converted into N^6-hydroxycytosine derivatives by elimination of hydroxylamine, effected by heat or by acid-catalysis. Uracil derivatives are formed in the last reaction.

We have for some time been interested in the action of simple reagents on nucleic acids, in order both to elucidate nucleotide sequence and to cast light on the nature of chemical mutagenesis. These interests could be complementary in that both might be served by the discovery of reagents of high specificity for one or other of the different types of base residue. Our interest in hydrazine, which reacts with pyrimidine but not purine nucleotides [2] and is itself mutagenic in bacterial [3] and bacteriophage systems,[4] led us to study the related compound hydroxylamine. Some of the conclusions detailed in this Paper have been given briefly elsewhere.[6] The mutagenic action of the reagent has been demonstrated by a number of workers.[7-11] Freese and his co-workers,[7] and Schuster [12] have also enquired into the chemical action of the reagent, but their conclusions differ in certain respects from ours which agree with those of Verwoerd *et al.*[13]

When a commercial sample of salmon sperm deoxyribonucleic acid was treated with 10N-hydroxylamine at 95° and the base ratios were studied after acid hydrolysis, the

[1] Part XLVII, *J.*, 1963, 1149.
[2] Baron and Brown, *J.*, 1955, 2855.
[3] Lingens, *Naturwiss.*, 1961, **48**, 480.
[4] A. O. Orgel, Ph.D. Thesis, Cambridge, 1960.
[5] Freese in "Molecular Genetics," ed. Taylor, Academic Press, New York, 1962, Part I, p. 227.
[6] Brown and Schell, *J. Mol. Biol.*, 1961, **3**, 709.
[7] Freese, Bautz, and Bautz-Freese, *Proc. Nat. Acad. Sci. U.S.A.*, 1961, **47**, 845.
[8] Schuster and Vielmetter, *J. Chim. phys.*, 1961, **58**, 1005.
[9] Champe and Benzer, *Proc. Nat. Acad. Sci. U.S.A.*, 1962, **48**, 532.
[10] Ito, Yamasaki, and Matsudaira, personal communication.
[11] Freese, Bautz-Freese, and Bautz, *J. Mol. Biol.*, 1961, **3**, 133; Freese and Strack, *Proc. Nat. Acad. Sci. U.S.A.*, 1962, **48**, 1796.
[12] Schuster, *J. Mol. Biol.*, 1961, **3**, 447.
[13] Verwoerd, Kohlhage, and Zillig, *Nature*, 1961, **192**, 1038; Zillig, Verwoerd, and Kohlhage, "Acid Ribonucléiques et Polyphosphates, Structure, Synthese et Fonctions," Colloques Internat. du C.N.R.S., Paris, 1962, p. 229.

results shown in Fig. 1 were obtained. Although no great accuracy is claimed for them, they show clearly that the cytosine residues in the deoxyribonucleic acid were largely affected, little if any change in adenine to thymine ratio being observed. Essentially similar results were obtained on using anhydrous hydroxylamine at 35°. It is hoped to make a detailed study of the reaction with polynucleotides the subject of a later communication. The present one records our observations with bases and nucleosides, but it is noteworthy that in comparable conditions simple cytosine derivatives react very much faster than the corresponding residues in the deoxyribonucleic acid macromolecule.

Since it appeared that cytosine residues were the main point of attack, the pH-dependence of the reaction with deoxycytidine was studied, and the rate maximum was found to

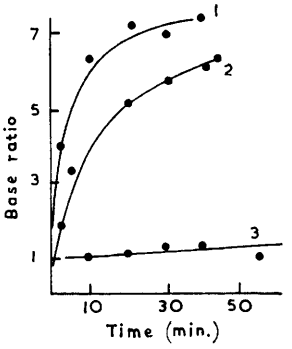


FIG. 1. Changes in base ratios, thymine : cytosine (1), adenine : cytosine (2) and total purines : thymine (3), obtained after treatment of deoxyribonucleic acid with 10N-hydroxylamine at 95°.

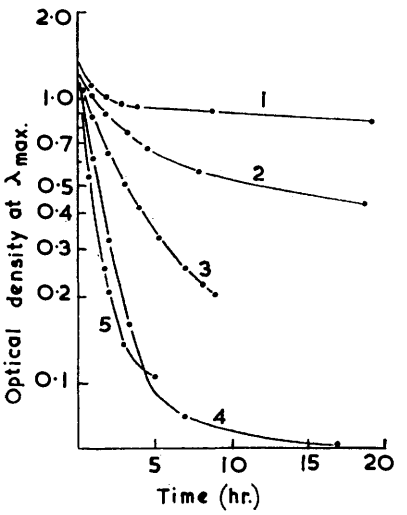


FIG. 2. Optical-density changes on treatment with 2N-hydroxylamine hydrochloride at pH 6·5 and 24° of uracil (1), uridine (2), 2′-deoxycytidine (3), 3-methylcytosine (4), and cytosine (5).

be approximately at pH 6·5. For the initial survey of bases and nucleosides, the compounds were treated with aqueous hydroxylamine at this pH and the fall in optical density followed, as a function of time (Fig. 2). Deoxyadenosine and deoxyguanosine were unaffected and in consequence were not further studied. Thymidine, too, appeared not to react although uridine did so slowly.

The reaction of cytosine and of 3-methylcytosine (a convenient nucleoside analogue) with hydroxylamine was examined in detail in aqueous solution at pH 6·5 and with the anhydrous base. Both conditions led to the same product, but the anhydrous reaction allowed quantitative isolation of the product by evaporation of the excess reagent *in vacuo*.

The product from 3-methylcytosine (I; R = Me) showed no selective absorption in the 270 mμ region, characteristic of the cytosine chromophore, but instead absorbed at 223 mμ (Fig. 3). This result was consistent with the addition of hydroxylamine to the 4,5-double bond. The substance, which showed no marked basic properties, gave analytical values and a molecular weight corresponding to the formula $C_5H_{10}N_4O_3$, indicating that two hydroxylamine molecules were involved. Warming with dilute hydrochloric acid gave a product in which ultraviolet absorption in the 270 mμ, region was restored, but which was not 3-methylcytosine. Its spectra (Fig. 3) at several pH values were almost identical

with those recorded for N^6-hydroxycytidine.[14] The hydrochloride, formed in high yield, was clearly that of N^6-hydroxy-3-methylcytosine (IV; R = Me). A small amount of 3-methyluracil (V; R = Me) was formed at the same time.

The structure of the hydroxylamine reaction product can be discussed in terms of structure (III; R = Me). Nuclear magnetic resonance spectroscopy in D_2O solution gave strong support for a structure involving addition to the 4,5-double bond. The spectrum of 3-methylcytosine showed two doublets to low field of the water peak which were due to protons on C-4 and C-5.[15] These resonances were not present in the bis-hydroxylamine compound in which a multiplet was superimposed on the sharp resonance

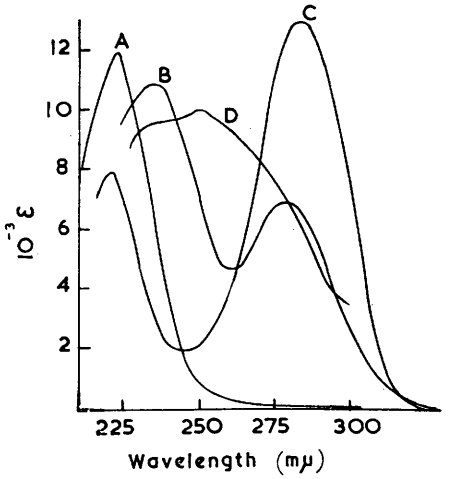


FIG. 3. Ultraviolet spectra in water of 4,5-dihydro-N^6-hydroxy-4-hydroxyamino-3-methylcytosine at pH 7 (A) and of N^6-hydroxy-3-methylcytosine at pH 7 (B), pH 1 (C), and pH 10 (D).

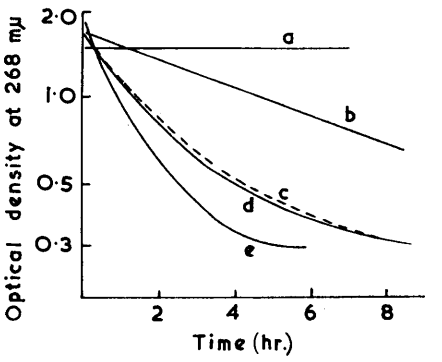


FIG. 4. Optical-density changes during reaction of cytosine with 1·66N-*O*-methylhydroxylamine hydrochloride at 50° and (a) pH 3, (b) pH 6·5, (c) pH 5·5, (d) pH 5·9, and (e) pH 4·9.

of the methyl group at 6·91 τ. This could be interpreted as the resonance of the AB protons of an ABX system with chemical shifts of about 6·85 and 7·25 τ. A quartet of lines to lower field at 5·43 τ corresponded to the resonance of the X proton. This spectrum is consistent with structure (III; R = Me) and not with one in which the hydroxylamine had added in the reverse direction. The resonance at 5·43 τ can clearly be interpreted as deriving from the proton at C-4 lying between the two more electro-negative nitrogen atoms.

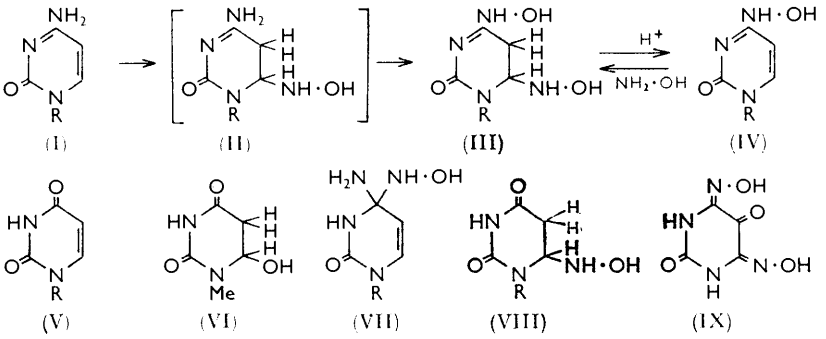
Cytosine behaved entirely analogously to its 3-methyl derivative, giving rise to a crystalline bishydroxylamine compound (III; R = H) which with acid afforded N^6-hydroxycytosine (IV; R = H), together with variable amounts of uracil which increased with increasing pH.[16] The n.m.r. spectrum was consistent with the structure (III; R = H). It was qualitatively similar to that of the 3-methyl compound, showing a triplet centred at 7·3 τ and a quartet, resolved into two doublets in dimethyl sulphoxide, at 5·39 τ.

As further evidence of structure (III), the easy acid-catalysed elimination of one mol. of hydroxylamine has its counterpart in the dehydration of the products obtained by

[14] Fox, van Praag, Wempen, Doerr, Cheong, Knoll, Eidinoff, Bendich, and Brown, *J. Amer. Chem. Soc.*, 1959, **81**, 178.
[15] Jardetzky and Jardetzky, *J. Amer. Chem. Soc.*, 1960, **82**, 222.
[16] Phillips, personal communication.

addition of water under ultraviolet irradiation, to uracil and cytosine derivatives.[17] These addition products have been shown to have the 4,5-dihydro-4-hydroxy-structure (*e.g.*, VI). They are prone to thermal dehydration; our compounds correspondingly lose hydroxylamine smoothly when heated *in vacuo*, providing an alternative means of forming the free N^6-hydroxycytosine bases. The latter, with hydroxylamine, readily re-form the bis-adducts.

In the parallel studies of the hydroxylamine reaction by others, referred to above, different conclusions were drawn as to the structure of the primary product having $\lambda_{max.}$ in the region of 220 mμ. Freese *et al.*,[7] working with cytosine, proposed structure (VII). This is clearly untenable on general grounds, and in particular is invalidated by the n.m.r.



evidence reported here. They did observe that a compound having the ultraviolet absorption characteristics of N^6-hydroxycytidine was formed on acid hydrolysis, which was reconverted into cytosine by reduction with titanous chloride. Schuster,[12] working with cytidine and cytidylic acid, concluded that the structures (VIII; R = ribofuranosyl and 5′-phosphoribosyl) accounted for the properties of the intermediates, particularly their conversion in acid into uracil derivatives. The formation, in addition, of N^6-hydroxycytosine derivatives necessitated the assumption of a rearrangement for which no analogy exists. Verwoerd *et al.*[13] have interpreted their observations on the hydroxylamine reaction with cytosine in the light of the present structural conclusions.

The apparently ready introduction of the 6-hydroxyamino-group would be surprising if it were due to an exchange of the amino-group in a cytosine residue. Base-catalysed hydrolysis of cytidylic to uridylic acid is known to occur, but this is very slow.[18] The corresponding hydrolysis noted with 4,5-dihydro-2′-deoxycytidine [19] is, on the contrary, very rapid, suggesting that the intermediate (II) is necessarily involved in the formation of (III).

Although it was unlikely, *a priori*, that the hydroxylamine had added by way of its oxygen atom (cf. ref. 20), we have eliminated this possibility by showing that *O*-methylhydroxylamine formed a compound with cytosine analogous to (III), which underwent easy acid-catalysed elimination to form N^6-methoxycytosine. The pH optimum for the addition reaction was 5, consistent with the lower pK_a value of 4·60 for *O*-methylhydroxylamine compared with the value [21] for hydroxylamine of 5·97. Some rate measurements are recorded in Fig. 4, in which it is seen that at the lower pH the rate apparently decreases

[17] Shugar, in " Nucleic Acids," ed. Chargaff and Davidson, Academic Press, New York, 1960, Vol. III, p. 73.
[18] Marrian, Spicer, Balis, and Brown, *J. Biol. Chem.*, 1951, **189**, 533; Brown, Dekker, and Todd, *J.*, 1952, 2715.
[19] Green and Cohen, *J. Biol. Chem.*, 1957, **225**, 397; 1957, **228**, 601; Cohn and Doherty, *J. Amer. Chem. Soc.*, 1956, **78**, 2863.
[20] Jencks, *J. Amer. Chem. Soc.*, 1958, **80**, 4581, 4585.
[21] King in " Technique of Organic Chemistry," ed. Bentley, Interscience Publ. Inc.. New York, 1963, Vol. XI, Part I, p. 339.

with time, a result we ascribe to the incursion of the acid-catalysed elimination. The same observation, although less marked, was made with the hydroxylamine reaction itself. *O*-Methylhydroxylamine, did not react at pH 6·5 or 8·9 with uridine or thymidine, confirming the observation of Kochetkov *et al.*[22] that the *O*-methyl compound has greater specificity than hydroxylamine itself; an investigation of its possible mutagenic activity is being made by Dr. T. Alderson.

The bishydroxylamino-compounds are readily oxidised. Thus compound (III; R = H) formed a red complex with ferric chloride solution, the colour appearing only after the preliminary reduction of two ferric ion-equivalents per mole. N^6-Hydroxycytosine (IV) on the other hand gave a blue complex, a reaction which could be used for its quantitative determination; no oxidation was involved in this case. Both compounds (III) and (IV) acted as reducing agents toward diphenylformazan. It was noted that the bishydroxylamino-compounds were very sensitive to air, becoming yellow in solutions of pH above 7. When compound (III; R = H) was aerated in dilute aqueous sodium hydroxide, the solution became yellow, then red. The isolated red product was the sodium salt of a yellow acid of pK_a 9·2. Analysis indicated the formula $C_4H_4N_4O_4$ and the sodium salt in D_2O solution gave no n.m.r. signal, indicating the absence of non-exchangeable protons. We assign, tentatively, the structure (IX) to the free acid.

The scope of the reaction with hydroxylamine has been extended to include a number of other cytosine derivatives. Cytidine and 2′-deoxycytidine reacted normally to give the corresponding bishydroxylamino-compounds. The former on acid hydrolysis afforded N^6-hydroxycytidine,[14] only a small amount of glycosidic hydrolysis being noted. Of greater interest was the fact that under milder acid conditions (pH 1·0) employed to minimise glycoside-bond hydrolysis, a large part of the product from the deoxycytidine adduct appeared as 2′-deoxyuridine, the remainder being the expected N^6-hydroxy-2′-deoxycytidine.

When hydroxylamine reacted with 5-methyl- and 5-hydroxymethyl-cytosine, bis-adducts were again formed, but at a very much reduced rate compared with cytosine. For the successful preparation of the compounds anhydrous hydroxylamine at 70° had to be used. The products had the expected properties and gave the corresponding 5-methyl- and 5-hydroxymethyl-N^6-hydroxycytosine on acid hydrolysis. Even thymidine, which was unreactive towards aqueous hydroxylamine at neutrality, underwent reaction under these conditions, with loss of selective ultraviolet absorption. Schuster [12] has already shown that uridine reacts readily with hydroxylamine at pH 8—9. We therefore conclude that substitution by methyl or hydroxymethyl at position 5 has a very marked effect on the addition, in both the uracil and the cytosine series.[5]

Schuster [12] and, later, Verwoerd *et al.*[13] showed that the reaction products from uridine and hydroxylamine were isoxazolone and ribose oxime. Cytidine, too, we found afforded ribose oxime when treated with the anhydrous reagent although only the bis-adduct was formed with aqueous hydroxylamine at pH 6·5. These reactions are clearly analogous to those of the nucleosides with hydrazine in which pyrazolone and aminopyrazole, respectively, are formed. The conclusion may be drawn that the hydrazine reaction also depends on an initial addition to the 4,5-double bond but that the subsequent attack at position 6 in which pyrimidine ring fission is effected proceeds readily in the hydrazinolysis and is, as expected, slow or absent in the hydroxylamine reaction with cytosine and its derivatives.

The present work does not provide an immediate explanation for the mutagenic effect of hydroxylamine. Further work [16] on this question is in progress and will be reported shortly.

Experimental

Ultraviolet absorption spectra were obtained by using a Cary recording spectrophotometer, model 14 M50. Reaction time and course studies were made on the same instrument with a constant-temperature cell. N.m.r. spectra were run in D_2O solution in a Varian A-40 apparatus.

[22] Kochetkov, Budowsky, and Shibaeva, *Biochim. Biophys. Acta*, 1963, **68**, 493.

For paper chromatography (ascending), Whatman No. 1 paper was used in the systems A (butan-1-ol saturated with water), B [propan-2-ol–HCl (*d*, 1·19)–water, 170 : 41 : 39], and C (water).

Anhydrous hydroxylamine was prepared by Brauer's method [23] and sublimed before use (m. p. 32°). Hydroxylammonium acetate was prepared by mixing ethanolic solutions of the free base and of acetic acid (excess) and evaporating the mixture to dryness *in vacuo*. The salt was then recrystallised from ethanol.

4,5-*Dihydro*-N^6-*hydroxy*-4-*hydroxyaminocytosine*.—Finely powdered anhydrous cytosine (1·0 g.) and anhydrous hydroxylamine (7 g.) were mixed in a flask and protected from atmospheric moisture by a drying-tube (P_2O_5). After 45 hours' heating at 35° with occasional shaking, the crystalline *product* (1·46 g., 95%) was collected, washed with a little absolute ethanol, and dried at room temperature. It had m. p. 157—158° (decomp.) (Found: C, 28·5; H, 5·3; N, 32·5. $C_4H_8N_4O_3,0·5H_2O$ requires C, 28·4; H, 5·3; N, 33·1%). It had R_F 0·072 in system A, $\lambda_{max.}$ in H_2O (pH 7) 220 mμ (ε 10,800), and formed a red complex, $\lambda_{max.}$ 525 mμ, with ferric chloride at pH 2.

The compound was also obtained from N^6-hydroxycytosine by the above treatment or by the method involving aqueous hydroxylamine acetate (pH 6·5) (see below). The product was in each case characterised by its ultraviolet spectrum.

4,5-*Dihydro*-N^6-*hydroxy*-4-*hydroxyamino*-3-*methylcytosine*.—(*a*) This was prepared from 3-methylcytosine as described above. The *product* (95%) was recrystallised twice from water, giving needles, m. p. 229—230° (decomp.) (Found: C, 34·6; H, 6·0; N, 31·8. $C_5H_{10}N_4O_3$ requires C, 34·5; H, 5·8; N, 32·2%), $\lambda_{max.}$ in H_2O (pH 7) 223 mμ (ε 11,990), R_F 0·124 in system A. It formed a red complex in solution with ferric ion.

(*b*) 3-Methylcytosine (250 mg.) in 10N-hydroxylamine acetate (12 ml.) (pH 6·6) was incubated for 24 hr. at 35°. The reaction mixture was evaporated and most of the hydroxylamine acetate removed by sublimation at room temperature. The residue was dissolved in the minimum amount of water and methanol (50 ml.) was added. The mixture was kept for 7 days at −15° and then the crystalline N^6-hydroxy-4,5-dihydro-4-hydroxyamino-3-methylcytosine was collected. It was identical (ultraviolet spectrum) with the product obtained by procedure (*a*).

4,5-*Dihydro*-N^6-*hydroxy*-4-*hydroxyamino*-5-*hydroxymethylcytosine*.—5-Hydroxymethylcytosine (160 mg.) and anhydrous hydroxylamine (2 g.) were kept together with exclusion of moisture at 70° for 19 hr. After cooling, the contents of the flask solidified and after removal of the hydroxylamine (19 hr. at 20°/10^{-4} mm.) the *product* was obtained in quantitative yield as a hygroscopic solid, m. p. 78° (Found: C, 32·6; H, 5·6; N, 30·0. $C_5H_{10}N_4O_4$ requires C, 31·6; H, 5·3; N, 29·5%), $\lambda_{max.}$ in H_2O (pH 7) 220 mμ (ε 9160), $\lambda_{infl.}$ 270 mμ (ε 980), R_F 0·72 in system C. It gave a violet complex, $\lambda_{max.}$ 570 mμ with ferric chloride.

4,5-*Dihydro*-N^6-*methoxy*-4-*methoxyaminocytosine*.—Cytosine (75 mg.) was dissolved in aqueous 2N-*O*-methylhydroxylamine acetate (7·5 ml.), pH 5·2. Reaction was complete after 15 hr. at 37°. The solution was taken to dryness *in vacuo*, the residue dissolved in the minimum amount of absolute methanol, and the solution kept in an atmosphere of ether. The *product* formed hexagonal crystals (93 mg., 78%), m. p. 136° (Found: C, 38·9; H, 6·8; $C_6H_{12}N_4O_3$ requires C, 38·3; H, 6·4%), $\lambda_{max.}$ 225 mμ (ε 11,900) in water (pH 7).

4,5-*Dihydro*-N^6-*hydroxy*-4-*hydroxyaminocytidine*.—Cytidine (1·0 g.) was dissolved in 5N-hydroxylamine acetate (14 ml.) at pH 6·5 and kept at 35° for 13 hr. The solution was taken to dryness *in vacuo* and kept at 10^{-4} mm. (to constant weight) to remove hydroxylamine acetate, and then the residue was dissolved in absolute methanol. The solution was filtered and then taken to dryness at room temperature, and the residue was dissolved in absolute ethanol. Partial evaporation in the cold under anhydrous conditions gave the crystalline *product* (950 mg.) which was collected by centrifugation and washed with absolute ether (Found: C, 37·2; H, 6·0; N, 18·4. $C_9H_{16}N_4O_7$ requires C, 37·0; H, 5·5; N, 19·2%); it had m. p. 130—135°, R_F, 0·158 in system A, $\lambda_{max.}$ 224 mμ (ε 11,190) in water (pH 7).

Formation of Ribose Oxime from Cytidine.—Cytidine (1·0 g.) and anhydrous hydroxylamine (5·0 g.) were kept at 45° for 26 hr. with exclusion of moisture. Hydroxylamine was removed from the clear yellow solution at 25°/10^{-4} mm. The ribose oxime formed needles (0·55 g., 80%), m. p. 143—144° (lit.,[24] 141°) (Found: C, 36·6; H, 6·0; N, 8·7. Calc. for $C_5H_{11}NO_5$, C, 36·4;

[23] Brauer, "Handbuch der Präperativen Anorganischen Chemie," Ferdinand Enke Verlag, Stuttgart, 1960, Vol. I, p. 450.

[24] Kuhn, Reinemund, Weygard, and Ströbele, *Ber.*, 1935, **68**, 1765.

H, 6·7; N, 8·5%). After mild acid treatment (N-HCl for 10 min. at 100°), ribose was detected in the hydrolysate by paper chromatography.

4,5-Dihydro-N⁶-hydroxy-4-hydroxylamino-2′-deoxycytidine.—Deoxycytidine (1·0 g.) was treated with hydroxylamine acetate as described above. Solvent and hydroxylamine acetate were removed by sublimation in a high vacuum and the residue was thrice precipitated from dry ethanol by dry ether. After two crystallisations from dry ethanol the *product* formed hygroscopic crystals (30% yield), m. p. 121—122° (Found: C, 38·8; H, 5·9; N,18·6. $C_9H_{16}N_4O_6$ requires C, 39·1; H, 5·8; N, 20·2%), R_F 0·175 in systems A, $\lambda_{max.}$ 222 mμ (ε 12,010) in water (pH 7).

N⁶-Hydroxycytosine.—(*a*) 4,5-Dihydro-*N*⁶-hydroxy-4-hydroxyaminocytosine (340 mg.) in N-hydrochloric acid (10 ml.) was heated at 100° for 1 hr. After evaporation to dryness under reduced pressure a crystalline residue (315 mg.) was obtained which was recrystallised from ethanol. *N⁶-Hydroxycytosine hydrochloride* formed needles, m. p. 220—221° (Found: C, 29·8; H, 3·7; N, 25·6. $C_4H_6ClN_3O_2$ requires C, 29·4; H, 3·7; N, 25·7%), $\lambda_{max.}$ in H_2O, 233, 273 mμ (ε 10,600, 5260), $\lambda_{min.}$ 259 mμ (ε 4770) at pH 7; $\lambda_{max.}$ 216 (8650), 276 (11,750), $\lambda_{min.}$ 240 (2780) at pH 1. The compound had pK_a 2·25 and formed a blue complex, $\lambda_{max.}$ 608 mμ, with ferric ion at pH 2, which was useful for detection of the compound on chromatograms. It had R_F 0·44 in system B and migrated at 1·7 times the rate of uracil on paper electrophoretograms at pH 3·5.

Uracil (R_F 0·66 in system B) was present in the mother-liquors.

(*b*) The bishydroxylamino-compound (340 mg.) was kept at 180—190° in an oil-bath under a vacuum for 45 min., during which the compound melted and hydroxylamine was evolved. The pale yellow residue was recrystallised (charcoal) from ethanol. *N⁶-Hydroxycytosine* formed colourless needles, m. p. 252° (Found: C, 37·9; H, 4·1; N, 33·1. $C_4H_5N_3O_2$ requires C, 37·5; H, 4·6; N, 32·8%).

Treatment with a slight excess of hydrochloric acid yielded a hydrochloride identical with that described above in infrared and ultraviolet spectra. The m. p. and mixed m. p. were identical. When the hydrochloride was dissolved in 30% aqueous ammonia under nitrogen and the solution was evaporated *in vacuo*, the free base was obtained in crystalline form and characterised by comparison with material prepared as above.

N⁶-Hydroxy-3-methylcytosine Hydrochloride.—4,5-Dihydro-*N*⁶-hydroxy-4-hydroxyamino-3-methylcytosine (350 mg.) was treated with N-hydrochloric acid (10 ml.) as described above. The *product* formed colourless crystals (320 mg., 90%) from 19 : 1 ethanol–ether or absolute ethanol, m. p. 218—219° (decomp.) (Found: C, 34·1; H, 4·2; N, 23·0. $C_5H_7N_3O_2$,HCl requires C, 33·8; H, 4·5; N, 23·6%), $\lambda_{max.}$ 234 mμ (ε 10,900), 279 (6960), $\lambda_{min.}$ 261 (4660) in water (pH 7); $\lambda_{max.}$ 221 (7960), 284 (12,300), $\lambda_{min.}$ 246 (1984) in HCl (pH 1). The compound had pK_a 2·3 (spectrophotometric) and in ferric chloride (pH 2) formed a blue complex, $\lambda_{max.}$ 620 mμ.

N⁶-Hydroxy-5-hydroxymethylcytosine.—4,5-Dihydro-4-hydroxyamino-5-hydroxymethylcytosine (100 mg.) was heated under reflux in N-hydrochloric acid (10 ml.) for 1 hr. and the solution was then brought to pH 5 by Dowex-2 (acetate). Thereafter the product was worked up as above. Crystallisation twice from methanol and once from ethanol gave the *hydrochloride* (0·07 g., 70%), m. p. 260° (decomp.) (Found: C, 31·8; H, 3·9; N, 22·2. $C_5H_7N_3O_3$,HCl requires C, 31·0; H, 4·1; N, 21·8%), $\lambda_{max.}$ in water 230 mμ (ε 9260), 273 (5570), $\lambda_{min.}$ 258 (4800) at pH 7, $\lambda_{max.}$ 217 (940), 279 (10,300), $\lambda_{min.}$ 245 (2600) at pH 1.

N⁶-Hydroxy-5-methylcytosine.—5-Methylcytosine (250 mg.) and hydroxylamine (4 g.) were heated together at 70° for 19 hr. with excusion of moisture. Working up in the usual way gave crude 4,5-dihydro-*N*⁶-hydroxy-4-hydroxyamino-5-methylcytosine as an amorphous product (280 mg.), $\lambda_{max.}$ 217 mμ (ε 6016). This was dissolved in the minimum amount of 0·25N-hydrochloric acid, heated for 1 hr. at 100° and the solution evaporated. The residue was a mixture of the hydrochloride and the very insoluble free base; attempted recrystallisation led to further dissociation of the hydrochloride. Sublimation gave the free *base*, m. p. 270°, in high yield (Found: C, 42·6; H, 4·9; N, 29·8. $C_5H_7N_3O_2$ requires C, 42·6; H, 5·0; N, 29·8%), $\lambda_{max.}$ in water 232 mμ (ε 8810), 272 (6650), $\lambda_{min.}$ 256 (5810) at pH 7; $\lambda_{max.}$ 217 (10,340), 283 (11,950) at pH 1. The substance had R_F 0·65 in system C and pK_a 2·6. It formed a blue complex with ferric ion.

N⁶-Hydroxycytidine.—The dihydrohydroxyamino-compound (200 mg.) was dissolved in N-hydrochloric acid (6 ml.) and kept at 100° for 8 min. The pH was adjusted to 5 (Dowex-2, acetate) and after filtration the volume of solution and washings was made up to 10 ml. The

solution was passed through a column (0·7 × 8 cm.) of Amberlite IR-120 (H^+), and the column washed with water until no further ultraviolet-absorbing material was eluted. The product was eluted with 3% aqueous ammonia. The solvent was quickly removed *in vacuo* and the residue (106 mg., 60%) crystallised from hot ethanol. Recrystallisation gave N^6-hydroxycytidine, m. p. 171—172° (lit.,[14] 169—172°), R_F 0·77 (system C), pK_a 2·3, $\lambda_{max.}$ in H_2O, 236 mμ (ε 11,820), 272 (6400) at pH 7 $\lambda_{max.}$ 221 (9150), 280 (14,530) at pH 1.

*2′-Deoxy-*N^6*-hydroxycytidine.*—4,5-Dihydro-N^6-hydroxy-4-hydroxyamino-2′-deoxycytidine (1·0 g.) was heated with dilute hydrochloric acid (pH 1; 30 ml.) for 8 min. and the mixture worked up as for N^6-hydroxycytidine. A neutral fraction (60%) passed through the Amberlite column; chromatography and ultraviolet spectroscopy showed it to contain mainly 2′-deoxyuridine. The basic fraction was dissolved in a little aqueous ethanol from which some N^6-hydroxycytosine crystallised. The residue, a pale purple gum (300 mg., 27%) was essentially *2′-deoxy-*N^6*-hydroxycytidine* which resisted crystallisation. In system C it had R_F 0·77 with a trace of material at 0·71 (Found: C, 41·7; H, 5·7; N, 17·0. $C_9H_{13}N_3O_5,H_2O$ requires C, 41·4; H, 5·8; N, 16·1%), (pH 7) $\lambda_{max.}$ in H_2O, 234 mμ (ε 11,100), 272 (6360) at pH 7 $\lambda_{max.}$ 219 (8500), 278 (13,000) at pH 1.

N^6*-Methoxycytosine.*—4,5-Dihydro-N^6-methoxy-4-methoxyaminocytosine (50 mg.) was treated with N-HCl as in the preparation of N^6-hydroxycytosine. The *hydrochloride* (30 mg., 63%) crystallised from ethanol–ether and had m. p. 195—196° (Found: C, 33·6; H, 4·9. $C_5H_8ClO_2N_3$ requires C, 33·8; H, 4·5%), $\lambda_{max.}$ in water 237 mμ (ε 9190), 273 (6070) at pH 7, $\lambda_{max.}$ 216 mμ (ε 7200), 279 (10,000) at pH 1. The compound gave no blue colour with ferric ion at pH 2.

Aerial Oxidation of 4,5-*Dihydro-*N^6*-hydroxy-4-hydroxyaminocytosine.*—The compound (120 mg.) was dissolved in N-sodium hydroxide (25 ml.), and oxygen was bubbled through the solution for 18 hr. Partial evaporation of the solution *in vacuo* gave red crystals which were recrystallised from hot dilute sodium hydroxide and then precipitated from ethanol by ether. They had $\lambda_{max.}$ in water 335 mμ at pH 7, and 400 mμ at pH 11·5. The sodium salt (100 mg., 79%) had pK_a 9·2 and Equiv. 200 (required for $C_4H_3N_4NaO_4$, 195) by titration. The salt was treated with dilute acetic acid, giving a yellow precipitate which was washed with water and dried. It had m. p. 265° (decomp.) (Found: C, 27·9; H, 2·8. $C_4H_4N_4O_4$ requires C, 27·9; H, 2·3%).

Reaction of Deoxyribonucleic Acid with Hydroxylamine.—(*a*) Portions (1 mg.) of highly polymerised salmon sperm deoxyribonucleic acid (Calbiochem) were shaken with anhydrous hydroxylamine (0·4 ml.) at 35° in sealed glass tubes until they dissolved. The samples were incubated at 35° for various times. After removal of the hydroxylamine by high-vacuum sublimation the material was hydrolysed with 98% formic acid at 180° for 75 min. The formic acid was removed *in vacuo* and the residue was dissolved in dilute hydrochloric acid (0·5 ml.) and chromatographed in system B. The spots were eluted with N-hydrochloric acid for 24 hr. and the optical density at 260 mμ measured.

(*b*) Deoxyribonucleic acid (12 mg.) was dissolved in water (0·4 ml.) and to the solution was added anhydrous hydroxylamine 0·8 g. in water (2 ml.). The mixture was kept at 95° and samples (1 mg.) were taken at intervals. The base ratios were estimated as described under (*a*). The results are recorded in Fig. 1. After treatment, samples from both experiments were dialysed against water at 4°. No ultraviolet-absorbing material passed through the membrane.

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