Reaction of Diethyl Pyrocarbonate with Nucleic Acid Components. Adenosine*

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ABSTRACT: Diethyl pyrocarbonate opens the pyrimidine ring of adenine and N^6-substituted adenines. By contrast, this reagent opens the imidazole ring of 9-substituted adenines, exemplified by the conversion of 9-propyladenine to three ring-opened products. Two of these were useful in the identification of comparable products obtained from the reaction of diethyl pyrocarbonate with adenosine: 4,5-dicarbethoxyamino-6-*N*-ribofuranosylaminopyrimidine and 4-amino-5-carbethoxyamino-6-*N*-ribofuranosylaminopyrimidine. Conditions have been found which make these reaction sequences with diethyl pyrocarbonate of preparative value. Under conditions used for ribonuclease inhibition by diethyl pyrocarbonate (dilute solution, pH 7, 23°), 50% of the total adenosine conversion occurs in about 10 min. Recognition of this facile reaction with a nucleic acid component is important to keep in mind when diethyl pyrocarbonate is used for nuclease inhibition in the preparation of biologically active RNA. The infectivity of tobacco mosaic virus RNA is destroyed completely by diethyl pyrocarbonate at 23 or 37° and reduced to near zero at 0°.

The demonstrated capacity of diethyl pyrocarbonate[1] (DEP) (ethoxyformic anhydride) to inactivate enzymes has been utilized in the isolation of RNA (Solymosy *et al.*, 1968; Abadom and Elson, 1970; Solymosy *et al.*, 1970; Summers, 1970). The acceptor activity of $tRNA^{Ala}$ isolated using DEP was reported unchanged (Abadom and Elson, 1970), whereas the phenylalanine-, lysine-, and alanine-acceptor activities of RNA were diminished when concentrations of DEP above 50 mM were used (Denić *et al.*, 1970). The transforming activity of preparations of DNA treated with DEP was retained (Fedorcsák and Turtóczky, 1966). Furthermore, diethyl pyrocarbonate did not inactivate the infectivity of the double-stranded replicative form of poliovirus RNA. By contrast, DEP destroyed the infectivity of single-stranded poliovirus RNA (Öberg, 1970) and TMV RNA (Gulyás and Solymosy, 1970; Oxelfelt and Årstrand, 1970). In order to be in a better position to interpret the reactions of this reagent with RNAs and with polynucleotides (Öberg, 1971[2]), we have been continuing a study (Leonard *et al.*, 1970) of the fate of various nucleic acid components in the presence of diethyl pyrocarbonate.

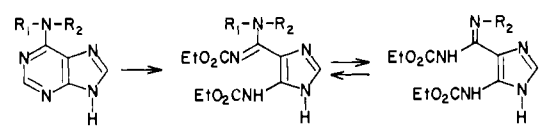
Experimental Section

Materials. Adenine, 4-amino-6-chloro-5-nitropyrimidine, 4,5,6-triaminopyrimidine sulfate, adenosine, and ethyl chloroformate were purchased from the Aldrich Chemical Co. 6-Ethylaminopurine and 6-diethylaminopurine were synthesized and their properties were found to be identical with those published (Elion *et al.*, 1952; Pal and Horton, 1964; Skinner *et al.*, 1956). 9-Propyladenine was prepared according to the method of Lambert (1967). The 5% palladium on carbon was purchased from Baker and Co., Inc. Diethyl pyrocarbonate was prepared by the method of Degering *et al.* (1950).

Methods. For analytical ultraviolet spectra, a solution of a known amount of compound in absolute ethanol was diluted with an appropriate amount of 2 N HCl, H_2O, or 2 N NaOH to a final strength of 95% ethanol, according to Leonard *et al.* (1965). Nuclear magnetic resonance spectra were measured with Varian 60-MHz instruments using tetramethylsilane as an internal standard. Infrared spectra were recorded on a Perkin-Elmer Model 337 instrument. Microanalyses were performed by Mr. Joseph Nemeth and associates, and mass spectra were determined by Mr. Joseph Wrona on an Atlas Model CH-5 low-resolution spectrometer at the School of Chemical Sciences, University of Illinois, Urbana, Ill.

Reaction of Diethyl Pyrocarbonate with Adenine (**1a**). To 250 ml of water acidified with 2 ml of concentrated hydrochloric acid was added 3.0 g (22 mmoles) of adenine. After complete solution the pH was adjusted to 4.5 with 2 N NaOH. To the efficiently stirred solution was added 17.8 g (0.11 mole) of diethyl pyrocarbonate slowly over several hours. A precipitate formed rapidly with liberation of carbon dioxide. Stirring was continued overnight. After chilling the mixture, the precipitate was filtered. A second crop was obtained by concentrating the filtrate and again filtering; upon neutralization of the filtrate with 2 N HCl, a third crop was obtained. Total combined yield of thoroughly dried product was 3.4 g (57%) of chro-

SCHEME I



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[1] Abbreviation used is: DEP, diethyl pyrocarbonate, diethyl dicarbonate.

[2] We are grateful to Dr. Öberg for sending us a preprint of this article.

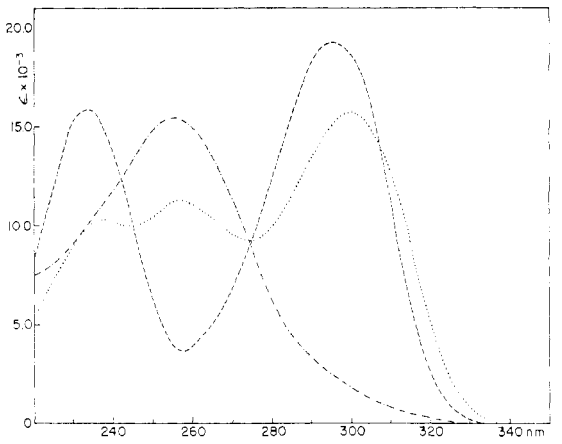


FIGURE 1: Quantitative ultraviolet spectra of imidazoles in 95% ethanol: (- - - - -) **2a**, (· · · · · · ·) **2b**, (—·—·—) **2c**.

matographically pure powder. A portion was recrystallized from aqueous ethanol for analysis as colorless, fluorescent needles of 5(4)-carbethoxyaminoimidazole-4(5)-*N'*-carbethoxycarboxamidine (**2a**) mp > 320° (Leonard *et al.*, 1970).

Reaction of DEP with Adenine-8-d. Adenine-*8-d*, prepared by refluxing a suspension of 10 g of adenine in 30 ml of 99.5% D_2O for 24 hr, followed by two successive evaporations *in vacuo* after treatment with D_2O (Fox, 1965), exhibited a single sharp resonance in the purine region of the pmr spectrum whereas adenine showed two sharp resonances separated by 7.5 Hz. Treatment of adenine-*8-d* with diethyl pyrocarbonate as before, but for less than 1 hr, led to a product (29% yield) identical with **2a** in chromatographic mobility and uv spectrum. The nmr spectrum of this preparation, however, was lacking in aromatic absorption near τ 2.5 (imidazole-H).

Preparation of 5-Carbethoxyamino-4,6-diaminopyrimidine Hydrochloride (**6**). To a stirred suspension of 1.25 g (10 mmoles) of 4,5,6-triaminopyrimidine in 50 ml of dry pyridine in an ice bath was added dropwise 2.16 g (20 mmoles) of ethyl chloroformate. Stirring was continued for 1.5 hr. The solvent was removed *in vacuo*, and the residue was extracted with absolute ethanol. The extracts yielded white needles (1.35 g, 58%): mp >320°; nmr (Me_2SO-d_6) τ 1.75 (s, 1, pyrimidine-H), 2.26 [broad s, 3, NH_2 (exchanged with D_2O), NHCO], 5.91 (q, J = 7 Hz, 2, CH_2), 8.78 (t, J = 7 Hz, 3, CH_3). *Anal.* Calcd for $C_7H_{12}ClN_5O_2$: C, 35.98; H, 5.18; Cl, 15.18; N, 29.97. Found: C, 35.98; H, 5.18; Cl, 15.41; N, 30.03.

Treatment of **6** *with Aqueous Ammonia.* A mixture of 0.80 g (3.4 mmoles) of **6** in 20 ml of concentrated aqueous ammonia was heated on a steam bath in a pressure bottle for 3 hr. Upon cooling, 0.43 g (63%) of the corresponding free base was deposited as chromatographically pure yellow plates: $\lambda_{max}^{95\% EtOH}$ (pH 1) 266 nm; nmr (Me_2SO-d_6) τ 2.29 (s, 1, pyrimidine-H), 5.95 (q, J = 7 Hz, 2, OCH_2), 8.80 (t, J = 7 Hz, 3, CH_3).

Reaction of DEP with **1b**. A solution of 1.63 g (10 mmoles) of **1b** in 200 ml of water containing 2 ml of concentrated HCl was adjusted to pH 4.5 with 2 N NaOH. To the rapidly stirred solution was added 14.0 g (86 mmoles) of DEP dropwise overnight. The reaction solution was extracted with ether.

ISOLATION OF 5(4)-CARBETHOXYAMINOIMIDAZOLE-4(5)-*N*-CARBETHOXY-*N'*-ETHYLCARBOXAMIDINE (**2b**). When the pH of the concentrated aqueous solution was adjusted to 6.5, **2b** precipitated; yield 0.65 g (22%). Recrystallization of a portion from ethyl acetate–hexane gave colorless needles, mp 150–150.5° (Leonard *et al.*, 1970).

ISOLATION OF 5(4)-CARBETHOXYAMINOIMIDAZOLE-4(5)-*N*-CARBETHOXY)CARBOXAMIDE (**8**). The dried ($MgSO_4$) ether extract (see above) was concentrated to dryness and chromatographed on a 30-g silica gel column using 2% ethanol in chloroform as eluent. The solvent was stripped from the early fractions, and the residue (**8**) was recrystallized from ethyl acetate–hexane: yield, 130 mg; mp 165–166°; nmr ($CDCl_3$) τ 0.83 and 0.94 (br s, 2, NH, exchanged with D_2O), 2.56 (s, 1, imidazole-H), 5.65 (q, J = 7 Hz, 4, NCH_2 and OCH_2), 8.66 (t, J = 7 Hz, 6, CH_3's); mass spectrum (70 eV) *m/e:* 270 (M^+), 224 (M^+ − EtOH), 178 (224^+ − EtOH), 152 (224^+ − $C_3H_4O_2$), 136 ($C_5H_2N_3O_2^+$), 109 (152^+ − HNCO), 81 (109^+ − CO). Metastable peaks were observed for the fragmentations: 270 → 224 (186) 224 → 178 (141.5), 178 → 136 (104), 224 → 152 (103), 152 → 109 (78), 109 → 81 (60.2). *Anal.* Calcd for $C_{10}H_{14}N_4O_5$: C, 44.44; H, 5.22; N, 20.73. Found: C, 44.43; H, 5.19; N, 20.76.

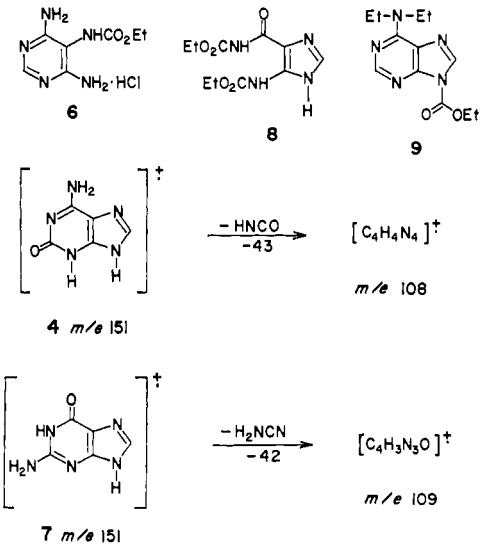
Reaction of DEP with **1c**. A solution of 2.2 g (11 mmoles) of **1c** in 150 ml of water acidified with 3 ml of concentrated hydrochloric acid was adjusted to pH 4.5 with 2 N NaOH. To the rapidly stirred solution was added 16 g (0.1 mmole) of DEP very slowly overnight. The clear solution was extracted with ether.

ISOLATION OF 5(4)-CARBETHOXYAMINOIMIDAZOLE-4(5)-*N*-CARBETHOXY-*N',N'*-DIETHYLCARBOXAMIDINE (**2c**). The aqueous phase, concentrated *in vacuo* to a small volume, was neutralized with 2 N NaOH, and then taken to dryness. Recrystallization of the solid residue from ethanol gave 0.79 g of **2c** as white needles: mp 180–182°; nmr (Me_2SO-d_6) τ 1.06 (br s, 1, NH, exchanged with D_2O), 2.49 (s, 1, imidazole-H), 5.92 and 6.17 (qq, J = 7 Hz, 4, OCH_2's), 6.64 (q, J = 7 Hz, 4, NCH_2's), 8.80, 8.89, and 8.99 (ttt, J = 7 Hz, 12, CH_3's); mass spectrum (70 eV) *m/e:* 325 (M^+), 296 (M^+ − C_2H_5), 279 (M^+ − EtOH), 250 (296^+ − EtOH), 233 (279^+ − EtOH), 207 (279^+ − $C_3H_4O_2$), 204 (250^+ − EtOH), 178 (250^+ − $C_3H_4O_2$). Metastable peaks were observed for the fragmentations: 325 → 296 (270), 325 → 279 (240), 296 → 250 (211), 279 → 233 (194.5), 279 → 207 (153.5), 250 → 204 (166.5), 250 → 178 (127). *Anal.* Calcd for $C_{14}H_{23}N_5O_3$: C, 51.68; H, 7.12; N, 21.53. Found: C, 51.55; H, 7.08; N, 21.35. The uv spectra of **2a**, **2b**, and **2c** are given in Figure 1.

ISOLATION OF 9-CARBETHOXY-6-DIETHYLAMINOPURINE (**9**). The dried ($MgSO_4$) ether extract (see above) was concentrated to an oil which was dissolved in ethyl acetate–hexane and deposited a small amount of **1c**. Concentration of the mother liquors and recrystallization of the residue from hexane yielded 270 mg of crude **9**. Attempted purification of a portion on silica gel led to the recovery of only **1c**. Recrystallization of the remainder gave yellowish needles: mp 96–97°; nmr ($CDCl_3$) τ 1.53 (s, 1, purine-H), 1.78 (s, 1, purine-H), 5.46 (q, J = 7 Hz, 2, OCH_2), 6.08 (q, J = 7 Hz, 4, NCH_2's), 8.55 and 8.75 (tt, J = 7 Hz, 9, CH_3's); $\lambda_{max}^{95\% EtOH}$ 272 nm, (H^+) 276, (OH^-) 283, sh 292. *Anal.* Calcd for $C_{12}H_{17}N_5O_2$: C, 54.74; H, 6.51; N, 26.60. Found: 54.98; H, 6.57; N, 26.19.

Conversion of **2a**–**2c** *to Isoguanine* (**4**). A solution of 1.0 g (3.7 mmoles) of **2a** in 75 ml of concentrated aqueous ammonia was heated in a sealed flask for 3 hr at 120°. Removal of the solvent was followed by two recrystallizations from water to yield 0.39 g (71%) of **4**, mp > 320°. Samples of **2b** (250 mg, 0.85 mmole) and of **2c** (150 mg, 0.46 mmole) were each treated with 20 ml of absolute ethanol saturated with ammonia at 0° in sealed tubes at 110° for 6 hr. After allowing the ammonia to evaporate at room temperature, the precipitated products were collected and recrystallized from water to yield 58 (44%) and 25 mg (36%) of **4**, respectively, from **2b** and **2c**, mp > 320°. The identity of these three samples with of **4** prepared by

SCHEME II



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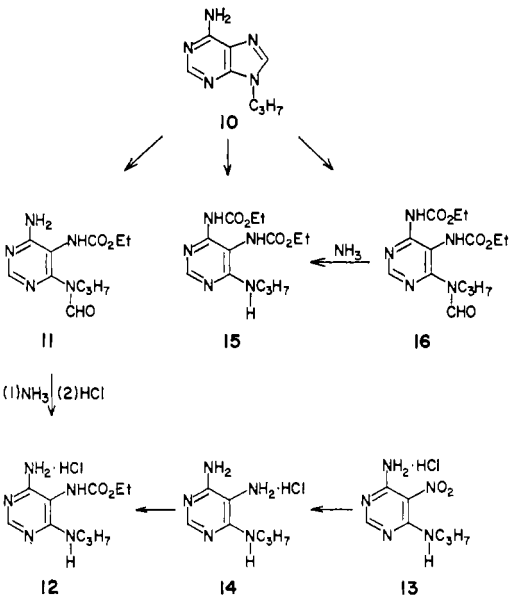


acid hydrolysis of isoguanosine was confirmed by ascending paper chromatogrphy in two systems: R_{Ade} 0.55 in 1:1.5 N aqueous acetic acid–1-butanol and R_{Ade} 1.05 in isopropyl alcohol 65 ml, concentrated HCl 16.7 ml, diluted to 100 ml with water; λ_{max} 283 nm in aqueous HCl and 285 in aqueous NaOH; mass spectrum (70 eV) *m/e:* 151 (M^+), 108 (M^+ − HNCO), with a metastable peak for the fragmentation 151 → 108 (77).

Reaction of DEP with 9-Propyladenine (**10**). A solution of 2.66 g (15 mmoles) of **10** in 150 ml of water acidified with 1 ml of concentrated HCl was adjusted to pH 4.5 with 2 N NaOH. To the rapidly stirred solution was added 14.0 g (85 mmoles) of DEP over several hours and stirring was continued overnight. The solution was concentrated to dryness *in vacuo* and applied to a 400-g silica gel column. Elution was begun with 2%, followed by 6%, and, finally, with 12% ethanol in chloroform.

ISOLATION OF 4-AMINO-5-CARBETHOXYAMINO-6-*N*-FORMYL-*n*-PROPYLAMINOPYRIMIDINE (**11**). The 12% fraction (see above) was concentrated to small volume and diluted with hexane. Upon cooling, 1.34 g (33%) of analytically pure white microneedles was deposited: mp 153–154° with bubbling; nmr ($CDCl_3$) τ 1.68 (s, 2, pyrimidine-H and CHO), 3.15 (br s, 1, NHCO, exchanged with D_2O), 4.45 (br s, 2, NH, exchanged with D_2O), 5.86 and 6.13 (q and t, both J = 7 Hz, 4, OCH_2 and NCH_2), 8.51 (m, propyl CH_2), 8.75 (t, J = 7 Hz, ethyl CH_3), 9.13 (t, J = 7 Hz, propyl CH_3), the last three patterns integrating for 8 protons; ir ($CHCl_3$) 1720 cm^{-1} (5-NHCO), 1680 (CHO). *Anal.* Calcd for $C_{11}H_{17}N_5O_3$: C, 49.43; H, 6.41; N, 26.20. Found: C, 49.46; H, 6.38; N, 26.08.

ISOLATION OF 4,5-DICARBETHOXYAMINO-6-*n*-PROPYLAMINOPYRIMIDINE (**15**). The 6% fraction (see above) was concentrated to dryness and taken up in some chloroform, and the solution was diluted with hexane and placed in a refrigerator, yielding white needles (0.25 g): mp 173–173.5° with bubbling; nmr ($CDCl_3$) τ −0.03 (br, 1, NH, exchanged with D_2O), 1.64 (s, 1, pyrimidine-H), 2.50 (s, 1, NH, exchanged with D_2O), 4.48 (t, $J \sim 6$ Hz, 1, $NHCH_2$, exchanged with D_2O), 5.72 and 5.81 (qq, J = 7 Hz, 4, OCH_2), 6.45 and 6.55 (tt, 2, $NHCH_2$, collapses to t at τ 6.49 with addition of D_2O), 8.3 (m, propyl CH_2), 8.68 (t, J = 7 Hz, ethyl CH_3), 8.75 (t, J = 7 Hz, ethyl CH_3), 9.03 (t, J = 7 Hz, propyl CH_3), with the last four patterns integrating for 11 protons; ir ($CHCl_3$) 1720 cm^{-1} (5-NHCO), 1705 (4-NHCO). *Anal.* Calcd for $C_{13}H_{21}N_5O_4$: C, 50.15; H, 6.80; N, 22.50. Found: C, 50.07; H, 6.69; N, 22.66.

ISOLATION OF 4,5-DICARBETHOXYAMINO-*N*-FORMYL-*n*-PROPYLAMINOPYRIMIDINE (**16**). The solvent was completely removed from the 2% fraction (see above) leaving 0.60 g of a gum which could not be crystallized or analyzed satisfactorily. Spectroscopic and chemical evidence established the structure sufficiently: nmr ($CDCl_3$) τ 1.02 (br, 1, NH, exchanged with D_2O), 1.35 (s, 2, pyrimidine-H and CHO), 2.59 (br, 1, NH, exchanged with D_2O), 5.69, 5.85, 6.10 (qqt, all J = 7 Hz, 6, OCH_2's and NCH_2), 8.3 (m, propyl CH_2), 8.65 (t, J = 7 Hz, ethyl CH_3), 8.76 (t, J = 7 Hz, ethyl CH_3), 9.12 (t, J = 7 Hz, propyl CH_3); ir ($CHCl_3$) 1720 cm^{-1} (5-NHCO), 1705 (4-NHCO), 1685 (CHO).

CONVERSION OF **16** TO **15** WITH ETHANOLIC AMMONIA. About 300 mg of **16** in 50 ml of absolute ethanol saturated with ammonia at 0° was allowed to stand at room temperature for 20 hr. Removal of the solvent *in vacuo* followed by recrystallization of the solid residue from ethyl acetate–hexane yielded 150 mg of white needles, mp 173–173.5°, with uv spectra and chromatographic mobility identical with those of **15**.

Treatment of **11** *with Ethanolic Ammonia.* A solution of 0.35 g (1.3 mmoles) of **11** in 50 ml of absolute ethanol saturated with ammonia at 0° was allowed to stand at room temperature for 4 days. The solvent was removed, the residue was redissolved in ethanol containing 1 ml of concentrated HCl, and the solution was again concentrated to dryness *in vacuo*. Recrystallization of the residue from ethanol–ethyl acetate yielded 0.28 g (77%) of white needles, mp 197–198°, with uv spectra and chromatographic mobility identical with those of **12** prepared by an unequivocal route.

Synthesis of 4-Amino-5-nitro-6-n-propylaminopyrimidine (**13**). A mixture of 3.0 g (17 mmoles) of 4-amino-6-chloro-5-nitropyrimidine and 6.0 g (0.1 mmole) of *n*-propylamine in 60 ml of water was heated on a steam bath for 1.5 hr. The solution was taken to dryness *in vacuo*. The solid residue was recrystallized from aqueous ethanol: mp 163–164°; yield 2.65 g (78%) of yellow needles; nmr ($CDCl_3$) τ 1.99 (s, 1, pyrimidine-H), 6.42 (m, 2, NCH_2), 8.30 (m, 2, CH_2), 8.98 (t,

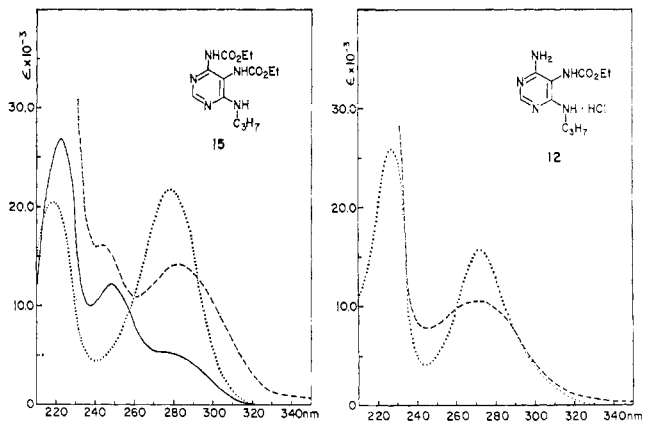


FIGURE 2: Quantitative ultraviolet spectra of propyl-substituted pyrimidines **15** and **12**: (—) 95% EtOH, (· · · · · ·) 0.1 M HCl in 95% EtOH, (- - - -) 0.1 M NaOH in 95% EtOH.

J = 7 Hz, 3, CH_3). *Anal.* Calcd for $C_7H_{11}N_5O_2$: C, 42.63; H, 5.62; N, 35.52. Found C, 42.69; H, 5.52; N, 35.55.

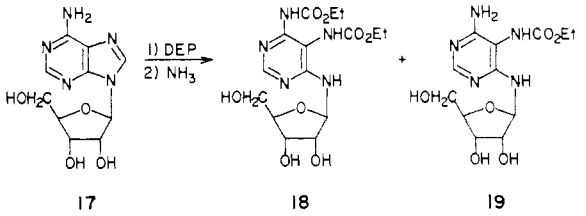
Synthesis of 4,5-Diamino-6-n-propylaminopyrimidine (**14**). A suspension of 2.5 g (12.7 mmoles) of **13** and 0.25 g of 5% palladium in carbon in 250 ml of absolute ethanol was hydrogenated for 7 hr at an initial pressure of 4 atm. The catalyst was filtered and washed with hot ethanol. Concentrated HCl (3 ml) was added to the combined filtrates, which were then taken to dryness *in vacuo*. Recrystallization of the solid residue from 95% ethanol yielded 1.6 g (62%) of yellow needles; mp 198–199°; nmr (Me_2SO-d_6) τ 1.88 (s, 1, pyrimidine-H), 2.43 and 2.60 (br s's, 4, NH_2's, exchanged with D_2O), 6.53 (br t sharpened by addition of D_2O, 2, NCH_2), 8.38 (m, 2, CH_2), 9.08 (t, 3, CH_3). *Anal.* Calcd for $C_7H_{14}ClN_5$: C, 41.28; H, 6.93; N, 34.39. Found: C, 41.81; H, 6.87; N, 34.43.

Synthesis of 4-Amino-5-carbethoxyamino-6-n-propylaminopyrimidine Hydrochloride (**12**). To a solution of 1.1 g (5 mmoles) of **14** in 50 ml of dry pyridine cooled in an ice bath was added slowly and dropwise 1.1 g (10 mmoles) of ethyl chloroformate. After 1 hr the solvent was removed *in vacuo*, and the residue was dissolved in water and acidified with 2 N HCl. After removal of the solvent again, a solid remained which was recrystallized from 95% ethanol to yield 0.65 g (44%) of crude product. Further recrystallization from ethanol–ethyl acetate gave hygroscopic white needles: mp 197–198°; nmr (Me_2SO-d_6) τ 1.72 (s, 1, pyrimidine-H), 1.80 (br s, 1, NH), 2.08 (br t, 1, $NHCH_2$, exchanged with D_2O), 2.41 (s, 2, NH_2, exchanged with D_2O), 5.93 (q, J = 7 Hz, 2, OCH_2), 6.60 (apparent q goes to t with addition of D_2O, J = 7 Hz, 2, $NHCH_2$), 8.5 (m, propyl CH_2), 8.78 (t, J = 7 Hz, ethyl CH_3), 9.15 (t, J = 7 Hz, propyl CH_3), the last three patterns integrating for 8 protons. *Anal.* Calcd for $C_{10}H_{18}ClN_5O_2$: C, 43.56; H, 6.58; N, 25.40. Found: C, 43.36; H, 6.55; N, 25.29. The uv spectra of **15** and **12** are shown in Figure 2.

Preparative Reaction of DEP with Adenosine (**17**). To an aqueous solution of 7.1 g (27 mmoles) of adenosine adjusted to pH 5 was added 45.8 g (283 mmoles) of DEP over an hour and stirring was continued overnight. The volatile material was removed *in vacuo*, and the residue was applied to a 200-g silica gel column and eluted with 3:1 chloroform–ethanol.

ISOLATION OF A MIXTURE OF MINOR COMPONENTS. Early fractions contained a mixture of components, as indicated by tlc, and an nmr spectrum (acetone-d_6, D_2O) of the evaporated residue indicated a methyl proton to aromatic proton ratio of *ca.* 9:1. Treatment of the mixture with ethanolic ammonia

SCHEME IV



produced a new mixture of compounds having mobilities on tlc the same as those of **18** and **19** (following).

ISOLATION OF 4,5-DICARBETHOXYAMINO-6-*N*-RIBOFURANOSYLAMINOPYRIMIDINE (**18**). The next eluate fractions contained a component which, after concentration to dryness, was recrystallized from ethanol–ethyl acetate. Entrapped ethyl acetate was removed by dissolution in a minimum of ethanol and precipitation by adding the solution to a rapidly stirred excess of diethyl ether. The fluffy white crystals of **18** (2.8 g), after initial contraction at 103°, expanded in volume and evolved gas at 122° and became liquid by 130°: nmr (Me_2SO-d_6) τ *ca.* 0.7 (br, 1, NHCO; exchanged with D_2O), 1.78 (s, 1, pyrimidine-H), *ca.* 1.9 (br, 1, NHCO; exchanged with D_2O), *ca.* 3 (v br, 1, NH), 3.8–4.8 (several m, 3, 1′-, 2′-, 3′-CH), *ca.* 5.2 (br, 3, OH; exchanged with D_2O), 5.82 (q, J = 7.5 Hz, 4, CH_2), 6.5 (br m, 3, 4′-CH, 5′-CH_2), 8.77 (t, J = 7.5 Hz, 6, ethyl CH_3); mass spectrum (70 eV) *m/e*: 401 (M^+). *Anal.* Calcd for $C_{15}H_{23}N_5O_8$: C, 44.89; H, 5.77; N, 17.45. Found: C, 44.91; H, 5.91; N, 17.31.

Further elution with chloroform–ethanol did not displace **19** from the column. It was easier to isolate **19** using an intervening ammonia treatment.

ISOLATION OF 4-AMINO-5-CARBETHOXYAMINO-6-*N*-RIBOFURANOSYLAMINOPYRIMIDINE (**19**). To a rapidly stirred solution of 1.0 g (3.7 mmoles) of adenosine in 100 ml of water at pH 5 was added 6.1 g (38 mmoles) of DEP, and the mixture was stirred for 5 hr. After concentration to dryness *in vacuo* the crude product was treated with 25 ml of ethanolic ammonia for 70 min, and the solution was again evaporated to dryness. The residue in 2:1 ethanol–diethyl ether was applied to a 130-g silica gel column packed using 1:1 ethanol–diethyl ether. Early fractions yielded 526 mg of **18** after recrystallization. Later fractions were evaporated to dryness, and the solid was recrystallized from ethanol–ethyl acetate and treated as with **18** to remove entrapped ethyl acetate, yielding 517 mg of fluffy white crystals of **19** which, after initial contraction at 100°, dampened at 115° and rapidly evolved gas at 133°: nmr (Me_2SO-d_6) τ 2.17 (s, 2, pyrimidine-H, NHCO; carbamate H exchanged with D_2O), *ca.* 3.6 (broad, 1, NH, exchanged with D_2O), 4.05 (broad s, 2, NH_2, exchanged with D_2O), 4.0–5.5 (several m, 3, 1′-, 2′-, and 3′-CH), *ca.* 5.2 (br, 3, OH; exchanged with D_2O), 5.96 (q, J = 7 Hz, 2, ethyl CH_2), 6.4 (br, 3, 4′-CH, 5′-CH_2), 8.79 (t, J = 7 Hz, 3, ethyl CH_3). *Anal.* Calcd for $C_{12}H_{19}N_5O_6$: C, 43.77; H, 5.82; N, 21.27. Found: C, 43.31; H, 6.20; N, 20.98. The uv maxima for **18** and **19** and related compounds in this series are given in Tables I and II (see also Figure 3).

Thin-Layer Chromatography of the Products of Adenosine and DEP. The data in Table III (R_F and R_{Ado}), obtained using Eastman silica gel plates with indicator, are useful for detecting the formation of **18** and **19** from adenosine and for distinguishing between them.

Reaction of DEP with Adenosine under Conditions Used for

TABLE I: Ultraviolet Spectra of Imidazoles.

Compound	0.1 N HCl λ_{max} (nm)	0.1 N HCl ϵ ($\times 10^{-3}$)	95% EtOH λ_{max} (nm)	95% EtOH ϵ ($\times 10^{-3}$)	0.1 N NaOH λ_{max} (nm)	0.1 N NaOH ϵ ($\times 10^{-3}$)
2a	300	11.0	233	16.1	322	23.0
			296	19.3		
2b	230	sh	237	10.3	246	11.6
	304	5.6	257	11.2	324	18.4
			300	15.7		
2c	230	13.1	255	15.5	255	16.9
	325	5.2				
8	276	14.5	281	17.7	305	19.0

Ribonuclease Inhibition. Concentrations of 8 mmoles/l. and 4 mmoles/l. of adenosine in pH 7.0 phosphate buffer and pH 8.5 Tris buffer were used to study its reactions with DEP under conditions used for ribonuclease inhibition by DEP. Zero time samples were taken once the stock adenosine solution was temperature equilibrated. Vigorous shaking caused solution of added DEP within 30 sec at 23 and 37°, and within 2 min at 0°. Aliquots were taken at frequent time intervals. The samples were diluted separately to 100 ml with buffer to give a 40 μmoles/l. solution which could be measured directly by uv spectroscopy. Dilution of the 0.5- and 1.0-ml aliquots in this fashion served to stop the reaction. We ascertained previously that a 40-μmole/l. solution of adenosine which was 10 mM in DEP showed essentially no decrease in absorption at 260 nm over 60 min in pH 7.0 buffer. Qualitative figures obtained for the times required to bring about 50% of the total adenosine conversion realized in any one run were as follows, at pH 7: 23°, concentration of adenosine: concentration of DEP = 8 mmoles/l.:10 mmoles/l., 10 min; 4:10, 11 min, 8:20, 10 min; 37°, 8:10, 8 min; 4:10, 8 min; 0°, 8:10, 80 min. Thus, the rate, as determined by following the decrease in OD_{260}, was increased in going from 23 to 37° and greatly decreased in going from 23 to 0°. From a study of the reaction at 23° and pH 8.5 it was concluded, since the reaction was over within 30 min but the extent of adenosine conversion was only about 4–5%, that DEP is rapidly hydrolyzed under these alkaline conditions (see also Öberg, 1970).

TABLE II: Ultraviolet Spectra of Pyrimidines.

Compound	0.1 N HCl λ_{max} (nm)	0.1 N HCl ϵ ($\times 10^{-3}$)	95% EtOH λ_{max} (nm)	95% EtOH ϵ ($\times 10^{-3}$)	0.1 N NaOH λ_{max} (nm)	0.1 N NaOH ϵ ($\times 10^{-3}$)
6	220	28.8			268	7.0
	266.5	11.3				
11	237.5	16.8	231	21.2	271	8.6
	287.5	7.0	280	4.4		
12	227	27.2			270	10.5
	272	15.6				
13	242	23.8	340	9.7	339	10.3
	295	3.6				
	336	6.7				
14	226	19.5			282	11.1
	307	11.8				
15	218	20.3	222	26.8	244	16.0
	278	21.6	248	12.2	282	14.1
			275 sh			
16[a]	226		226		285	
	269		267			
18	221	22.8	221	35.3	240 sh	
	279	20.7	245 sh		283	13.2
			269	6.4		
19	226	25.5	222	38.1	275	8.8
	272	12.3	262	6.1		

[a] Qualitative spectra.

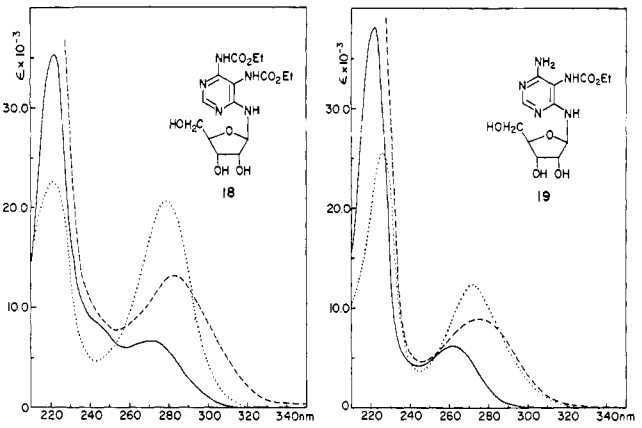


FIGURE 3: Quantitative ultraviolet spectra of ribosyl-substituted pyrimidines **18** and **19**: (—) 95% EtOH, (· · · ·) 0.1 M HCl in 95% EtOH, (- - - -) 0.1 M NaOH in 95% EtOH.

Effect of DEP on the Infectivity of TMV RNA. TMV was purified from tobacco leaves by the method of Boedkter and Simmons (1958). TMV RNA was prepared by phenol extraction of the purified virus in the presence of 0.5% sodium dodecyl sulfate. After ether extraction of the aqueous phase sodium acetate was added to 0.1 M, the RNA was precipitated with 2 volumes of cold ethanol, and the RNA pellet was resuspended in 0.01 M Tris-acetate buffer (pH 7.0). The final RNA concentration was 27 OD_{260}/ml. Aliquots of 0.5 ml of this RNA preparation were shaken on a Vortex mixer for 2 min in the presence of 2, 20, and 200 μl of DEP, or in the absence of DEP. Duplicate reactions were carried out at 0°, 23°, and 37°. Each reaction mixture was subsequently extracted 6 times with ether saturated with cold water to remove any unreacted DEP. Residual ether was blown off under a steam of nitrogen. The ultraviolet absorption spectra of these treated samples were practically identical with that of the untreated RNA, and the RNA concentration of each treated sample was 25 OD_{260}/ml.

TABLE III

System	R_F (**18**)	R_F (**19**)	R_F (Ado)	R_{Ado} (**18**)	R_{Ado} (**19**)
$CHCl_3$–EtOH (4:1)	0.39	0.14	0.23	1.7	0.61
Et_2O–EtOH (1:1)	0.57	0.36	0.40	1.4	0.90
EtOAc–*i*-PrOH–H_2O (103:47:23)	0.49	0.26	0.39	1.3	0.67

TABLE IV: Effect of Diethyl Pyrocarbonate on Infectivity of TMV RNA at Different Temperatures.

Temp of Reaction (°C)	RNA Concn[a]	Concentration of Diethyl Pyrocarbonate: 0 μl	2 μl	20 μl	200 μl
		No. of Lesions			
0	1	C[b]	50	13[c]	0
	5	132[c]	0	0	0
25	1	C[b]	0	0	0
	5	186[c]	0	0	0
37	1	C[b]	0	0	0
	5	260[c]	0	0	0

[a] The first concentration for any given temperature is given in milligrams per milliliter; the second concentration is given in micrograms per milliliter. [b] C = lesions confluent. [c] Numbers represent averages of 3–6 leaves.

Infectivity tests were carried out by inoculating the leaves of *Nicotiana tobaccum* var *xanthi* n.c. with aliquots of the various samples to which Celite had been added. Since undiluted control aliquots yielded confluent lesions, inoculations using 1:200 dilutions of all samples were also made. The results are given in Table IV.

When the reaction was carried out at 23° or at 37° the RNA was completely inactivated by all concentrations of DEP tested. On the other hand, when the reactions were carried out at 0° the infectivity of the RNA was reduced to 0.25% by 2 μl of DEP, to 0.05% by 20 μl of DEP, and destroyed completely by 200 μl of DEP.

The absence of undegraded virus in the RNA preparation was demonstrated by comparing the infectivity of an aliquot of the RNA at the concentration of 27 OD_{260}/ml to that of an identical aliquot which was digested with pancreatic ribonuclease. The undigested aliquot gave confluent lesions while no lesions appeared on the plant infected with the digested aliquot.

Mass Spectral Correlations. The mass spectra at 70 eV of **2a–2c** and **8** all displayed substantial peaks for the molecular ions. A prominent fragmentation pathway, in which the appropriate metastable peaks were observed for all four molecular ions, involved two successive losses of the neutral ethanol molecule. A second fragmentation common to all four compounds involved the loss of 72 mass units, $C_3H_4O_2$, from the $M^+ - EtOH$ radical ion giving an ion of mass $M^+ - 118$. This eliminated fragment must arise from the ethoxycarbonyl group and carries no charge, judging from the complete absence of a peak at *m/e* 72 in the spectra of **2a** and **2b**. Metastable peaks at *m/e* 102, 127, and 103 are observed for these $M^+ - 46 \rightarrow M^+ - 118$ fragmentations in **2a**, **2b**, and **8**. The base peak for the spectrum of **2c**, however, appears at *m/e* 72 and is probably due to the ion $(C_2H_5)_2N^+$.

Increasing ethyl substitutions of the amidine function as in **2b** and **2c** cause variations in the fragmentation patterns which are present in addition to those mentioned above. In fact, the main further fragmentation of the $M^+ - 46$ ion of **2b** involves loss of 43 mass units, probably CH_3CHNH, to give the $M^+ - 89$ ion at *m/e* 208. Loss of 72 mass units then occurs to give the ion at *m/e* 136. Metastable peaks at 173 and 89 provide evidence for these fragmentations. Abundant ions follow the loss of 29 mass units ($CH_3CH_2\cdot$) from the molecular ion at *m/e* 325 of **2c**. The ion at *m/e* 296 undergoes characteristic fragmentation involving loss of EtOH and $C_3H_4O_2$ to give the abundant ions at *m/e* 250, 178, and 204 with accompanying metastable peaks.

Results and Discussion

Adenine and N⁶-Substituted Adenines. As a preliminary to studying the reaction of diethyl pyrocarbonate with nucleosides, the reaction with adenine and substituted adenines was undertaken first as an indication of the types of reactions that might be involved with adenosine and related nucleosides.

The reaction of an aqueous solution of adenine (**1a**) with excess DEP resulted in the formation of a precipitate and generation of carbon dioxide within several minutes. When agitation and dropwise addition of DEP proceeded overnight, the best crude yield of homogeneous product was 57%, obtained with a 5:1 molar ratio of DEP to adenine. Complete reaction of adenine was not achieved even with a higher ratio of DEP to adenine. The yield of recrystallized product using a 2.5:1 ratio and shorter reaction time was 35% (Leonard *et al.*, 1970). The precipitated product was a colorless, blue-fluorescing solid, $C_{10}H_{15}N_5O_4$, mp > 320°, which was assigned structure **2a** (Scheme I).

The loss of the C-2 rather than the C-8 proton from the original purine ring system during the reaction was demonstrated by deuterium labeling. Adenine-*8-d*, prepared by complete exchange at the C-8 of adenine in refluxing D_2O (Fox, 1965), was treated with DEP under the same conditions as **1a** to yield a product identical with **2a**, as determined by uv and tlc, except for the absence of absorption at τ 2.51 in the nmr spectrum.

The formation of **2a** can probably be accounted for by the initial nucleophilic attack of N-1 or N-3 of adenine on the pyrocarbonate carbonyl with the generation of carbon dioxide and ethanol. Ring opening following the addition of the elements of water, loss of the original C-2 as formate by hydrolysis, and subquent carbethoxylation of the liberated amino group by a second molecule of DEP complete the sequence. Carbethoxylation of either N^6 or N-9 as the initial step in the reaction has been eliminated (Leonard *et al.*, 1970).

There is ample precedent for the opening of 1-substituted adenine in aqueous acid. In particular, Brookes *et al.* (1968) have demonstrated that the hydrolytic ring opening with loss of C-2 of 1-methyladenine to 5-aminoimidazole-4-*N*-methylcarboxamidine proceeded with a half-life of 350 min in 1 N HCl at 80°. Substitution of the more electron-withdrawing benzyl group hastened the formation of 5-aminoimidazole-4-*N*-benzylcarboxamidine by reducing the half-life to 35 min. It is to be expected that the substitution of a group such as carbethoxy, with greater electron-withdrawing capability, would accelerate the solvolysis to an even greater extent. The rapid production of **2a** would seem to corroborate this progression.

Alkyl substitution at N^6 does not interfere with the ring opening reaction, since both 6-ethylaminopurine (**1b**) and 6-diethylaminopurine (**1c**) react with DEP to give the corresponding imidazoles **2b** and **2c**. The nmr spectra of **2b** and **2c** show characteristic imidazole resonances at τ 2.69 and 2.49, respectively. Resonances for all exocyclic NH's are present and disappear upon exchange with D_2O. The carbethoxylations with loss of a carbon atom were indicated in each case by molecular formulas for $C_{12}H_{19}N_5O_4$ and $C_{11}H_{23}N_5O_4$, con-

firmed by identification of molecular ions at *m/e* 297 and 325 in the mass spectra.

The ultraviolet spectra of imidazoles **2a–2c** in 95% ethanol reproduced in Figure 1 reveal the presence of tautomers. Increasing substitution on the amidine moiety is matched by the collapse of the two absorption peaks in the spectrum of **2a** to a single absorption at 255 nm for **2c**. The spectrum of **2b** is a combination of the other two. Since there can be only one amidine tautomer for **2c**, the tautomer **3a** must predominate by far over **2a**. The intermediate spectrum can then be accounted for by the presence of both tautomers **2b** and **3b** in solution. In both acidic and basic solution (see Table I) increasing alkyl substitution causes an increase of absorption at shorter wavelengths at the expense of the longer wavelength band. For convenience the product will continue to be referred to as pictured in **2** without necessarily designating tautomeric forms. Protonation of the amidine moiety allowing conjugation of charge with the aromatic ring can account for the absorption at long wavelength in acid solution. Conversely, removal of a proton on basification would lead to an extension of the chromophore. The absence of the long-wavelength absorption in the basic spectrum of **2c** can be explained by the impossibility of suitable ionization of the amidine moiety. The ultraviolet spectra of **2a–2c** provide a clear-cut example of the principle enunciated by Albert (1959) concerning the assessment of the presence of tautomers in solution by the comparison of the ultraviolet spectra of the appropriately alkyl-substituted compounds. Further proof of pyrimidine ring opening in the original bases **1a–1c** and similarity of the imidazole products was demonstrated by the conversion of **2a–2c** to isoguanine by heating with concentrated aqueous or ethanolic ammonia. In the case of **1a**, the reaction probably involves basic hydrolysis of a carbethoxyl group followed by ring closure between the liberated amine group and the remaining carbethoxyl. For **1b** and **1c**, ammonolysis of the amidine group is a prerequisite for completion of the process. The observed ultraviolet maxima for the isoguanine samples, 283 nm in aqueous acid and 285 nm in aqueous base, compare favorably with the reported values of 282 and 285 nm (Cavalieri *et al.*, 1948; Bendich *et al.*, 1948). Confirmation of structure was provided by the molecular ion at *m/e* 151 and comparison of the mass spectrum with the previously reported spectrum of guanine (**7**) (Rice and Dudek, 1967). The principle fragmentation of **4** is the loss of HNCO (43 mass units) to give $C_4H_4N_4^+$, *m/e* 108. The previously described major fragmentation of **7** involves loss of H_2NCN (42 mass units) to give $C_4H_3N_3O^+$, *m/e* 109 (Scheme II). Indeed, the spectra are nearly identical except for a shift of one unit to lower *m/e* of the fragmentation pattern for **4** in the area *m/e* 107–109.

The conversion of adenine to **2a** to isoguanine actually constitutes a useful synthesis of isoguanine. An attempt was made to effect similar ring closure with a carbethoxylated aminopyrimidine. Treatment of 4,5,6-triaminopyrimidine (**5**) with ethyl chloroformate gave 5-carbethoxyamino-4,6-diaminopyrimidine hydrochloride (**6**), parallel to the N^5 locus of acetylation (Brown, 1962). Liberation of the corresponding free base of **6** but not ring closure to 6-amino-8-hydroxypurine, was realized on heating with aqueous ammonia.

Minor products were isolated from ether extracts of the crude reaction mixtures of **1b** and **1c** with DEP. The ether-extractable product from **1b** was purified on a silica gel column. The nmr spectrum displayed absorption for two protons exchangeable with D_2O, two equivalent ethyl groups, and the characteristic absorption for an imidazole C-2 proton at τ 2.56. The shift of absorption from 281 nm to 305 nm upon basification was indicative of extended conjugation (Table I). Observation of a molecular ion at *m/e* 270 agrees with the molecular formula of $C_{10}H_{14}N_4O_5$ from microanalysis. These data are consistent with structure **8** for the ether-extractable product. Lack of a bathochromic shift in the acidic ultraviolet spectrum indicates, as would be expected, that the exocyclic nitrogens of **8** are not basic enough to be protonated, in contrast to **2**, under the experimental conditions. Formation of **8** could reasonably result from aqueous hydrolysis of **2b**.

The minor, ether-extractable product from the crude reaction mixture of **1c** was found by microanalysis to have the molecular formula $C_{12}H_{17}N_5O_2$, suggesting a single carbethoxylation with no loss of carbon. The nmr spectrum revealed that the purine ring remained intact by the presence of resonances at τ 1.53 and 1.78 characteristic of purine protons. Also present was absorption for three ethyl groups. An attempt to purify the compound on a silica gel column demonstrated the lability of the carbethoxyl group in that only **1c** could be recovered. These data are consistent with the structure **9** for the purine. Enough material was collected for the qualitative ultraviolet spectra recorded in the Experimental Section.

9-n-Propyladenine. The reaction of DEP with 9-propyladenine (**10**) (Scheme III) provided useful models for the reaction with adenosine. It was felt that the lack of the potentially hydrolyzable *N*-ribosyl linkage would be advantageous during first experience in the manipulation of the products. The crude reaction mixture of **10** with DEP was separated into three components by silica gel chromatography. The major component was identified as the pyrimidine **11**. The nmr spectrum showed absorption for propyl and ethyl, and for three protons exchangeable with D_2O. Resonances for the pyrimidine C-2 proton and the formyl proton were observed as a single peak integrating for two protons, τ 1.68. A molecular ion at *m/e* 267 confirmed the formula of $C_{11}H_{17}N_5O_3$ from microanalysis. Upon treatment with ethanolic ammonia, **11** underwent deformylation to **12**, which was isolated as the hydrochloride salt. Proof that the original N^6 had remained unmasked was provided by the nmr spectrum. When the sample was treated with D_2O, the broad triplet at τ 2.08 disappeared, and the apparent quartet centered at τ 6.60 collapsed to an apparent triplet, indicating coupling of the amino proton with the *N*-methylene group.

An unambiguous synthesis of **12** was accomplished by first treating 4-amino-6-chloro-5-nitropyrimidine with *n*-propylamine to form **13**. The nitro group of **13** was catalytically reduced to form **14**, isolated as the hydrochloride. The nmr spectra of both **13** and **14** showed evidence of coupling of the N^6-hydrogen with *N*-methylene. The substituted triaminopyrimidine **14** was carbethoxylated with ethyl chloroformate in pyridine to yield a compound with properties identical with those of **12**.

It is likely that **11** results from the attack of the N-7 of 9-propyladenine on the carbonyl of DEP followed by hydrolytic opening at C-8 of the 7-carbethoxylated intermediate. The possibility of a six-membered-ring cyclic process involving effective addition of COOEt to N-7 and OEt to C-8 with loss of CO_2 is not ruled out. A hydrolysis step would complete this sequence. The conversion of **10** to **11** is analogous to the ring opening of 9-substituted adenines under certain Schotten–Baumann conditions observed by Altman and Ben-Ishai (1962, 1968).

The other two components were present in much lower yield. The compound of molecular formual $C_{13}H_{21}N_5O_4$ was assigned structure **15** because the nmr spectrum showed three widely shifted resonances for D_2O-exchangeable protons, one

of which, appearing as a triplet, was coupled with the *N*-methylene. The third product, homogeneous by tlc, could be converted into **15** by treatment with ethanolic ammonia and was assigned structure **16**. The nmr spectrum, which displayed magnetic equivalence of the pyrimidine C-2 and formyl protons, was consistent with this structure.

Adenosine. As expected, adenosine (**17**) was susceptible to the same type of ring-opening reaction as the model **10** (Scheme IV). After the reaction with DEP, the products were collected by evaporation of the aqueous solution. The dried residue was treated with ethanolic ammonia to remove any extraneous carbethoxylation of the hydroxyl groups by DEP. Although this treatment will hydrolyze *N*-formyl groups such as those of **11** and **16**, the chromatographic mobilities of the two main products were unchanged. Some very minor products, however, were destroyed by the ammonia treatment.

Separation of the two products was achieved by elution from a silica gel column. The compound obtained crystalline from the first fractions was assigned structure **18** on the basis of microanalysis for $C_{15}H_{23}N_5O_8$, the mass spectrum containing a molecular ion at *m/e* 401, and the nmr spectrum. The ultraviolet spectra of **18** (Figure 3) were closely analogous to those of **15** (Figure 2), confirming the structure assignment of the adenosine product. The compound that crystallized from later fractions was assigned structure **19** on the basis of microanalysis for $C_{12}H_{19}N_5O_6$ and the nmr spectrum. The ultraviolet spectra of **19** (Figure 3) were correspondingly very similar to those of **12** (Figure 2).

The first experiments with adenosine and DEP were designed on a preparative scale to establish the structures of the primary products and then to improve the reaction conditions so as to obtain good yields of 4,5-dicarbethoxyamino-6-*N*-ribofuranosylaminopyrimidine (**18**) and 4-amino-5-carbethoxyamino-6-*N*-ribofuranosylaminopyrimidine (**19**). The next experiments were designed to follow the reaction of adenosine with DEP under the conditions used in inhibition of ribonuclease activity. Under such conditions (dilute solution, pH 7), 50% of the total adenosine conversion realized took place in about 10 min at 23° and in about 8 min at 37°. Lowering the temperature to 0° increased by approximately tenfold the time required for 50% of the total adenosine conversion.

The chemical modification of adenosine as reported in this paper is of importance in structural studies of nucleic acids. The facts that the imidazole ring of adenosine is opened by DEP and that both monocarbethoxyamino (**19**) and dicarbethoxyamino (**18**) derivatives are produced suggest that any analytical reliance upon simple monocarbethoxylation of adenosine derivatives is unwarranted (Öberg, 1971). Moreover, the results indicate the potential usefulness of determining the products of reaction of DEP with bases such as adenine and with ribonucleosides such as adenosine in understanding and assessing the behavior of this reagent with polynucleotides and nucleic acids (Denić *et al.*, 1970; Solymosy *et al.*, 1971). It should be noted that in RNA sequence studies oligonucleotides have been prepared by cleavage with enzymes showing specificity to all bases except adenine. A chemical modification of this base which could be utilized for a specific cleavage of the phosphodiester bond involving adenine would therefore be very useful in sequence determination. The reaction of DEP leading to ring opening of the adenosine moiety suggests one approach to potentially selective attack on an RNA molecule, with resulting localized change in susceptibility to ribosyl cleavage in the modified structure.

Finally, a number of questions are raised by the inactivation of TMV RNA with this reagent. How does the modified adenine result in a lethal event? Is it transcribed at all or does the substitution result in a frame shift or in the termination of the transcription? These problems are being investigated further.

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