254. *The Methylation of Cytosine and Cytidine.*

By P. Brookes and P. D. Lawley.

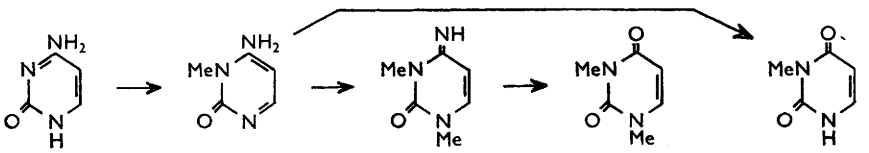
Methylation of cytidine by dimethyl sulphate in dimethylformamide gave 1-methylcytidine which on hydrolysis with perchloric acid yielded 1-methylcytosine. Methylation of cytosine gave 1-methyl- and 1,3-dimethyl-cytosine. The ultraviolet absorption spectra and pK_a values of these products are given and their conversion by alkali into the corresponding methylated uracils has been established.

Of the groups in nucleic acids reactive towards alkylating agents those of guanine at $N_{(7)}$,[1] and of adenine at $N_{(1)}$ and $N_{(3)}$,[2] have been identified. The remaining reactive site, that in cytosine, appeared most likely to be the unsubstituted ring nitrogen atom $N_{(1)}$. A previously reported synthesis of 1-methylcytosine [3] involving as the final stage pyrolytic decarboxylation of 5-carboxy-1-methylcytosine appears to be incorrect: Brown [4] showed that this reaction yielded 2-hydroxy-6-methylaminopyrimidine, *i.e.*, on pyrolysis net migration of the methyl group from ring nitrogen to extranuclear nitrogen had occurred. 1-Methylcytosine was therefore not known. The identification of the reactive group of cytidine as $N_{(1)}$ therefore required that the product obtained by removal of the ribose moiety from the methylated cytidine should be isolated and shown to be 1-methylcytosine.

Methylation of cytidine by dimethyl sulphate in dimethylformamide gave a monomethylcytidine in good yield and on hydrolysis with perchloric acid this gave a monomethylcytosine, again in good yield. The absorption spectrum of this compound in the range pH 2—5 was closely similar to that of cytosine in acid solution, but markedly different in alkaline solution. The pK_a' value, determined spectroscopically, was 7·4. These physical properties are in agreement with those expected for 1-methylcytosine since the attachment of a proton to cytosine at $N_{(1)}$ would be expected to change the

[1] Brookes and Lawley, *J.*, 1961, 3923.
[2] Brookes and Lawley, *J.*, 1960, 539.
[3] Whitehead and Traverso, *J. Amer. Chem. Soc.*, 1955, **77**, 5867.
[4] Brown, *J. Appl. Chem.*, 1955, **5**, 358.

absorption spectrum in the same way as methylation at $N_{(1)}$, whereas the spectrum of the methylcytosine in alkali would differ markedly from that of cytosine as a free base since the molecule no longer possesses an NH·CO group. The absence of this acidic group is further reflected by the increase of pK_a from 4·6 to 7·4 from cytosine to 1-methylcytosine.



(I) (II) (III)

Further proof of the structure of this compound was obtained from its conversion by alkali into the known 1-methyluracil, obtained by methylation of uridine,[5] the configuration of which has been rigorously established by Brown, Hoerger, and Mason.[6]

Methylation of cytosine by dimethyl sulphate in dimethylformamide was attempted at both 37° and 100°. In each case an excess of the reagent was added until all the cytosine had dissolved. Paper chromatography showed that much cytosine remained unchanged but two products were present. These were 1-methylcytosine, identified by comparison with the product obtained as above, and the known 1,3-dimethylcytosine. No 3-methylcytosine was found, which provides additional confirmation that the original methylation was at $N_{(1)}$. This suggests that the conventional representation of cytosine as (I) is correct, methylation being confined to the $N_{(1)}$ position with no tendency for the NH·CO group to react. However, once the 1-methylcytosine (II) is formed, further reaction at $N_{(3)}$ can occur to give 1,3-dimethylcytosine (III).

Hilbert [7] reported that 1,3-dimethylcytosine was readily deaminated by dilute acid yielding 1,3-dimethyluracil. This has not been confirmed and no appreciable deamination of 1-methylcytosine, 1,3-dimethylcytosine, or 1-methylcytidine was obtained on acid treatment. However, alkali readily caused deamination and yielded the corresponding substituted uracils. When the products were 1,3-disubstituted uracils the conversion was accompanied by overall loss of ultraviolet absorption which is in keeping with their reported instability in alkali.[8]

This behaviour of 1- and 1,3-disubstituted cytosines in alkali contrasts with that of 1-methyladenine,[2] 1,2-dihydro-2-imino-1-methylpyrimidine and certain other pyrimidines [9] with *N*-methyl substituents in the ring adjacent to extranuclear amino-groups, which undergo rearrangement with net migration of the methyl group. The 1-methylcytosines are converted into a more stable ring system by deamination, as occurs with 3-methyladenine [10] and 1,4-dihydro-4-imino-1-methylpyrimidine.[11]

Experimental

M. p.s were observed on a microscope hot stage. Absorption spectra were measured for aqueous solutions with a Unicam S.P. 500 spectrophotomer. Paper chromatography was carried out on Whatman No. 1 filter paper (ascending), the following solvents being used: (1) methanol–concentrated hydrochloric acid–water (7 : 2 : 1); (2) ethanol–water–aqueous ammonia (*d* 0·88) (80 : 18 : 2); (3) butan-1-ol–water (86 : 14); (4) 5% aqueous disodium hydrogen phosphate saturated with 3-methylbutan-1-ol.

[5] Levene and Tipson, *J. Biol. Chem.*, 1934, **104**, 385.
[6] Brown, Hoerger, and Mason, *J.*, 1955, 211.
[7] Hilbert, *J. Amer. Chem. Soc.*, 1934, **56**, 190.
[8] Shugar and Fox, *Biochim. Biophys. Acta*, 1952, **9**, 199.
[9] Brown, *Nature*, 1961, **189**, 828.
[10] Elion, "Ciba Foundation Symposium on the Chemistry and Biology of Purines," J. and A. Churchill Ltd., London, 1957, p. 39.
[11] Brown, Hoerger, and Mason, *J.*, 1955, 4035.

1-*Methylcytidine*.—Cytidine (0·5 g.; 2 mmoles) was treated in dimethylformamide (5 c.c.) with dimethyl sulphate (2 c.c., 20 mmoles) at 37° for 30 min. The clear solution was diluted to 20 c.c. with methanol and ethyl acetate was added to make it cloudy. 1-*Methylcytidine methosulphate* slowly separated as needles (86%), m. p. 193—194° (Found: C, 35·6; H, 5·3; N, 11·4. $C_{11}H_{19}N_3O_9S$ requires C, 35·8; H, 5·2; N, 11·4%).

TABLE 1.

Properties of products from the methylation of cytosine and cytidine.

	pH	$\lambda_{max.}$ (mμ)	$10^{-3}\varepsilon$	$\lambda_{min.}$ (mμ)	$\varepsilon_{260}/\varepsilon_{280}$	pK_a'	R_F in solvent * (1)	(2)	(3)	(4)
1-Methylcytosine (II)	4	274	9·4	240	1·33	7·4 †	0·67	0·67	0·15	0·79
	12	294	11·9	250	5·7					
1,3-Dimethylcytosine (III)	4	280·5	10·3	243	2·4	9·4 ‡	0·80	0·80	0·20	0·89
	12	272	8·9	246·5	1·2					
1-Methylcytidine	4	278	11·8	243	1·9	8·7 ‡	0·71	0·74	0·08	0·91
	12	266	9·0	243	0·69					

* For solvents see p. 1349. † *I* 0·1 at 25°. ‡ *I* 0·1 at 20°.

1-*Methylcytosine*.—1-Methylcytidine methosulphate (0·5 g.) in 72% perchloric acid (5 c.c.) was heated at 100° for 1 hr. After dilution to 50 c.c. with water, the solution was filtered and applied to a column of Dowex-50 (H^+-form; 30 × 3 cm.; equilibrated with water). The column was washed with 0·1N-hydrochloric acid (500 c.c.) and eluted with 0·67N-hydrochloric acid; fractions (50 c.c.) were collected and the optical densities at 260 and 280 mμ measured. Fractions 45—65, which contained 1-*methylcytosine*, were evaporated and the resulting hydrochloride recrystallised from methanol–ethyl acetate as prisms which sublimed above 200° and melted at 242—245° (Found: C, 37·8; H, 5·05; N, 25·6. $C_5H_8ClN_3O$ requires C, 37·2; H, 4·95; N, 26·0%). A derived *picrate* crystallised from water as needles, m. p. 244—246° [Found: *M* (spectroscopic method of Cunningham, Dawson, and Spring [12]), 120. $C_5H_7N_3O$ requires *M*, 125].

Methylation of Cytosine.—Cytosine (5 mmoles) in dimethylformamide (10 c.c.) was treated with dimethyl sulphate (10 mmoles) at 100° for 1 hr.; all the cytosine dissolved. The solvent was evaporated and the resulting solid was dissolved in 0·2N-hydrochloric acid (30 c.c.) and applied to a column of Dowex-50 (H^+-form equilibrated with 0·2N-hydrochloric acid). The column was washed with 0·2N-acid (500 c.c.) and eluted with 0·7N-acid. Fractions 15—35 contained an unresolved mixture of cytosine and 1-methylcytosine, and fractions 40—50 contained a product with $\lambda_{max.}$ 281 mμ. The latter fractions were evaporated, and the resulting solid redissolved in water (2 c.c.), made alkaline (NaOH), and immediately extracted with chloroform (3 × 5 c.c.). Light petroleum (b. p. 40—60°) was added to this extract to yield microcrystals, which on sublimation gave 1,3-dimethylcytosine, m. p. 145° (lit.,[7] 144°).

In further experiments the yields of 1-methylcytosine and 1,3-dimethylcytosine were determined by paper chromatography of the reaction mixture, elution of the spots, and measurement of their ultraviolet absorption. With cytosine (0·3 mmole) and dimethyl sulphate (3 mmoles) in dimethylformamide (5 c.c.) the yields were: at 100° for 1 hr., 1-methylcytosine, 25%; 1,3-dimethylcytosine, 5%; and at 37° for 20 hr., 1-methylcytosine, 27%; 1,3-dimethylcytosine, 7%.

Action of Alkali on Methylation Products.—A solution of 1-methylcytosine hydrochloride (0·02 g./l.) in 0·04N-sodium hydroxide was heated at 96° and the absorption spectrum measured after 0·3, 0·9, and 6 hr. The spectrum changed to that of 1-methyluracil, *i.e.*, $\lambda_{max.}$ 282 mμ at pH 12, and 258 mμ at pH 2—7, the time of half-change being 0·9 hr. In another experiment, 1-methylcytosine hydrochloride (0·1 mmole) was treated with 40% potassium hydroxide (0·2 ml.) at 98° for 2 hr. and the solution (0·05 c.c.) chromatographed on paper. This showed one ultraviolet-absorbing product, with R_F in solvent (1), 0·82; (2), 0·80; (3), 0·70; (4), 0·72, and identical with authentic 1-methyluracil.

In similar experiments with 1,3-dimethylcytosine and 1-methylcytidine the ultraviolet absorption changed completely to that of the corresponding uracils in 15 min. at 100° in 0·1N-sodium hydroxide and was accompanied at the maxima by a decrease in optical density to 10% of the original value. With 1-methylcytidine the conversion was complete in 48 hr. at 37°,

[12] Cunningham, Dawson, and Spring, *J.*, 1951, 2305.

with a decrease in ultraviolet absorption to 40% of the original. In both cases the products were identified by paper chromatography by comparison with authentic 1,3-dimethyluracil [R_F in solvent (1), 0·94; (2), 0·90; (3), 0·77; (4), 0·82], and 1-methyluridine [R_F in solvent (1), 0·83; (2), 0·78].

Action of Acid on Methylation Products.—1-Methylcytosine, 1-methylcytidine, and 1,3-dimethylcytosine (2 g./l.) were separately heated in N-hydrochloric acid (5 c.c.) at 100° and the ultraviolet absorption determined, after dilution to 1/100, initially and after 1, 2, and 20 hr. No changes in absorption spectrum were found.

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