in order to surround the complex to the same extent with nonpolar amino acid side chains. The alternative substitution of larger amino acids for smaller ones would require a large fraction of aromatic amino acids relative to aliphatic amino acids which is not evident from the reported amino acid analyses of the *c*-type cytochrome from bacteria.[13] With regard to a heme environment which is less polar than that in cytochrome *c*, it has been observed that the heme–ligand complex in cytochrome *c* sits in a crevice, the walls of which are composed of nonpolar amino acid side chains.[10] Yet there appears to be channels from the complex to the surface of the protein which are available to the solvent. It has been suggested on the basis of nmr spectra and sequence homologies between cytochrome *c* and bacterial cytochromes c_2 that additional sequences of amino acids in the bacterial cytochromes might fill one of the channels to the heme–ligand complex.[14] Such a structure would have the effect surrounding the complex to a greater extent with nonpolar groups, which would contribute to a heme environment which is less polar than that in cytochrome *c*. Consistent with this analysis is the observation that these cytochromes exhibit redox potentials which are significantly more positive than cytochrome *c*.

The influence of structural parameters other than the local nonpolar environment on the redox potential of cytochromes is not without notice. Significant free-energy changes may be associated with conformational changes attendant to oxidation and reduction. Such changes may include the transfer of apolar groups from an aqueous to a nonaqueous environment[8] or the transfer of charged groups from a polar to a nonpolar environment. The relationship between changes in conformation and the energetics of the redox process have been emphasized by Takano, *et al.*[15] Charged groups on the surface of the protein may also influence the redox potential, yet the high redox potentials of *c*-type cytochromes are not restricted to basic proteins but have also been observed for an acidic protein.[16] The ability of structural forces in the protein to affect the extent of coordination of the methionine sulfur to the heme iron in the oxidized state has also been discussed.[6,7] Yet the present theoretical treatment suggests that the high redox potentials of many *c*-type cytochromes may be accounted for by a heme environment of a nonpolar nature without structural changes attendant to the redox process, and that such an environment may have a profound effect on the redox potentials of all heme proteins.

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Reaction of Diethyl Pyrocarbonate with Nucleic Acid Components. Bases and Nucleosides Derived from Guanine, Cytosine, and Uracil

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Abstract: Diethyl pyrocarbonate, an enzyme inhibitor and bactericidal agent, reacts in aqueous solution with purine ribonucleosides to form products in which the imidazole ring has been opened, *e.g.*, compound **2** from adenosine, as previously reported, and compound **4**, 2-amino-5-carbethoxyamino-4-hydroxy-6-*N*-ribofuranosylaminopyrimidine, from guanosine. Unlike adenine, which does react, the free base guanine was not modified under the conditions employed. Cytosine and the "minor base" 5-hydroxymethylcytosine react with diethyl pyrocarbonate in aqueous solution to give respectively 1-carbethoxycytosine (**9**) and an unstable compound which appears to be the 1-carbethoxylated derivative **11**. The ribonucleoside cytidine reacted under the same conditions to give N^4-carbethoxycytidine (**13**). Uracil and thymine, in slightly basic aqueous media, give closely related ring-substituted products 1-carbethoxyuracil (**15**) and 1-carbethoxythymine (**17**). The ribonucleosides uridine and ribothymidine, by contrast, react in slightly basic solution to form unstable products which spectral data suggest to be 3-carbethoxyuridine (**19**) and 3-carbethoxyribothymidine (**21**). Pseudouridine C, a modified nucleoside found in transfer RNA, is converted by diethyl pyrocarbonate to an unstable compound which data suggest to be the 1-acylated **23**. Biological consequences of the use of diethyl pyrocarbonate as a nuclease inhibitor are considered, in view of the observed reactions of the pyrocarbonate with the individual nucleic acid components.

Diethyl pyrocarbonate (ethoxyformic anhydride or diethyl dicarbonate) has been used as a preservative in wines and other beverages.[1] The reagent is used to deactivate enzymes, presumably by blocking exposed nucleophilic functions,[2–7] thereby changing the chemical and structural integrity. Recent reports indicate,

(1) O. Pauli and H. Genth, *Z. Lebensm. Unters. Forsch.*, **132**, 216 (1966).

(2) T. Boehm and D. Mehta, *Chem. Ber.*, **71**, 1797 (1938).

(3) (a) J. Larrouquere, *Bull. Soc. Chim. Fr.*, 1543 (1964); (b) *ibid.*, 382 (1965); (c) *ibid.*, 2972 (1965).

(4) C. G. Rosen and I. Fedorcsák, *Biochim. Biophys. Acta*, **130**, 401 (1966).

(5) A. G. Mühlárd, A. G. Hegyi, and G. Tóth, *Acta Biochim. Biophys.*, **2**, 19 (1967).

(6) J. Ovádi, S. Libor, and P. Elödi, *ibid.*, **2**, 455 (1967).

(7) J. Ovádi and T. Keleti, *ibid.*, **4**, 365 (1969).

however, that diethyl pyrocarbonate reacts with ammonia in certain beverages to form the carcinogen urethan, although there is disagreement as to the amount produced.[8]

Reaction of diethyl pyrocarbonate in aqueous medium with adenine[9,10] and its riboside[10] and ribotides[11] was found to produce ring-opened products. As a result, we have urged caution in using the reagent as a nuclease inhibitor during nucleic acid purification procedures. Although several reports have suggested that the compound did not interfere with the function of some nucleic acid preparations,[12-19] evidence to the contrary in at least some systems has suggested deactivation and chemical modification of the nucleic acids.[10,19-28] One of the reports indicated that treatment of rat liver ribosomal RNA with diethyl pyrocarbonate resulted in greater resistance to hydrolysis by several nucleases.[26] We have recently reported that conversion of model adenosine-containing dinucleoside phosphates [adenylyl-(3′→5′)-adenosine, adenylyl-(3′→5′)-uridine, and uridylyl-(3′→5′)-adenosine] to the corresponding dicarbethoxyaminopyrimidine derivatives also results in greatly reduced susceptibility to nuclease-induced hydrolysis.[11]

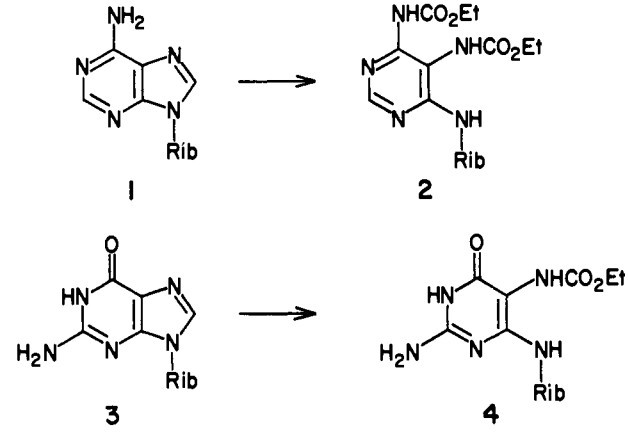
We are now able to report the results of treatment of the major, and some minor, nucleic acid bases and nucleosides with diethyl pyrocarbonate in aqueous media. Rather than attempting to maximize yields by utilizing other solvents or raised temperature, we sought to demonstrate the products that can be formed in aqueous solution and in the pH range employed when the reagent is customarily used for nuclease inhibition and nucleic acid isolation.

Results and Discussion

In order to simulate conditions encountered when diethyl pyrocarbonate is used for the isolation or deactivation of various nucleic acids, all the reactions reported here were carried out in aqueous media. Since the reagent has been added to solutions varying from neutral or slightly acidic to pH 8.5, we have performed all test reactions of nucleic acid components in this pH range. Although the purine ribosides (**1** and **3**) and the cytosine derivatives (**6**, **10**, **12**) react within the entire pH range, uracil and its derivatives react *only* in basic solution. Moreover, the products of the reaction of $(EtOCO)_2O$ with uracil were susceptible to hydrolysis in water. It was necessary to base the structural assignments of the unstable products on spectral evidence and comparison with other compounds.

Purines. Adenosine (**1**) reacts with excess diethyl pyrocarbonate in water to give the ring-opened dicarbethoxylated derivative **2** as the major product. Reports on the reactions of the pyrocarbonate with adenine,[9,10] adenosine,[10] and several adenosine-containing nucleotides[11] provide more complete discussions of the ring openings observed earlier in this laboratory.

Guanosine (**3**) can now be reported to undergo imidazole ring opening in a reaction like that described for adenosine. The exocyclic N^2-amino group of guanosine, in contrast to the N^6-amino group of adenosine, does not appear to be sufficiently reactive to acylate in water, even after modification by opening has occurred. A compound which is structurally similar to the guanosine product, 2-amino-4-hydroxy-5-*N*-methylformamido-6-*N*-ribofuranosylaminopyrimidine (or 5-*N*-methylformamido-6-*N*-ribofuranosylaminoisocytosine (**5**)) is formed during basic hydrolysis of 7-methylguanosine.[29,30] The striking similarity between the ultraviolet spectra (Table I) of **5** and the major guanosine product greatly facilitated assignment of structure **4** to the product.



Rib = β-D-Ribofuranosyl

Unlike adenine free base, guanine as the free base did not react with diethyl pyrocarbonate in aqueous solution in the pH range studied.

Cytosine and Derivatives. Cytosine (**6**) has been reported to acylate under anhydrous conditions at the exocyclic amino position, giving, for example, N^4-acetylcytosine (**7**).[31] Reaction with ethyl chloroformate in anhydrous pyridine has similarly afforded carbethoxylation of cytosine to give a compound, **8**,

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(14) F. Solymosy, I. Fedorcsák, A. Gulyás, G. L. Farkas, and L. Ehrenberg, *Eur. J. Biochem.*, **5**, 520 (1968).
(15) I. Fedorcsák, A. T. Natarajan, and L. Ehrenberg, *ibid.*, **10**, 450 (1969).
(16) P. N. Abadom and D. Elson, *Biochim. Biophys. Acta*, **199**, 528 (1970).
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(18) F. Solymosy, G. Lázár, and G. Bagi, *ibid.*, **38**, 40 (1970).
(19) B. Öberg, *Biochim. Biophys. Acta*, **232**, 107 (1971).
(20) B. Öberg, *ibid.*, **204**, 430 (1970).
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(22) P. Oxelfelt and K. Årstrand, *Biochim. Biophys. Acta*, **217**, 544 (1970).
(23) B. Öberg and L. Philipson, *J. Mol. Biol.*, **58**, 725 (1971).
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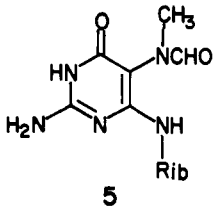
(25) B. Öberg, *Eur. J. Biochem.*, **19**, 496 (1971).
(26) F. Solymosy, P. Hüvös, A. Gulyás, I. Kapovits, Ö. Gaál, G. Bagi, and G. L. Farkas, *Biochim. Biophys. Acta*, **238**, 406 (1971).
(27) B. J. Ortwerth, *ibid.*, **246**, 344 (1971).
(28) D. G. Humm, J. H. Humm, and L. H. Shoe, *ibid.*, **246**, 458 (1971).
(29) J. A. Haines, C. B. Reese, and Lord Todd, *J. Chem. Soc.*, 5281 (1962).
(30) L. B. Townsend and R. K. Robins, *J. Amer. Chem. Soc.*, **85**, 242 (1963).
(31) D. M. Brown, A. R. Todd, and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).

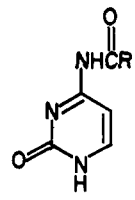
Table I. Ultraviolet Spectra of Compounds **4** and **5**, Derived from Guanosine; **2**, Derived from Adenosine; and Acylated Compounds Derived from Cytosine

Compd	0.1 *N* HCl: λ_{max}, nm ($\epsilon \times 10^{-3}$)	0.1 *N* HCl: λ_{min}, nm	H_2O: λ_{max}, nm ($\epsilon \times 10^{-3}$)	H_2O: λ_{min}, nm	0.1 *N* NaOH: λ_{max}, nm ($\epsilon \times 10^{-3}$)	0.1 *N* NaOH: λ_{min}, nm
4	215 (19.9) 272 (16.8)	245	216 (30.8) 273 (15.7)	248	266 (11.8)	245
5[a]	271 (22.3)	246	273	247	265 (16.3)	239
2[b]	219 (25.5) 277 (22.3)	242	221 (35.3) 270 (7.6)	255	283 (13.2)	257
N^4-AcCyt (**7**)[c,d]	211 *ca.* 225 (sh) 305	259	213 242 292	225 267	294	252
8[e]	*ca.* 220 (sh) 298 (13)	254	211 (21) 236 (13) 285 (6)	223 260	293 (10) *ca.* 315 (sh)	256
9	211 (9.8) 271 (10.4)	240	247 (9.7) *ca.* 270 (w sh)	221	281 (7.3)	248
N^4-AcCyd[d,g]	214 (12.4) 236 308 (13.8)	226 267	212 (18.2) 245 (15.1) 294 (8.6)	225 268	302	241
13	211 (14.2) 227 (9.5)[f] 302 (14.2)	262	212 (18.2) 240 (14.4) 291 (8.7)	224 265	292 (19.8)	239

[a] Reference 29. [b] Reference 11. [c] For quantitative spectral data in neutral 95% ethanol see ref 31. [d] IUPAC–IUB Commission on Biochemical Nomenclature recommendations, *J. Mol. Biol.*, **55**, 299 (1971). [e] Low solubility required individual weighings for each solution. [f] Flat shoulder. [g] Reference 33.



5



7, R = CH_3
8, R = OC_2H_5

which exhibited ultraviolet absorption spectra very much like those of **7**. Reaction of diethyl pyrocarbonate with cytosine in water, on the other hand, produced a compound having ultraviolet and pmr spectral properties not at all like those of **8** (see Tables I and II).

Table II. Comparison of 60-MHz Pmr Spectrum of N^4-Carbethoxycytosine (**8**) with Those of 1-Carbethoxycytosine (**9**) and 1-Carbethoxy-5-hydroxymethylcytosine (**11**)

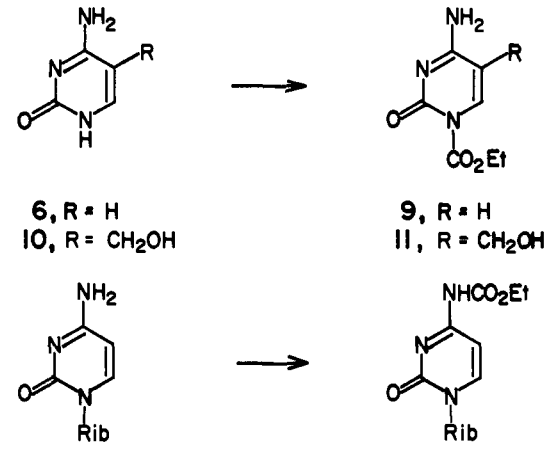
Compd	Ethyl CH_3	Ethyl CH_2	Cyt 5-H	Cyt 6-H	N^4H_2[a]	5-CH_2O	5-OH[a]
	Chemical shift, ppm (mult, *J* (Hz))						
8[b]	1.47 (t, 7)	4.56 (q, 7)	6.71 (d, 7)	8.31 (d, 7)			
9[c]	1.30 (t, 7)	4.30 (q, 7)	5.88 (d, 8)	7.80 (d, 8)	7.24 (br s)		
11[c]	1.30 (t, 7)	4.26 (q, 7)		7.75 (s)	7.24 (br s)	4.17 (s)	4.95 (br s)

[a] Proton exchanged with D_2O. [b] CF_3COOH. In $(CD_3)_2SO$, Cyt 5-H *ca.* 7.0 and 6-H *ca.* 8.1. [c] $(CD)_3SO$.

Significantly, the pmr spectrum included a D_2O exchangeable signal integrating for two protons, those of an intact N^4-amino group. On the basis of spectral data, therefore, the product of reaction of cytosine with diethyl pyrocarbonate in water has been assigned the structure **9**, 1-carbethoxycytosine.

A modified base found in *E. coli*-even phage DNA,[32] 5-hydroxymethylcytosine (**10**), appears to be converted to a 1-carbethoxy derivative, **11**, which is the hydroxymethyl analog of the cytosine product. Although **11** is extremely unstable and could not be purified sufficiently for proper elemental analysis, it could be compared directly with 1-carbethoxycytosine using pmr data given in Table II.

The nucleoside cytidine (**12**) reacts with diethyl pyrocarbonate in unbuffered aqueous medium to form the derivative **13**, the result of carbethoxylation of the exo-



6, R = H
10, R = CH_2OH

9, R = H
11, R = CH_2OH

12

13

cyclic amino group. Trace quantities of minor products can be observed by thin-layer chromatography but are not seen after hour-long, room-temperature treatment with ammonia in ethanol. Since the major product is not affected by the ammonia treatment, as observable by uv absorption and tlc, the minor products are believed to arise by reaction of diethyl pyrocarbonate with ribose hydroxyl groups, as observed in the reaction of $(EtOCO)_2O$ with adenosine.[10] Evidence for the reported course of the reaction can be supplied by pmr spectral data and by comparison of ultraviolet

(32) G. R. Wyatt and S. S. Cohen, *Nature (London)*, **170**, 1072 (1952).

data with those reported for N^4-acetylcytidine.[33] (For uv data, see Table I.)

In summary, cytosine and 5-hydroxymethylcytosine are most reactive toward diethyl pyrocarbonate in aqueous solution at the pyrimidine N-1 position. By contrast, the ribose moiety of cytidine acts as a blocking group and thereby forces any carbethoxylation to occur at the exocyclic amino position.

Uracil and Derivatives. Pyrimidine bases and nucleosides which can be collectively classed as uracil derivatives do not react observably with diethyl pyrocarbonate in neutral or slightly acidic aqueous solutions. Since, under these conditions, the compounds were not sufficiently reactive to acylate, they were activated by partial conversion to anionic forms in basic solution. Since strong base induces rapid hydrolysis not only of diethyl pyrocarbonate but of the reaction products, reactions were performed at somewhat lower pH (7.5–8.5) than first acidic pK_a's of the reactants might suggest as optimum for ionization. Nevertheless, adequate concentrations of anionic species permitted reaction to proceed at the ring nitrogens.

In the range of pH 7.5–8.5, diethyl pyrocarbonate reacted with aqueous solutions of uracil (**14**), adding a single carbethoxy group, as seen by pmr, mass spectrometry, and elemental analysis. Thymine (**16**), which has a somewhat higher first acidic pK_a than uracil (9.9 *vs.* 9.5),[34,35] underwent a similar reaction with the reagent, but only in slightly more basic solution (pH 8.0–8.5). Products derived from each compound were unstable even in neutral aqueous solution, but they could be separated from salts by acetone extraction. Others[36] have reported reaction of ethyl chloroformate with uracil in basic solution to give a product suggested to be 1-substituted. This has now been compared with the product obtained from uracil and diethyl pyrocarbonate and found to be identical. A similar product was prepared from thymine using ethyl chloroformate and was identical with that formed using the pyrocarbonate. Mass spectra (10 and 70 eV) of the crude uracil product indicated addition of a single carbethoxy group, exhibiting a molecular ion at *m/e* 184. Correspondingly, the thymine product exhibited a molecular ion at *m/e* 198, indicative of one carbethoxy group. Ultraviolet and pmr spectra of the uracil and thymine products were compared with those of 1-acetyluracil and 1-acetylthymine[37] (Tables III and IV), and these data were strongly suggestive of N-1, rather than N-3, carbethoxylation. In order to demonstrate more rigorously that 1-carbethoxylated pyrimidines were formed, 3-methyluracil[38] was converted to its carbethoxy derivative using diethyl pyrocarbonate, both in aqueous base and in pyridine. Ultraviolet spectra of 1-carbethoxy-3-methyluracil were found to be very nearly identical to those of the product resulting from uracil and diethyl pyrocarbonate, *i.e.*, **15** (Table III); moreover, *shifts* in pyrimidine pmr signals upon conversion of 3-methyluracil to 1-carbethoxy-3-methyluracil parallel the shifts observed upon reaction of uracil and thymine with diethyl pyrocarbonate (Table IV). The products formed by reaction of diethyl pyrocarbonate with uracil and thymine, therefore, are proposed to be the 1-carbethoxy derivatives **15** and **17**, respectively.

Uridine (**18**), which has a first acidic pK_a of 9.2,[39] reacted with diethyl pyrocarbonate in water maintained at pH 7.5–8.5 to form a carbethoxylated product, as shown by a pmr spectrum of the lyophilized reaction mixture (Table IV). Two sets of pyrimidine-5 and -6 proton signals, one set attributable to uridine itself, were detected, however, indicating either incomplete reaction with the uracil moiety or hydrolysis of the

Table III. Ultraviolet Spectra of Uracil and Thymine Derivatives[a]

Compd	pH 3 λ_{max}, nm ($\epsilon \times 10^{-3}$)	pH 3 λ_{min}, nm	pH 7 λ_{max}, nm ($\epsilon \times 10^{-3}$)	pH 7 λ_{min}, nm
1-AcUra[b]	257 (10.7)		205 (10.2) 257 (10.4)	226
1-EtOCO-3-MeUra	*ca.* 212 (sh) 249 (9.9)	230	212 (9.2) 249 (9.9)	230
15	250 (10.9)	227	250 (11.0)	227
1-AcThy[b]	265 (10.4)		265 (10.4)	231
17	255 (11.9)	231	256 (12.0)	232
19[c]	257	228	257	228
21[c]	257	238	258	232
23[c]	252[d]	240[d]	253	230

[a] Aqueous solution, neutral and acidic. All carbethoxylated derivatives are unstable at pH 10. [b] IUPAC–IUB Commission on Biochemical Nomenclature recommendations, *J. Mol. Biol.*, **55**, 299 (1971). [c] Qualitative spectra for samples containing small amounts of respective unreacted nucleosides. [d] Aqueous solution at pH 4.5.

Table IV. Comparison of 60-MHz Pmr Spectra ($(CD_3)_2SO$) of Uracil, Uridine, Thymine, and Ribosylthymine with Selected Pmr Signals of Acylated Derivatives

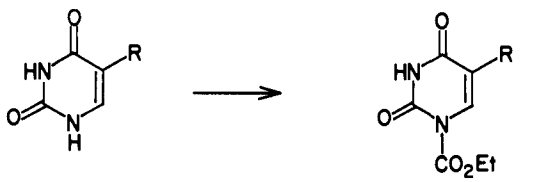
Compd	Chemical shift, ppm (mult, *J* (Hz)) Ethyl CH_3	Ethyl CH_2	Ura 5-H	Thy CH_3	Pyr 6-H	NH[a]
Ura			5.43 (d, 8)		7.32 (d, 8)	>9 (broad)
3-MeUra			5.60 (d, 8)		7.40 (d, 8)	
1-EtOCO-3-MeUra	1.34 (t, 7)	4.41 (q, 7)	5.88 (d, 9)		7.96 (d, 9)	
1-AcUra			5.75 (d, 8)		8.00 (d, 8)	11.2 (br s)
15	1.30 (t, 7)	4.32 (q, 7)	5.68 (d, 8)		7.85 (d, 8)	11.1 (br s)
Urd			5.63 (d, 8)		7.80 (d, 8)	
19[b]	1.2 (t)	4.2 (q)	5.75 (d, 8)		7.90 (d, 8)	
Thy				1.75 (d, 1)	7.17 (q, 1)	10.5 (br s)
1-AcThy				1.82 (d, 1)	7.89 (q, 1)	11.2 (br s)
17	1.35 (t, 7)	4.43 (q, 7)		1.81 (d, 1)	7.73 (q, 1)	11.2 (br s)
rThd				1.78 (s)	7.73 (s)	11.2 (br s)
21[b]	1.2 (t)	4.2 (q)		1.81 (br s)	7.63 (br m)	

[a] Proton exchanged with D_2O. [b] 220-MHz spectra of samples contaminated with starting material.

(33) "Handbook of Biochemistry," H. A. Sober, Ed., Chemical Rubber Co., Cleveland, Ohio, 1968, p G-29, and references cited therein.
(34) P. A. Levene and N. Goldsmith, *J. Phys. Chem.*, **30**, 1683 (1926).
(35) D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9**, 199 (1952).
(36) E. Dyer, M. L. Stulz, and E. J. Track, *J. Org. Chem.*, **27**, 982 (1962).
(37) L. B. Spector and E. B. Keller, *J. Biol. Chem.*, **232**, 185 (1958).
(38) D. J. Brown, E. Hoerger, and S. F. Mason, *J. Chem. Soc.*, 211 (1955).
(39) J. J. Fox and D. Shugar, *Biochim. Biophys. Acta*, **9**, 369 (1952).

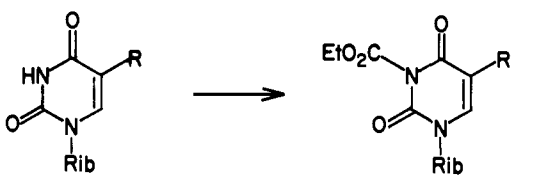
product during manipulations. Rapidly recorded qualitative ultraviolet spectra (Table III) were obtained for several freeze-dried reaction mixtures and maxima and minima were reproducible to 1 nm, but very weak shoulders in the spectra *may* be attributable to unmodified uridine. Attempts to prepare analytical samples of product resulted in complete loss of the carbethoxyl group. The relatively small shifts observed in the ultraviolet and pmr spectra suggest relatively minor structural change in the uracil moiety by reaction at N-3 rather than at an exocyclic oxygen, but the structure of the highly unstable product can only tentatively be assigned that shown as **19**.

Ribosylthymine (**20**), likewise, was converted to an unstable carbethoxylated product upon reaction with diethyl pyrocarbonate in slightly alkaline solution. A 220-MHz pmr spectrum (Table IV) and qualitative uv spectra (Table III) obtained using the crude lyophilized product were strongly suggestive of reaction with the pyrimidine nucleus, presumably giving **21**.



14, R = H
16, R = CH_3
22, R = β-D-Ribofuranosyl

15, R = H
17, R = CH_3
23, R = β-D-Ribofuranosyl



18, R = H
20, R = CH_3

19, R = H
21, R = CH_3

Pseudouridine C (**22**), an important modified nucleoside found in transfer ribonucleic acid,[40] was predicted to behave much like thymine in reacting with diethyl pyrocarbonate in water. With a first acidic pK_a of 8.9,[41,42] pseudouridine C reacted rapidly at pH 8 to give a carbethoxylated compound, as determined from a 220-MHz pmr spectrum, which exhibits an ultraviolet absorption spectrum very much like that observed for carbethoxyuracil (**15**) and carbethoxythymine (**17**). The small quantity of pseudouridine available, coupled with the lability of the product, precluded more complete elucidation of structure, but evidence strongly suggests conversion to **23**.

Uracil and thymine are most reactive toward diethyl pyrocarbonate in aqueous base at the N-1 position. Nevertheless, substitution by ribofuranose at N-1 apparently allows carbethoxylation at N-3, but the products formed are quite unstable and difficult to isolate.

(40) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press, New York, N. Y., 1963, p 12, and references cited therein.

(41) R. W. Chambers, *Progr. Nucl. Acid Res. Mol. Biol.*, 5, 349 (1966).

(42) J. Ofengand and H. Schaffer, *Biochemistry*, 4, 2832 (1965).

Summary

Although nucleic acid components related to uracil were found to react with diethyl pyrocarbonate in water only near pH 8 and formed rather unstable products, all the other products reported could be prepared over a much wider pH range, extending into slightly acid solution, and were considerably more stable than the uracil products. Perhaps most significant were the conversion of guanosine to a ring-opened product, **4**, in a manner like that previously reported for adenosine, and the acylation of the exocyclic amino group of cytidine. Conversion of adenosine-containing dinucleoside phosphates to modified counterparts has been shown to affect greatly hydrolysis by several nucleases, and modification of guanylate and cytidylate, both important nucleic acid components, may be predicted to affect enzymatic processes in which they would normally participate. Uridylate or thymidylate modification, on the other hand, may be predicted to be of less consequence enzymatically, since reaction with diethyl pyrocarbonate is restricted to basic solution and the products are extremely sensitive to hydrolysis at any pH.

The reactions of diethyl pyrocarbonate with every major ribonucleic acid base and corresponding ribosides, at least under some conditions, further emphasize the careful consideration which must be given to use of the reagent as a nuclease inhibitor for the isolation of unaltered nucleic acids. Possible use of diethyl pyrocarbonate as a nucleic acid probe, to effect specific modification, is receiving further consideration, but the traditional application as a nuclease inhibitor seems to be ill-advised in view of these and earlier studies.

Experimental Section

Materials and Methods. Nucleic acid bases and nucleosides were purchased from Sigma Chemical Co., Calbiochem, and Papierwerke "Waldhof-Aschaffenburg" Aktiensgesellschaft (PWA). Diethyl pyrocarbonate and ethyl chloroformate were purchased from Aldrich Chemical Co.

All ultraviolet spectra were recorded on a Cary 15 spectrophotometer on aqueous solutions prepared by one of the following methods. Solutions listed as 0.1 *N* HCl or 0.1 *N* NaOH were prepared according to the method of Leonard, *et al.*[43] Those listed at a given pH were prepared using buffers or using HCl or NaOH in conjunction with a Corning pH meter just before final dilution in volumetric flasks. Proton magnetic resonance spectra were measured with Varian 60-, 100-, and 220-MHz instruments in $(CD_3)_2SO$ with $(CH_3)_4Si$ internal standard. Mass spectra were obtained by Mr. J. Carter Cook and associates on Varian MAT spectrometers, and microanalyses were performed by Mr. Josef Nemeth and his associates.

2-Amino-5-carbethoxyamino-4-hydroxy-6-*N*-ribofuranosylaminopyrimidine (4). To 1.0 g (3.5 mmol) of guanosine (**3**) suspended in 75 ml of H_2O at *ca.* pH 6 was added dropwise 10.4 g (64 mmol) of $(EtOCO)_2O$. After 14 hr and 28 hr, additional $(EtOCO)_2O$, 9.1 g (56 mmol) and 6.1 g (38 mmol), respectively, was added to the stirred solution. Undissolved and unreacted **3** (0.43 g) was removed by filtration, and the filtrate was concentrated to dryness *in vacuo*. Treatment of the residue with concentrated ethanolic ammonia for 30 min destroyed a minor component but did not affect the major product, as observed by tlc (2:1 EtOH–EtOAc on Eastman silica gel plates). After removal of ammonia, **4** was separated from unreacted, dissolved starting material by passage through a Sephadex G-15 column packed with H_2O. Analytically pure **4** was collected in *ca.* 30% yield by recrystallization from EtOH–Et_2O. A pmr spectrum indicated ring opening with loss of the concomitantly formed formyl group and addition of one carbethoxy group.

(43) N. J. Leonard, K. L. Carraway, and J. P. Helgeson, *J. Heterocycl. Chem.*, 2, 291 (1965).

Ultraviolet spectral data are listed in Table I. *Anal.* Calcd for $C_{12}H_{19}N_5O_7$: C, 41.74; H, 5.55; N, 20.28. Found: C, 41.51; H, 5.64; N, 19.66.

A similar reaction in which the reaction mixture was warmed just enough to dissolve **3**, but also enough to thermally decompose $(EtOCO)_2O$, which was then added in larger quantities over the course of reaction, brought the yield up to 40%.

N^4-Carbethoxycytosine (8). A suspension of 1.1 g (10 mmol) of cytosine (**6**) was dissolved in 100 ml of dry pyridine cooled and stirred in an ice bath. EtOCOCl (2.2 g, 20 mmol) was added slowly over 30 min and then allowed to warm to room temperature for an additional 30 min of stirring. After concentration to dryness *in vacuo*, the residue was suspended in *ca.* 150 ml of H_2O to which was added aqueous HCl until the odor of pyridine nearly disappeared. Analytically pure **8** was recrystallized from ethanol as 635 mg of pure white crystals, which decompose to a yellow solid above 290°. A second crop of 185 mg raised the overall yield to 45%. Ultraviolet and pmr spectral data are listed in Tables I and II. *Anal.* Calcd for $C_7H_9N_3O_3$: C, 45.90; H, 4.95; N, 22.94. Found: C, 45.69; H, 5.10; N, 23.01.

1-Carbethoxycytosine (9). To 0.55 g (5 mmol) of cytosine (**6**) dissolved in 100 ml of H_2O was added 2.4 g (15 mmol) of $(EtOCO)_2O$, and the mixture was stirred for 5 hr, at which time the mixture was concentrated *in vacuo* to dryness. The product was separated from unreacted cytosine by passage through a cellulose column packed with 9:1 $CHCl_3$–CH_3OH. The first eluate fractions produced a material which was recrystallized from CH_3CN to give 18 mg of analytically pure **9**, which undergoes physical change at 200–203°, followed by decomposition above 280°. Ultraviolet and pmr spectral data are included in Tables I and II. *Anal.* Calcd for $C_7H_9N_3O_3$: C, 45.90; H, 4.95; N, 22.94. Found: C, 45.61; H, 5.01; N, 23.12.

Similar reactions performed over a longer time and not followed by column chromatography produced less pure **9**, but with less decomposition, as by hydrolysis, during manipulation and thus in crude yields approaching 75%.

1-Carbethoxy-5-hydroxymethylcytosine (11). To 705 mg (5 mmol) of 5-hydroxymethylcytosine (**10**) suspended in 30 ml of H_2O at pH 7.5 was added 2.6 g (16 mmol) of $(EtOCO)_2O$ in five portions over 30 min. After a total of 45 min, unreacted starting material was removed by filtration, and the filtrate was dried thoroughly by lyophilization to give 600 mg of a white solid. The pmr spectrum of crude **11** is compared with that of 1-carbethoxycytosine in Table II.

N^4-Carbethoxycytidine (13). To 1.0 g (4.1 mmol) of cytidine (**12**) dissolved in 75 ml of H_2O at pH 6.4 was added dropwise 14 g (86 mmol) of $(EtOCO)_2O$. After 14 hr a further 11 g (68 mmol) of $(EtOCO)_2O$ was added, and the mixture was stirred an additional 22 hr before evaporation to dryness *in vacuo*. The residue was treated for 1 hr with ethanol saturated with ammonia to destroy only minor components, observed on tlc (1:1 EtOH–Et_2O on Eastman silica gel plates), attributed to reaction of the pyrocarbonate with the ribose hydroxyl groups, and the ammonia was removed *in vacuo*. Chromatography on Davison silica gel packed with 1:1 ethanol–diethyl ether separated unreacted cytidine from the major product, which was eluted first. Recrystallization from hot ethanol produced 670 mg of analytically pure **13**, which contracted at 125–127° to a milky liquid, evolved gas above 137°, and became a clear liquid by 145°. A second crop of 170 mg raised the overall yield to 65%: pmr ($(CD_3)_2SO$) δ (ppm) 1.23 (t, J = 7.5 Hz, 3, ethyl CH_3), 3.69 (br, 2, 5′-CH_2), 4.0 (br, 3, 2′-, 3′-, 4′-CH), 4.15 (q, J = 7.5 Hz, 2, ethyl CH_2), 5.0 (br m, 2, 2′-, 3′-OH, exchange with D_2O), 5.3 (br, 1, 5′-OH, exchanges with D_2O), 5.77 (d, J = 2.5 Hz, 1, 1′-CH), 6.98 (d, J = 7.5 Hz, 1, pyrimidine 5-H), 8.33 (d, J = 7.5 Hz, 1, pyrimidine 6-H), 10.4 (br s, 1, NHCO, exchanges with D_2O). Ultraviolet spectral data are included in Table I. *Anal.* Calcd for $C_{12}H_{17}N_3O_5$: C, 45.71; H, 5.44; N, 13.33. Found: C, 45.55; H, 5.57; N, 13.61.

1-Carbethoxyuracil (15). (a) **Diethyl Pyrocarbonate Method.** To 1.12 g (10 mmol) of uracil (**14**) suspended in 10 ml of H_2O was added 0.5 N NaOH to adjust the pH to 8. $(EtOCO)_2O$ (3.4 g, 21 mmol) was added in *ca.* 0.5-ml portions over 1 hr, and the pH was maintained throughout between 7.5 and 8.5 by controlled addition of 0.5 N NaOH. After 1 hr unreacted uracil was removed by filtration, and the filtrate was dried by lyophilization and then separated from inorganic salts with 150 ml of chilled, dry acetone. The acetone solution was concentrated to a small volume of solvent from which precipitated 550 mg (30%) of analytically pure **15**, which melts and resolidifies at 120°. Ultraviolet and pmr spectral data are listed in Tables III and IV. *Anal.* Calcd for $C_7H_8N_2O_4$: C, 45.66; H, 4.38; N, 15.21. Found: C, 45.43; H, 4.36; N, 15.51.

(b) **Ethyl Chloroformate Method.** A product prepared by the method of Dyer, *et al.*,[36] using EtOCOCl was found to be identical with **15**, as prepared using $(EtOCO)_2O$.

1-Carbethoxythymine (17). (a) **Diethyl Pyrocarbonate Method.** To 378 mg (3 mmol) of thymine (**16**) suspended in 15 ml of H_2O was added 0.5 N NaOH to adjust the pH to 8.5. $(EtOCO)_2O$ (2.7 g, 17 mmol) was added in 0.3-ml portions over 1 hr, during which time controlled addition of 0.5 N NaOH was used to maintain a pH of at least 8.0. During the final addition of $(EtOCO)_2O$ the pH was allowed to drop to equilibrium at *ca.* 7, and unreacted thymine was subsequently removed by filtration. As with carbethoxyuracil, the filtrate was lyophilized and then triturated with dry acetone, from which was recovered 130 mg (22%) of analytically pure **17**, mp 180°. Ultraviolet and pmr spectral data are listed in Tables III and IV. *Anal.* Calcd for $C_8H_{10}N_2O_4$: C, 48.48; H, 5.09; N, 14.13. Found: C, 48.65; H, 5.04; N, 14.22.

(b) **Ethyl Chloroformate Method.** Using the method of Dyer, *et al.*,[36] a product prepared by reaction of thymine with EtOCOCl was found to be identical with **17**, as prepared using $(EtOCO)_2O$.

1-Carbethoxy-3-methyluracil (Isomer of 17, CH_3 on N-3). To 261 mg (2.07 mmol) of 3-methyluracil[38] dissolved in 10 ml of dry pyridine was added 2.7 g (17 mmol) of $(EtOCO)_2O$. The mixture was stirred 4 hr and was then concentrated to dryness *in vacuo*. After complete drying under vacuum for 2 hr the residue was recrystallized from acetone, giving two crops (60%) of analytically pure 1-carbethoxy-3-methyluracil, mp 70–72. Ultraviolet and pmr spectral data are listed in Tables III and IV. *Anal.* Calcd for $C_8H_{10}N_2O_4$: C, 48.48; H, 5.09; N, 14.13. Found: C, 48.79; H, 5.11; N, 14.06.

Reaction of Uridine (18) with Diethyl Pyrocarbonate. To 370 mg (1.5 mmol) of uridine dissolved in 7 ml of H_2O, adjusted to pH 8 with 0.5 N NaOH, was added dropwise 1.1 g (7 mmol) of $(EtOCO)_2O$. After 1 hr the pH of the reaction mixture was allowed to drop, at which time the solution was dried by lyophilization. No analytical sample could be isolated, but a qualitative uv spectrum (Table III) and a pmr spectrum (Table IV) obtained for the dried product suggest structure **19** for the unstable adduct.

Reaction of Ribosylthymine (20) with Diethyl Pyrocarbonate. A qualitative reaction with ribosylthymine was performed at pH 8 using the method described above for uridine. Ultraviolet spectra (Table III) and a 220-MHz pmr spectrum (Table IV) of the lyophilized product suggest that $(EtOCO)_2O$ reacted with the thymine nucleus to give **21**.

Reaction of Pseudouridine C (22) with Diethyl Pyrocarbonate. To 34 mg (0.14 mmol) of pseudouridine dissolved in 5 ml of H_2O, adjusted to pH 8.5 with 0.5 N NaOH, was added in portions 0.5 g (3 mmol) of $(EtOCO)_2O$. After 45 min at pH 8.0–8.5, maintained by addition of 1 N NaOH, the reaction mixture was dried by lyophilization. No analytical sample could be isolated, but a qualitative uv spectrum (Table III) and a 220-MHz pmr spectrum, discussed in Results and Discussion, obtained on the lyophilized product, suggest formation of **23**.

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