Acylation of Cystosine by Ethyl *N*-Hydroxycarbamate and its Acyl Derivatives and the Binding of these Agents to Nucleic Acids and Proteins

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Ethyl *N*-hydroxycarbamate and several of its acyl derivatives specifically acylated the primary amino-group of cytosines; no reaction occurred at other sites or with the other nucleic acid bases. Compared with cytosine, the *N*(4)-acylcytosines showed (*a*) a bathochromic shift in u.v. absorption and (*b*) an increase in the polarity of the carbon–nitrogen bond resulting in greater ease of hydrolysis into uracils. [1-^{3}H]Ethyl *N*-hydroxycarbamate and ethyl [*acetyl*-^{3}H]*N*-acetoxycarbamate reacted with nucleic acids and proteins. Incubation of ethyl *N*-acetoxy-*N*-acetylcarbamate with DNA, followed by enzymatic hydrolysis of the nucleic acid, produced 2′-deoxyuridine and 2′(5′)-deoxyuridylic acid. Ethyl carbamate and acetylcarbamate, and several other acyl derivatives of ammonia, hydrazine, and hydroxylamine did not react with cytosine.

ETHYL CARBAMATE is a carcinogen which is metabolised by mammals into the approximately equally carcinogenic [1] ethyl *N*-hydroxycarbamate, and into ethyl *N*-acetoxycarbamate and other products.[2,3] Reasons for believing that ethyl carbamate is active *via* a metabolite have been discussed.[4] It has been suggested [5,6] that the biological effects of hydroxyurea may be mediated by metabolic conversion into ethyl *N*-hydroxycarbamate; this is unlikely, since hydroxyurea is not a carcinogen.

Ethoxycarbonylation appears to be a characteristic reaction of ethyl *N*-hydroxycarbamate. *In vivo*, the agent ethoxycarbonylates tissue mercapto-groups [2] and the cytosine ring of RNA; [7] when oxidised it ethoxycarbonylates itself, other hydroxamic acids, and ammonia.[8] The present work shows that, *in vitro*, ethyl *N*-hydroxycarbamate and its acyl derivatives specifically acylate the primary amino-group of cytosines and do not react with the other nucleic acid bases. Ethyl carbamate and acetylcarbamate, and several other acyl compounds did not react with cytosine. The enzyme-

[1] L. Boiato, S. S. Mirvish, and I. Berenblum, *Internat. J. Cancer*, 1966, **1**, 265.
[2] E. Boyland and R. Nery, *Biochem. J.*, 1964, **91**, 362.
[3] S. S. Mirvish, *Biochem. Biophys. Acta*, 1966, **117**, 1.
[4] R. Nery, *Biochem. J.*, 1968, **106**, 1.
[5] R. S. Schmidt, R. D. Pollock, and H. S. Rosenkranz, *Biochem. Biophys. Acta*, 1967, **138**, 645.
[6] G. M. Timmis and D. C. Williams in 'Chemotherapy of Cancer,' Butterworth, London, 1967, p. 119.
[7] E. Boyland and K. Williams, *Biochem. J.*, 1969, **111**, 121.
[8] E. Boyland and R. Nery, *J. Chem. Soc.* (*C*), 1966, 354.

catalysed transacetylation of amino-groups by *N*-hydroxyacetamidoarenes, which occurs with arylamines [9] as substrates, does not occur with aminopurines or aminopyrimidines.[10]

Compounds of type $R^1ONR^2R^3$, where at least one of the substituents is an acyl group (*e.g.* methyl *N*-methoxycarboxycarbamate, ethyl *N*-hydroxycarbamate, ethyl *N*-acetoxycarbamate, and ethyl *N*-acetoxy-*N*-acetylcarbamate) react with cytosine under physiological conditions, or more rapidly on heating, to give varying amounts (Table 2) of uracil and *N*(4)-acylcytosines. This acylation occurs whether the acyl group is attached to the nitrogen or oxygen atom of hydroxylamine, but occurs more readily in the latter case.

The mechanism of the acylations differs from that described [11] for the arylation of guanine residues by carcinogenic esters of *N*-arylhydroxamic acids involving arylamidinium ions. These acylations probably result from electrophilic attack by acyl carbon on the amino-nitrogen of cytosine; the *O*-acyl esters, because of the greater ease of transfer of the *O*-acyl group, have the acylating properties of, though they are more selective than, the corresponding acid anhydrides.

Reaction of benzyl *N*-benzyloxycarboxycarbamate with cytosine gave uracil, presumably formed from the unstable *N*(4)-benzyloxycarbonylcytosine, since benzyl chloroformate in pyridine also converted cytosine into uracil. Two acetamides (ethyl acetylcarbamate and *N*-methyl-5-acetamidovaleric acid), and several acyl derivatives of hydroxylamine or hydrazine did not react with cytosine at pH 5·7 and 80° in 4 hr. Ethyl *N*-acetoxy-*N*-acetylcarbamate acylated cytosine, cytidine, 2′-deoxycytidine, 2′(5′)-deoxycytidylic acid, and 5-methylcytosine in 16 hr. at 37° at neutral or near neutral pH, but did not react with uracil, uridine, thymine, thymidine, guanosine, 5′-guanylic acid, 2′(5′)-deoxyguanylic acid, adenine, adenosine, 2′-deoxyadenosine, or 2′(5′)-deoxyadenylic acid under similar conditions.

Acylation of the amino-group of cytosine caused (i) a bathochromic shift in u.v. absorption as compared with cytosine (Table 1) and (ii) labilisation of the acylamino group, so that hydrolysis to uracil readily occurred. In acid solution at 25°, the *N*(4)-acylcytosines gave varying amounts, increased in hot solutions, of uracil. In hot neutral solutions, mixtures of cytosine and several acylhydroxylamines also gave varying amounts of uracil (Table 2) which was probably formed from hydrolysis of the *N*(4)-acylcytosines since cytosine was stable under these conditions.

The present results show that ethyl *N*-hydroxycarbamate, ethyl *N*-acetoxycarbamate, and ethyl *N*-acetoxy-*N*-acetylcarbamate bind to biological macromolecules under physiological conditions, but do not explain the nature of the binding. Boyland and Williams have shown that *in vivo*, the RNA of rats dosed with ethyl carbamate contain a 5-ethoxycarbonylcytosine group,[7] and that the uracil content of RNA treated with aged samples of ethyl *N*-hydroxycarbamate, which probably contain ethyl *N*-ethoxycarboxycarbamate (an oxidation product [8]), is increased above normal levels.[12] If *N*(4)-ethoxycarbonylation of cytosine in nucleic acids occurred *in vivo*, this would not have been detected by Boyland and Williams [7] because of the instability of *N*(4)-ethoxycarbonylcytosine (*a*) in the presence of liver and blood enzymes and (*b*) under the acidic conditions used for the degradation of the nucleic acids. The results of Boyland and Williams [7] and those described here suggest that (*a*) ethyl carbamate (after metabolic *N*-oxidation [2]) and ethyl *N*-hydroxycarbamate react *in vivo* with the 4-amino-group and the 5,6-double bond of cytosine in nucleic acids (*cf.* hydroxylamine [13]) and (*b*) ethoxycarbonylation of intermediates of pyrimidine biosynthesis, *e.g.* by formation of ethoxycarbonyl phosphate which is a carcinogen [14] and which may act competitively with carbamyl phosphate, may modify the *de novo* biosynthesis of pyrimidines. Although no ring-substituted cytosines have been detected in the present experiments the following results suggest that the increase in the uracil content of nucleic acids results from prior *N*(4)-acylation of cytosine in these macromolecules: (i) the ease of hydrolysis of the *N*(4)-acylcytosines to form uracil, (ii) the ability of liver and blood enzymes to convert the *N*(4)-acylcytosines into uracil, and (iii) the formation of 2′-deoxyuridine and 2′(5′)-deoxyuridylic acid from incubates of DNA and ethyl *N*-acetoxy-*N*-acetylcarbamate after treatment of the products with deoxyribonuclease and snake venom phosphodiesterase.

EXPERIMENTAL

Ethyl cytosine-5-carboxylate, which was a gift from Dr. K. Williams, was prepared from cytosine-5-carboxylic acid (supplied by Sigma Chemical Co., St. Louis, Missouri, U.S.A.) by the method of Wheeler and Johns.[15] The other purines and pyrimidines and their ribosides, ribotides, and 2-deoxyribosyl derivatives, and highly polymerised yeast RNA and crystallised bovine albumin, were purchased from B.D.H. Hydroxylamine derivatives were prepared as described by Boyland and Nery [16] unless otherwise stated. [1-^{3}H]ethanol (specific activity 250 mc/mmole) and [^{3}H]-acetic anhydride (specific activity 100 mc/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks. Highly polymerised salmon sperm DNA (B.D.H.), after deproteinisation by the method of Kay, Simmons, and Dounce [17] contained 0·17% (w/v) of residual protein and was a gift from Dr. P. L. Grover. Ribonuclease A was obtained from Sigma Chemical Co. and deoxyribonuclease 1(D) and

[9] J. Booth, *Biochem. J.*, 1966, **100**, 745.
[10] J. Booth, *Ann. Report Inst. Cancer Res.: Royal Cancer Hosp.*, 1966, p. 22.
[11] E. C. Miller and J. A. Miller, *Pharmacol. Rev.*, 1966, **18**, 805.
[12] E. Boyland and K. Williams, Ann. Report Inst. Cancer Res.: Royal Cancer Hospital, 1965, p. 32.
[13] D. M. Brown and P. Schell, *J. Chem. Soc.*, 1965, 208.
[14] I. Berenblum, D. Ben-Ishai, N. Haran-Ghera, A. Lapidot, E. Simon, and N. Trainin, *Biochem. Pharmacol.*, 1959, **2**, 168.
[15] H. L. Wheeler and C. O. Johns, *Amer. Chem. J.*, 1908, **40**, 233.
[16] E. Boyland and R. Nery, *J. Chem. Soc.* (*C*), 1966, 346.
[17] E. R. M. Kay, N. S. Simmons, and A. L. J. Dounce, *J. Amer. Chem. Soc.*, 1952, **74**, 1742.

Crotalus adamanteus venom phosphodiesterase were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Whole histone, which is a heterogeneous mixture of five major basic proteins, was prepared [18] from calf thymus nuclei and was a gift from Dr. E. W. Johns.

U.v. spectra were determined with a Unicam SP 800 spectrometer and radioactivity was measured with a Packard Tricarb liquid-scintillation spectrometer.

Unless otherwise stated, Whatman No 1 paper was used for overnight descending development in the following solvents: A, butanol–acetic acid–water (12 : 3 : 5); B, butanol–acetic acid–water (2 : 1 : 1); C, butanol–propanol–0·1N-ammonia (2 : 1 : 1). For t.l.c., glass plates were coated with films (0·25 mm.) of silica gel G (Merck) and the chromatograms were developed in D, butanol–acetone–acetic acid–2N-ammonia (7 : 5 : 3 : 5); and E, 2-methylbutan-2-ol–formic acid–water (3 : 2 : 1). Spots were detected with u.v. light.

[1-^{3}H]*Ethyl* N-*Hydroxycarbamate*.—[1-^{3}H]Ethanol (25 mc) in ethanol (4 ml.) was added dropwise to a stirred solution of phosgene (20 g.) in ether (50 ml.) at 0°. After 2 hr. at 0° and 16 hr. at 25°, the ether and excess of phosgene were blown off in a stream of nitrogen. The residual oil was treated with hydroxylamine hydrochloride (10 g.) in water (10 ml.) containing sodium hydrogen carbonate (15 g.) at 0°. The mixture was stirred for 2 hr. at 0° and 3 hr. at 25° and extracted with ether (3 × 50 ml.). The extracts were combined, dried (Na_2SO_4), and distilled *in vacuo* to yield [1-^{3}H]*ethyl* N-*hydroxycarbamate* (3·2 g.; specific activity 0·4 mc/mmole) as a colourless oil, b.p. 72—74°/0·1 mm., n_D^{20} 1·448 (Found: N, 13·2. $C_3H_5NO_3$ requires N, 13·1%), indistinguishable from ethyl *N*-hydroxycarbamate in chromatographic behaviour and colour reactions described by Boyland and Nery.[2]

Ethyl [*acetyl*-^{3}H]-N-*Acetoxycarbamate*.—[^{3}H]Acetic anhydride in benzene (25 mc) was diluted with acetic anhydride (10 ml.) and added to a solution of ethyl *N*-hydroxycarbamate (12 g.) in pyridine (30 ml.) at 0°. After 16 hr. at 25°, the solvents were distilled off *in vacuo*, the residue was dissolved in ether (100 ml.), and the ethereal solution was washed with water (2 × 10 ml.), dried (Na_2SO_4), and distilled to give *ethyl* N-[^{3}H]*acetoxy*-N-[^{3}H]-*acetylcarbamate* (0·2 g.) as a colourless liquid, b.p. 32°/0·15 mm., n_D^{20} 1·4332 and *ethyl* [acetyl-^{3}H]N-*acetoxycarbamate* (14 g.; specific activity 60 μc/mmole) as a colourless oil, b.p. 64—66°/0·15 mm., n_D^{20} 1·4180 (Found: C, 40·7; H, 6·3; N, 9·6. $C_5H_9NO_4$ requires C, 40·8; H, 6·2; N, 9·5%). The [^{3}H]diacetyl derivative was indistinguishable from ethyl *N*-acetyl-*N*-acetoxycarbamate by chromatography and colour reactions [16] and was not further characterised.

Ethylene 1,2-*Bis*-(*chloroformate*).—Prepared as described by Oespar, Broker, and Cook,[19] this was obtained as a

TABLE 1

Acylcytosines

	Cytosine substituent	Method of Prep.*	M.p.†	Found (%) C	H	N	Required (%) C	H	N	U.v. spectra (0·1N–HCl) $\lambda_{max.}$ (mμ) ($10^{-3}\varepsilon_{max.}$)	$\lambda_{max.}$ (mμ) ($10^{-3}\varepsilon_{min.}$)	R_C ‡ in solvent A	B	C
(I)	*N*(4)-Acetyl	*a*, *b*	304—306 [a]	46·7	4·9	27·0	47·05	4·6	27·4	239(11·8) 300(11·0)	225(8·8) 265(2·5)	1·35	1·20	1·60
(II)	*N*(4)-Methoxycarbonyl	*a*, *b*	275—280 [b]	42·4	4·0	25·3	42·6	4·2	24·8	233(10·3) 293(8·6)	222(9·8) 258(2·2)	1·45	1·25	1·70
(III)	*N*(4)-Ethoxycarbonyl	*a*, *b*	302—308 [c]	46·0	5·0	22·9	45·9	4·95	22·9	233(9·9) 294(9·0)	223(9·3) 259(3·0)	1·64	1·32	1·90
(IV)	*N*(4)-n-Hexyloxycarbonyl [f]	*a*	285—288 [d]	54·8	7·3	17·6	55·2	7·2	17·6	227(3·6) 298(3·2)	253(0·8)	2·10	1·61	2·10
(V)	*N*(4),*N*(4′)-Bis(ethylene-1,2-dioxy carbonyl) [f]	*a*	240 [e]	42·9	4·1	24·4	42·9	3·6	25·0	220(16·0) 300(23·6)	247(0·8)	0·78		0·05
(VI)	*N*(1)-(1′-D-Ribosyl)-*N*(4)-acetyl	*b*	195 [e]	46·3	5·3	14·7	45·9	5·3	15·1	242(10·0) 307(12·4)	227(8·2) 269(2·8)	1·12	1·08	1·22
(VII)	5-Ethoxycarbonyl									227(7·8)	247(1·7)	1·55	1·46	1·80

* For details see text. † Colourless solids melting with decomposition. ‡ Relative to cytosine, which had R_F 0·40, 0·27, 0·34 in solvents A, B, and C, respectively.

[a] Needles from water. [b] Plates from dimethylformamide. [c] Needles from dimethylformamide. [d] Amorphous powder obtained by acidification to pH 2·0 with 2N-HCl of a solution of the compound in N-NaOH. [e] Needles from 90% aqueous ethanol. [f] U.v. spectra for solution in 2N-HCl.

General Preparation of Acylcytosines.—(*a*) A solution (0·25% w/v) of cytosine in anhydrous pyridine was stirred at 100° and acetic anhydride or the appropriate alkyl chloroformate (1·5—2·0 equiv.) was added during 20—30 min. After 2—5 hr., the mixture was stored overnight at 4°, any precipitate formed was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The precipitate and residue were separately recrystallised from an appropriate solvent (see Table 1) to yield *N*(4)-acylcytosines (45—70%) (see Table 1). Lower yields were obtained when water was present.

(*b*) A solution (1·2% w/v) of cytosine in 0·1M-phosphate buffer (pH 6·5) was treated with ethyl *N*-acetoxy-*N*-acetylcarbamate (1·5 equiv.) or the appropriate ethyl *N*-alkoxycarbonyl-*N*-alkoxycarboxycarbamate (1·5 equiv.) dissolved in an equal volume of ethanol. The resulting mixture was heated under reflux for 3—6 hr. and worked up as described in (*a*) to yield *N*(4)-acylcytosines (60—80%) (see Table 1). Varying amounts of *N*(4)-ethoxycarbonylcytosine and uracil were also formed.

[18] E. W. Johns, *Biochem. J.*, 1964, **92**, 55.
[19] R. E. Oespar, W. Broker, and W. A. Cook, *J. Amer. Chem. Soc.*, 1925, **47**, 2609.

colourless oil, b.p. 102°/14 mm. (Found: C, 26·2; H, 2·0; Cl, 37·6. Calc. for $C_4H_4Cl_2O_4$: C, 25·7; H, 2·2; Cl, 37·9%).

Ethylene 1,2-*Bis*-(N-*hydroxycarbamate*).—Ethylene 1,2-bis(chloroformate) (55·8 g.) was added dropwise during 1 hr. to a stirred mixture of hydroxylamine hydrochloride (49 g.) and sodium carbonate decahydrate (200 g.) in water (200 ml.) at 25°. After 20 hr., the pH of the mixture was adjusted to 3 with 10N-hydrochloric acid at 0°, the mixture was evaporated to dryness *in vacuo*, and the residue was extracted with propan-1-ol (2 × 100 ml.) at 60°. The extract was evaporated to small bulk *in vacuo* and stored at 4° to yield hydroxylamine hydrochloride (2 g.), m.p. and mixed m.p. 165°, and *ethylene* 1,2-*bis*-(N-*hydroxycarbamate*) (30 g., 55%) as colourless prisms, m.p. 114° (from propan-1-ol) (melts to a red liquid which effervesces at 124°) (Found: C, 26·7; H, 4·8; N, 16·0. $C_4H_8N_2O_6$ requires C, 26·7; H, 4·5; N, 15·55%).

Hydroxyamidinoacetohydrazide.—A solution of hydroxylamine hydrochloride (4·5 g.) in methanol (70 ml.) was added to a solution of potassium hydroxide (3 g.) in water (5 ml.) at 0°; the resulting mixture was filtered and washed with cold methanol (10 ml.). Solutions of hydroxylamine in methanol used in subsequent experiments were similarly prepared. The filtrate and washing were added to a solution of cyanoacetohydrazide (5 g.) in water (15 ml.). After 1 hr. at 60° and 16 hr. at 4°, the mixture deposited *hydroxyamidinoacetohydrazide* (5·4 g., 80%), as colourless needles, m.p. 146° (from aqueous ethanol) (decomp. to a red liquid) (Found: C, 27·6; H, 6·3; N, 42·2. $C_3H_8N_4O_2$ requires C, 27·3; H, 6·1; N, 42·4%).

N-*Hydroxy*-N′-(4-*piperidinosulphonylphenyl*)*thiourea*.— A solution of hydroxylamine (1·4 g.) in methanol (35 ml.) was treated with a solution of 4-piperidinosulphonylphenyl thiocyanate (8·4 g.) in benzene (80 ml.). After 16 hr. at 4°, this deposited pale yellow plates (6·2 g.), m.p. 98—100° (decomp.). A cold solution of this substance in methanol was treated with benzene until milky and stored at 4° to yield N-*hydroxy*-N′-(4-*piperidinosulphonylphenyl*)*thiourea* as colourless plates (6·0 g.), m.p. 103° (decomp.) (Found: C, 45·8; H, 5·3; N, 12·6; S, 20·9%; *M*, 315. $C_{12}H_{17}N_3O_3S_2$ requires C, 45·7; H, 5·4; N, 13·3; S, 20·3%; *M*, 315). Attempts to recrystallise this compound from hot chloroform gave two uncharacterised substances: (*a*) colourless prisms, m.p. >300°, and (*b*) pale yellow needles, m.p. 123°. The compound decomposed into benzene and cyanide and a sticky yellow residue on storage in a desiccator over calcium chloride for several days.

5-*Dimethylaminovaleric Acid*.—A solution of 5-aminovaleric acid (5 g.) in water (300 ml.) containing 36% formalin (13 ml.) and 5% palladised charcoal (6 g.) was hydrogenated overnight (uptake 1900 ml. at S.T.P.), treated with more catalyst (1 g.), and hydrogenated again (1 hr.; additional uptake 48 ml. at S.T.P.). The mixture was filtered, the residue was washed with hot water (50 ml.), and the filtrate and washings were combined and evaporated *in vacuo*. The residue was dissolved in water (200 ml.) and evaporated *in vacuo* several times until the odour of formalin no longer persisted, and finally dissolved in N-hydrochloric acid (100 ml.) and evaporated *in vacuo* to yield 5-*dimethylaminovaleric acid hydrochloride* as colourless plates (4·2 g.) from ethanol–acetone, m.p. 165° (Found: C, 64·1; H, 8·75; Cl, 19·7; N, 7·9. $C_7H_{16}ClNO_2$ requires C, 64·3; H, 9·1; Cl, 19·5; N, 7·7%).

5-(N-*Methylacetamido*)*valeric Acid*.—A solution of *N*-methyl-2-piperidone (10 g.) in 10N-hydrochloric acid (50 ml.) was heated under reflux for 16 hr., then evaporated *in vacuo*; the residue was recrystallised from ethanol–acetone to yield colourless plates of 5-methylaminovaleric acid hydrochloride (13·5 g.), m.p. 93° (lit.,[20] 93°) (Found: C, 42·6; H, 8·4; Cl, 21·35; N, 8·1. Calc. for $C_6H_{14}ClNO_2$: C, 43·0; H, 8·4; Cl, 21·2; N, 8·4%). A solution of 5-methylaminovaleric acid hydrochloride (5 g.) in pyridine (20 ml.) containing triethylamine (5 ml.) was treated with acetic anhydride (8 ml.). After 16 hr., the mixture was filtered and the filtrate distilled to yield 5-(N-*methylacetamido*)*valeric* acid (4·6 g.) as a colourless oil, b.p. 50—52°/0·05 mm., n_D^{23} 1·4458 (Found: C, 55·7; H, 9·15; N, 8·1. $C_8H_{15}NO_3$ requires C, 55·5; H, 8·7; N, 8·1%).

4-(*Hydroxyamidino*)*pyridine*.—A solution of hydroxylamine (1 g.) in methanol (20 ml.) was added to a solution of 4-cyanopyridine (2·1 g.) in methanol (15 ml.). After 16 hr. at 4°, the mixture was evaporated *in vacuo* and the residue gave 4-(*hydroxyamidino*)*pyridine* as colourless plates (1·4 g.), m.p. 196—198° (decomp.) (from ethanol) (Found: C, 52·6; H, 4·9; N, 31·0. $C_6H_7N_3O$ requires C, 52·5; H, 5·15; N, 30·6%).

NN′-*Diethoxycarbonylhydrazine*.—A solution of hydrazine dihydrochloride (105 g.) in water (20 ml.) containing potassium hydroxide (11·2 g.) at 0° was diluted with ethanol (50 ml.), filtered, and the filtrate was treated with ethyl chloroformate (65 g.). The mixture was heated under reflux for 1 hr. and evaporated *in vacuo* to yield *NN*′-diethoxycarbonylhydrazine (3·5 g.) as colourless needles, m.p. 131° (from water) (lit.,[21] 131°) (Found: C, 41·0; H, 6·8; N, 16·0. Calc. for $C_6H_{12}N_2O_4$: C, 40·9; H, 6·9; N, 15·9%).

N-*Ethoxycarbonyl*-N′-*methylhydrazine*.—A solution of methylhydrazine (4·5 g.) in a mixture of ethanol (15 ml.), ether (30 ml.), and triethylamine (8 ml.) was stirred rapidly at 0° and treated with ethyl chloroformate (7·5 g.), dropwise, during 30 min. After 4 hr., the mixture was filtered, the filtrate was evaporated *in vacuo*, and the residual oil dissolved in ethanol (2 ml.) was diluted with ethyl acetate (18 ml.), saturated with dry hydrogen chloride, and stored at 4° to yield N-*ethoxycarbonyl*-N-*methylhydrazine hydrochloride* as colourless prisms (7·8 g.), m.p. 106° (decomp.) (Found: C, 31·3; H, 6·7; Cl, 23·0; N, 18·0. $C_4H_{11}ClN_2O_2$ requires C, 31·1; H, 7·2; Cl, 22·9; N, 18·1%).

N′-*Ethoxycarbonyl*-NN-*dimethylhydrazine*.—Ethyl chloroformate (20 g.) was added during 30 min. to a stirred solution of *NN*-dimethylhydrazine (15 g.) in a mixture of ether (150 ml.), ethanol (100 ml.), and triethylamine (33 ml.) at 0°. After a further 30 min. at 0° and 16 hr. at 23°, the mixture was filtered, and the residue was washed with cold acetone (25 ml.). The filtrate and washings were combined and distilled to yield N′-*ethoxycarbonyl*-NN-*dimethylhydrazine* as a colourless oil (23 g.), b.p. 79—80°/10 mm., which solidified and gave colourless needles, m.p. 37° [from light petroleum (b.p. 40—60°)] (Found: C, 45·2; H, 8·8; N, 21·4. $C_5H_{12}N_2O_2$ requires C, 45·4; H, 9·15; N, 21·2%).

Acetone O-*Ethoxycarbonyloxime*.—Ethyl chloroformate (15 g.) was added during 1 hr. to a stirred solution of acetone oxime (10·6 g.) in a mixture of water (25 ml.), acetone (5 ml.), and sodium hydrogen carbonate (12 g.) at 0°. The ice–water bath was removed and the mixture was stirred for 2 hr. The oily upper layer which separated was combined with an ethereal (2 × 20 ml.) extract of the

[20] L. Ruzicka, *Helv. Chim. Acta*, 1921, **4**, 474.
[21] C. K. Ingold and S. D. Weaver, *J. Chem. Soc.*, 1925, 378.

aqueous layer and distilled to give *acetone O-ethoxycarbonyloxime* as a colourless oil (18·9 g.), b.p. 66—67°/0·4 mm. (Found: C, 49·9; H, 7·6; N, 9·65%; *M*, 149. $C_6H_{11}NO_3$ requires C, 49·6; H, 7·6; N, 9·65%; *M*, 145).

Reaction between Ethyl N-*Acetoxy*-N-*acetylcarbamate and Various Nucleic Acid Bases.*—A 0·05M-solution of the carbamate in ethanol (1 ml.) was added to a 0·02M-solution of each of the following bases in 0·1M-phosphate buffer (pH 6·5) (1 ml.): (i) 2′-deoxycytidine, (ii) 2′(5′)-deoxycytidylic acid, (iii) 5-methylcytosine, (iv) uracil, (v) uridine, (vi) thymine, (vii) thymidine, (viii) guanosine, (ix) 5′-guanylic acid, (x) 2′(5′)-deoxyguanylic acid, (xi) adenine, (xii) adenosine, (xiii) 2′-deoxyadenosine, and (xiv) 2′(5′)-deoxyadenylic acid. Fourteen control mixtures contained each base in buffer–ethanol (1 : 1 v/v; 2 ml.). After 16 hr. at 37°, the mixtures were examined by chromatography in solvent A. Areas visible under u.v. light on the developed and dried chromatograms were eluted with 0·1N-hydrochloric acid and the u.v. spectra of the eluted materials were determined. Mixtures (i), (ii), and (iii) each formed one major product, probably the corresponding *N*(4)-acetylcytosine, R_F 0·60, 0·17, and 0·64, $\lambda_{max.}$ 306, 306, and 309 mμ, respectively. The parent cytosines had R_F 0·40, 0·12, and 0·45, $\lambda_{max.}$ 280, 280, and 283 mμ, respectively. No reaction occurred in the remaining mixtures.

Failure of Cytosine to react with Acylamides and Related Compounds.—Solutions of cytosine (20 μmoles) in 0·5M-phosphate buffer (pH 6·7; 0·2 ml.) were treated, severally, with a solution of each of the following compounds (100 μmoles) in ethanol (0·2 ml.): (i) ethyl acetylcarbamate, (ii) *NN*′-diethoxycarbonylhydrazine, (iii) *N*-ethoxycarbonyl-*N*′-methylhydrazine, (iv) *N*′-ethoxycarbonyl-*NN*-dimethylhydrazine, (v) acetone *O*-ethoxycarbonyloxime, (vi) 5-(*N*-methylacetamido)valeric acid, (vii) hydroxyamidinoacetohydrazide, (viii) 4-(hydroxyamidino)pyridine, (ix) ethylene 1,2-bis-(*N*-hydroxycarbamate), and (x) *N*-hydroxy-*N*′-(4-piperidinosulphonylphenyl)thiourea. The mixtures were heated in stoppered tubes at 80° for 4 hr. and examined by chromatography in solvents A and B. Compounds visible under u.v. light on the dried chromatograms were eluted with 0·1N-hydrochloric acid and the u.v. spectra were determined. No reaction was seen in any of the mixtures.

Reaction between [1-^{3}H]*Ethyl* N-*Hydroxycarbamate and Cytosine.*—A solution of the [^{3}H]carbamate (10·5 mg.) in ethanol (4 ml.) was added to a solution of cytosine (11·1 mg.) in 0·2M-phosphate buffer (pH 6·7) (4 ml.). After 4 hr. at 70°, the mixture was evaporated *in vacuo* and the residue was suspended in ethanol (20 ml.), filtered off, washed with ethanol (50 ml.), and mixed with *N*(4)-ethoxycarbonylcytosine (18·3 mg.). After four crystallisations from dimethylformamide a chromatographically pure fraction (10 mg.) of *N*(4)-ethoxycarbonylcytosine, m.p. 302—308° (decomp.), was obtained, activity 4·7 μc/mmole.

Reaction between [1-^{3}H]*Ethyl* N-*Hydroxycarbamate and some Biological Macromolecules.*—A 0·2M-solution of the carbamate in 0·001M-phosphate buffer (pH 7·0) (0·5 ml.) was incubated at 37° for 16 hr. with a solution of each of the following (2 mg.) in the same buffer (10 ml.): (i) DNA, (ii) RNA, (iii) bovine albumin, and (iv) whole histone. Four control mixtures, which contained all ingredients except the carbamate, were also incubated. Each mixture was diluted with ethanol (40 ml.) and centrifuged after 1 hr. at 0°; the precipitates were redissolved in buffer (10 ml.), re-precipitated, and centrifuged twice more. The precipitates were dried to constant weight in an evacuated desiccator (P_2O_5). Samples (0·1 mg.) of precipitates (i) and (ii) were each dissolved in 5% perchloric acid (3 ml.); the mixtures were heated at 100° for 20 min., and the extinctions at 260 mμ were compared with those of solutions obtained from DNA (0·1 mg.) and RNA (0·1 mg.) treated similarly. This showed that precipitates (i) and (ii) contained 70 and 76% (w/v) of DNA and RNA, respectively, the remainder being inorganic salts. Precipitate (i) (1 mg.) in water (0·5 ml.) was treated with 0·5% deoxyribonuclease in water (0·1 ml.) and 1% magnesium chloride in water (0·1 ml.); precipitate (ii) (1 mg.) in water (0·5 ml.) was treated with similar solutions of ribonuclease and magnesium chloride; each resulting mixture was added to the scintillation fluid (10 ml.) after 15 min. at 37°. Precipitates (iii) and (iv) were each dissolved in water (4 ml.) and samples (0·3 ml.) were treated with 2N-sodium hydroxide (0·1 ml.), heated at 70° for 10 min., cooled, and treated with 2N-acetic acid (0·1 ml.) and scintillation fluid (10 ml.). The radioactivities of precipitates (i)—(iv) in mμc/mg. nucleic acid or protein, were (i) 0·6, (ii) 1·4, (iii) 4·5, and (iv) 12·1.

Reaction between Ethyl[*acetyl*-^{3}H]N-*Acetoxycarbamate and Nucleic Acids.*—As described in the foregoing experiment, mixtures containing (i) DNA, (ii) RNA, or (iii) DNA (heated at 70° for 15 min. and rapidly cooled to 0°), and the carbamate were analysed for radioactivity after 16 hr. at 37°. The hydrolysates of the products from the three mixtures showed the following activities (mμc/mg. nucleic acid): (i) 1·8, (ii) 0·3, (iii) 0·8. In another experiment in which the mixtures were incubated at 37° for 2 hr., the products showed the following activities: (i) 0, (ii) 0·008, and (iii) 0·02 mμc/mg. of nucleic acid.

Reaction between DNA and Ethyl N-*Acetoxy*-N-*acetylcarbamate.*—A solution of DNA (10 mg.) in 0·01M-phosphate buffer (pH 7·0; 10 ml.) was treated with a solution of the carbamate (0·2 g.) in ethanol (5 ml.) and the mixture was incubated at 37° for 16 hr. A similar mixture, in which the carbamate was omitted, was incubated similarly. The mixtures were treated with ethanol (30 ml.), and the precipitates of DNA were removed and washed with ethanol (2 × 10 ml.) and ether (2 × 10 ml.) and dried in an evacuated desiccator (P_2O_5). A solution of the dried DNA (5 mg.) in 0·01M-ammonium hydrogen carbonate (pH 7·0) (1 ml.) was treated with 0·015M-magnesium chloride (1 ml.) and the resulting mixture was incubated with 0·5% deoxyribonuclease (0·02 ml.) for 1 hr. at 37°. The pH of the mixture was adjusted to 8·5 with 2N-ammonia; the mixture was then treated with a 0·5% solution of snake venom phosphodiesterase (0·02 ml.), incubated for 2 hr. at 37°, evaporated *in vacuo*, and the residue was examined by chromatography and u.v. spectroscopy. Two components, which did not occur in the control mixture, were eluted with 0·1N-hydrochloric acid from the chromatogram developed in solvent system A, and their u.v. spectra were determined; these were (i) 2′(5′)-deoxyuridylic acid ($\lambda_{max.}$ 262 mμ; 3·5 μg formed/mg. DNA; R_F 0·20, 0·26, and 0·54 in systems A, D, and E, respectively) and (ii) 2′-deoxyuridine ($\lambda_{max.}$ 262 mμ; trace; R_F 0·36 in system A).

Acid Hydrolysis of N(4)-*Acylcytosines.*—Samples (5 mg.) of each of the compounds (I)—(VI) (Table 1) were dissolved in 2N-hydrochloric acid (1 ml.); the resulting solutions were heated at 80° for 2 hr. and examined by chromatography in system A. Substances visible under u.v. light were eluted from the chromatograms with 0·1N-hydrochloric acid and

their u.v. spectra were determined. Compounds (I)—(V) gave mainly uracil (R_F 0·50, $\lambda_{max.}$ 259 mμ) and traces of cytosine (R_F 0·41, $\lambda_{max.}$ 274 mμ); compound (VI) gave mainly uracil and small amounts of cytosine, uridine (R_F 0·36; $\lambda_{max.}$ 262 mμ), and cytidine (R_F 0·33, $\lambda_{max.}$ 280 mμ). When the mixtures were kept at 25° for 16 hr. or similar mixtures in 50% aqueous acetic acid were heated at 80° for 3 hr., uracil and cytosine in approximately equal amounts were formed from compounds (I)—(IV) and in a ratio of about 2 : 1 from compound (V). Compound (VI) gave uridine, cytidine, cytosine, and uracil.

Quantitative aspects of the Reaction between Cytosine and Hydroxamic Acids.—A 0·15M-solution of cytosine in 0·2M-phosphate buffer (pH 6·5; 0·4 ml.) was treated with a 0·4M-solution of each of the following compounds in ethanol (0·4 ml.): (i) ethyl *N*-hydroxycarbamate, (ii) *N*-ethoxycarboxyacetamide, (iii) ethyl *N*-acetoxycarbamate, (iv) ethyl *N*-acetoxy-*N*-acetylcarbamate, (v) ethyl *N*-acetyl-*N*-ethoxycarboxycarbamate, (vi) ethyl *N*-acetoxy-*N*-ethoxycarbonylcarbamate, (vii) ethyl *N*-acetoxy-*N*-methylcarbamate, (viii) ethyl *N*-acetyl-*N*-methoxycarbamate, (ix) ethyl *N*-ethoxycarboxycarbamate, and (x) benzyl *N*-benzyloxycarboxycarbamate. A 0·15M-solution of cytosine in Krebs–Ringer phosphate buffer (pH 7·4; 0·4 ml.) containing washed human erythrocytes (R_H^+ 0·02 ml.) was treated with a 0·1M-solution of (xi) ethyl *N*-hydroxycarbamate or of (xii) ethyl *N*-acetoxycarbamate in the same buffer (0·4 ml.). The mixtures containing compounds (i)—(x) were heated in stoppered tubes at 70° for 5 hr.; those containing (xi) and (xii) were heated at 37° for 4 hr. and centrifuged. Portions (0·2 ml.), in triplicate, of each mixture were applied as streaks to Whatman 3MM paper and bands visible under u.v. light on the chromatograms after development in solvent A were eluted with 0·1N-hydrochloric acid (10 ml.). The acylcytosines and uracil formed were determined by comparison of their u.v. spectra with those shown for the corresponding products in Table 1 and with the u.v. spectrum of uracil. The results from mixtures (i)—(x) are shown in Table 2. Mixtures (xi) and (xii), containing erythrocytes, gave uracil (8 and 15 mole %, respectively); *N*(4)-acylcytosines were not detected.

The Effect of Mouse Liver on the Reaction between Cytosine and Ethyl N-Acetoxycarbamate.—A solution of cytosine (14·0 mg.) in 0·55M-phosphate buffer (pH 7·0; 3 ml.) containing ethyl *N*-acetoxycarbamate (50 mg.) was treated with (i) the buffer (1·0 ml.), (ii) liver (0·2 g. wet wt.) from a female adult CBA/H mouse homogenised in the buffer (1 ml. of homogenate), or (iii) liver slices (0·2 g. wet wt.) from the same source and the buffer (1 ml.). Mixtures (ii) and (iii) also contained (per ml.) nicotinamide (8·75 mg.), glucose-6-phosphate (1·25 mg.), $NADP^+$ (0·19 mg.), NADH (0·21 mg.). The carbamate was omitted from three control mixtures prepared similarly. The mixtures were shaken in air for 1 hr. at 37°. Those containing liver preparations were treated with cold ethanol (10 ml.) and centrifuged; the sediment was shaken with 70% aqueous ethanol (2 × 5 ml.) and centrifuged. The two supernatants from each mixture were combined and evaporated *in vacuo*, and the residue was dissolved in the buffer (4 ml.). Samples (0·2 ml.) of each of the six mixtures were applied as streaks on sheets of Whatman 3MM paper; the chromatograms developed in system A

TABLE 2

Quantitative aspects of the reaction between cytosine and hydroxamic acids

Reaction mixtures, which contained cytosine (60 μmoles) and $R^1NR^2OR^3$ (160 μmoles) in a 50% mixture (0·8 ml.) of ethanol and 0·2M-phosphate buffer (pH 6·5), were heated at 70° for 5 hr. For other details, see text.

Reaction mixture	$R^1NR^2OR^3$			Products (mole %)			
					N(4)-Acylcytosines		
	R^1	R^2	R^3	Uracil	Acyl=R^1	R^2	R^3
i	CO_2Et	H	H	4·0	3·0		
ii	Ac	H	CO_2Et	6·4	5·6		15·4
iii	CO_2Et	H	Ac	7·1	5·3		16·4
iv	CO_2Et	Ac	Ac	8·2	10·2		56·0
v	CO_2Et	Ac	CO_2Et	6·4		2·8	38·0
vi	CO_2Et	CO_2Et	Ac	7·2		2·2	42·6
vii	CO_2Et	Me	Ac	7·6	5·5		16·0
viii	CO_2Et	Ac	Me	5·5	2·7	2·9	
ix	CO_2Et	H	CO_2Et	7·7	16·6		
x	CO_2CH_2Ph	H	$CO_2{\cdot}CH_2Ph$	18·0		None	

and the uracil and *N*(4)-acetylcytosine contents of each mixture were determined as previously described. The molar ratios, cytosine : uracil : *N*(4)-acetylcytosine, in the mixtures were (i) 1·0 : 0 : 0·05, (ii) 1·0 : 0·34 : 0·01, and (iii) 1·0 : 0·35 : 0·02. Mixture (i), after 0, 1, 2, 4, 6, 7, and 8 hr., was 3150, 3127, 3080, 3042, 2975, 2952, and 2889 × 10^{-5}M with respect to cytosine.

The author thanks Professor E. Boyland for his interest, Drs. K. Williams, P. L. Grover, and E. W. Johns for gifts of ethyl cytosine-5-carboxylate, deproteinised DNA, and calf thymus histone, respectively, and Mr. P. Bell for technical assistance. This investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the U.S. Public Health Service.

[9/228 *Received, February 7th,* 1969]