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Reaction of Diethyl Pyrocarbonate with Nucleic Acid Components, I. Adenine*

Nelson J. Leonard, Jerome J. McDonald, and M. E. Reichmann

DEPARTMENTS OF CHEMISTRY AND CHEMICAL ENGINEERING AND BOTANY, UNIVERSITY OF ILLINOIS, URBANA 61801

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Abstract. The use of diethyl pyrocarbonate as a nuclease inhibitor in the preparation of RNA of high molecular weight has prompted a study of the possible reactions of this compound with nucleic acid components under the conditions generally employed for providing inhibition. The first substrate investigated was adenine, which has been found to undergo ring opening with the formation of 5(4)-*N*-carbethoxyaminoimidazole-4(5)-*N'*-carbethoxycarboxamidine (II). This product was converted efficiently to isoguanine by treatment with ammonia. The structure of II was established by spectroscopy.

For comparisons of reactivity and of spectroscopic and chromatographic properties with the adenine–diethyl pyrocarbonate product, the compounds 9-carbethoxyadenine, 6-*N*-carbethoxyaminopurine (V), and 6-ethylaminopurine were made; compound V was made by employing the 1-ethoxyethyl protecting group in the synthetic sequence. Purine compounds can be converted to 9-(1-ethoxyethyl) derivatives simply by refluxing in acetal.

The facile reaction of adenine with diethyl pyrocarbonate illustrates the importance of gaining information as to the fate of various nucleic acid components in the presence of diethyl pyrocarbonate.

The successful preparation of high molecular weight, biologically active RNA depends on the rapid inactivation of nucleases in the early steps of the isolation. Conservation of biological activity upon prolonged storage also requires the total absence of nuclease activity in these preparations. Interest in specific nuclease inhibitors was often motivated by these considerations. Recently the inhibitory activity of diethyl pyrocarbonate (DEP) (ethoxyformic anhydride) was utilized in the isolation of high molecular weight RNA.[1,2] This bactericidal agent[3] was shown to convert primary and secondary amines into carbamic acid esters (urethans).[4] More recent investigations have demonstrated that amino acids and proteins reacted in a similar way and were converted into *N*-carbethoxy derivatives at either free α-amino groups or the ϵ-amino group of lysine.[5,6] In spite of these substitutions, it was concluded that irreversible inactivation of enzymatic activity was most probably due to conformational changes resulting in insolubility.[5] Subsequently, it was shown by others that the insolubility of proteins after reaction with DEP may be attributed to formation of intermolecular covalent bonds, possibly as a result of a much more complicated reaction involving both amino and carboxylic groups.[7,8] In addition to these findings it was also reported

that the imidazole ring of histidine, the guanidino group of arginine, the phenolic-hydroxyl group of tyrosine, and the sulfhydryl group of cysteine were carbethoxylated by DEP.[6,9,10] A detailed study of some of these reactions, using ^{14}C-labeled DEP, has been made.[11]

The reactivity of DEP with amines, and with the imidazole ring of histidine, raised the question of possible substitutions of the nucleotide bases accompanied by a loss of biological activity.[8] In this connection it was reported that the transforming activity of DNA remained unimpaired[12] and that the acceptor activity of $tRNA^{Ala}$ was unchanged[2] in preparations obtained in the presence of DEP. On the other hand, the infectivity of tobacco mosaic virus RNA solutions (1 mg/ml) was completely destroyed after a 1 min shaking with DEP (0.2 ml/ml).[13] Similar results have been obtained by others.[14] It is therefore possible that the lack of reactivity of DNA and tRNA might be attributed to their double-stranded nature. However, Fedorcsák and Ehrenberg reported that the susceptibility of single-stranded yeast RNA to pancreatic RNase was unaffected by treatment of RNA with DEP.[15] It must be recognized that such a finding indicates only that the pyrimidine ribonucleotides were essentially unchanged in the process. On the basis of the accumulated data, and in view of the increasing use of DEP as a nuclease inhibitor in the isolation of biologically active RNA, we have investigated the reaction of DEP with the natural purines and pyrimidines. The present communication describes the results with adenine.

Materials and Methods. 5(4)-Carbethoxyaminoimidazole-4(5)-*N'*-carbethoxycarboxamidine (II): To a solution of 10 g (74 mmol) of adenine in water, prepared by dissolving adenine in 1 liter of dilute HCl (0.1 N) and adjusting the pH to 4.5 with concentrated NaOH, was added 30 g (180 mmol) of DEP in portions with shaking at room temperature. After the generation of gas had ceased, the precipitated product was collected by filtration and was recrystallized as colorless, fluorescent needles from aqueous ethanol, mp > 320°C, yield 6.8 g (35%) of analytically pure material; nmr (DMSO-d_6, 60 Hz) τ 1.01 and 1.68 (br, 2, NH), 2.51 (s, 1, imidazole-H), 5.71 and 5.88 (overlapping qq, J = 7 Hz, 4, OCH_2), 8.20 and 8.76 (overlapping tt, J = 7 Hz, 6, CH_3); $\lambda_{max}^{95\%\ EtOH}$ 233 nm (ϵ 16,100), 296 (19,300); (0.1 N HCl) 300 (11,000); (0.1 N NaOH) 322 (23,000); mass spectrum (70 eV) m/e: 269 (M^+), 223 (M^+–EtOH), 177 (233^+–EtOH), 151 (223^+–$C_3H_4O_2$), 135 ($C_5H_3N_4O^+$), 134 ($C_5H_2N_4O^+$), 108 (151^+–CHNO). Metastable peaks were observed for the fragmentations: 269 → 223 (185), 223 → 177 (141), 223 → 151 (102), 151 → 108 (77). Anal. Calcd. for $C_{10}H_{15}N_5O_4$: C, 44.60; H, 5.62; N, 26.01. Found: C, 44.36; H, 5.40; N, 25.95.

9-(1-Ethoxyethyl)adenine (III): *Method A:* A suspension of 16 g (100 mmol) of 6-chloropurine in 300 ml of acetal (Aldrich) was heated at reflux for 5 hr, when complete solution had occurred and no starting material was observed by thin layer chromatography (TLC). The solvent was removed under reduced pressure. The residue (VI) was dissolved in 300 ml of absolute ethanol, the solution was cooled in an ice bath, then saturated with anhydrous ammonia, and heated for 3 hr in a sealed tube at 110°C. The solution was concentrated under reduced pressure; the solid residue was extracted with dry $CHCl_3$, and the $CHCl_3$ extracts were concentrated to dryness. The product was recrystallized from ethanol as colorless needles, mp 172–173°C, yield 15.4 g (74%); nmr ($CDCl_3$) τ 1.58 and 1.88 (ss, 2, purine H's), 3.15 (s, 2, NH_2), 3.97 (q, J = 6 Hz, 1, NCHO), about 6.5 (m, 2, OCH_2), 8.21 (d, J = 6 Hz, 3, $CHCH_3$), 8.82 (t, J = 7 Hz, 3, CH_2CH_3). Anal. Calcd. for $C_9H_{13}N_5O$: C, 52.16; H, 6.32; N, 33.80. Found: C, 52.15; H, 6.30; N, 33.76. *Method B:* A suspension of 0.20 g (1.5 mmol) of adenine in 25 ml of acetal (Eastman) was heated at reflux for 24 hr. After cooling, the solid (adenine) was removed by filtration and the filtrate was concentrated under reduced pressure

to a small volume and diluted with petroleum ether. Crystals of III separated on cooling. Recrystallization from absolute ethanol yielded 70 mg (22%) as colorless needles, mp 172–173°C.

6-*N*-carbethoxyamino-9-(1-ethoxyethyl)purine (IV): To a cooled suspension of 0.12 g (5 mmol) of hexane-washed NaH in 20 ml of dimethoxyethane, under nitrogen, was added dropwise a solution of 1.0 g (5 mmol) of 9-(1-ethoxyethyl)adenine (III) in dimethoxyethane. Stirring was continued for 30 min after complete addition. Then 4.7 g (40 mmol) of diethyl carbonate was added dropwise. Heating at 60°C for 30 min caused the formation of a precipitate, which dissolved with bubbling upon the addition of water at room temperature. After neutralization with 25 ml of 0.2 N HCl, concentration under reduced pressure at 30°C, extraction with ethyl acetate, and concentration, colorless needles crystallized from ethyl acetate–hexane, mp 146–148°C, yield 1.0 g (72%); nmr ($CDCl_3$) τ 0.99 (br, 1, NH), 1.18 and 1.70 (ss, 2, purine H's), 3.93 (q, 1, NCHO), 5.62 (q, 2, CH_2OOC), 6.5 (m, 2 CH_2OC), 8.21 (d, 3, $CHCH_3$), 8.63 and 8.83 (overlapping tt, 6, CH_2CH_3's); $\lambda_{max}^{95\%\ EtOH}$ 266 nm (ϵ 17,800); (0.1 N HCl) 272 (15,000); (0.1 N NaOH) 292 (28,400). Anal. Calcd. for $C_{12}H_{17}N_5O_3$: C, 51.60; H, 6.14; N, 25.08. Found: C, 51.59; H, 6.13; N, 25.10.

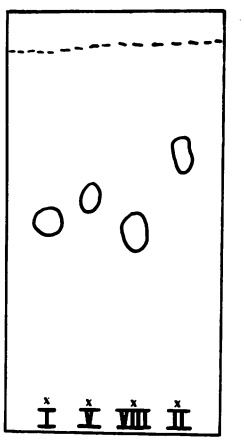


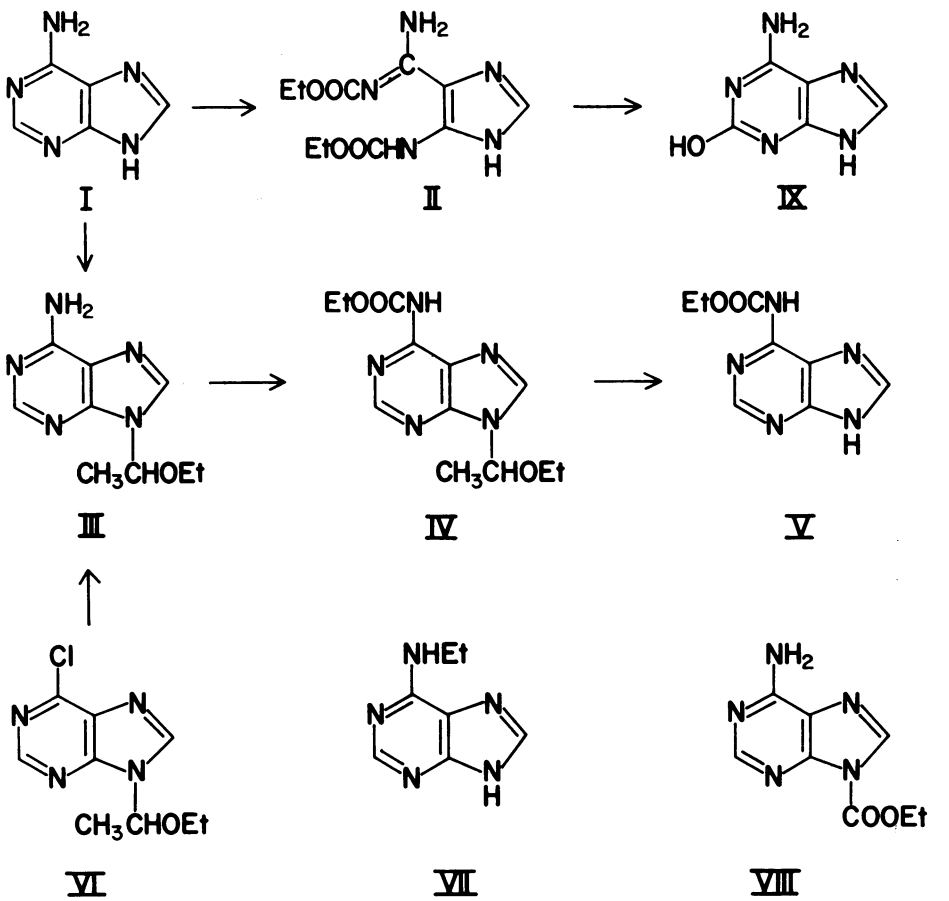
FIG. 1.—Thin-layer chromatography of carbethoxylated compounds on Silica Gel F-254 with indicator (E. Merck) in water-saturated butanol.

6-*N*-carbethoxyaminopurine (V): A solution of 0.8 g (2.9 mmol) of IV in 25 ml of 50%-aqueous acetic acid was stirred at room temperature for 1 hr to effect complete hydrolysis. Removal of the solvent under reduced pressure at 35°C and recrystallization of the residue from 95% ethanol yielded 0.59 g (98%) of colorless needles, mp 225°C (reported: 225°C[16]).

Treatment of 6-ethylaminopurine (VII) with DEP: The reaction conditions were similar to those employed with adenine. No precipitate formed during the reaction, and the solution was concentrated and extracted with ethyl acetate. Concentration of the ethyl acetate solution, fractionation on a silica gel column in 5% methanol in chloroform, and recrystallization of the first fractions from ethyl acetate–hexane gave colorless needles, mp 150–150.5°C; nmr ($CDCl_3$) τ −1.2 (br, 2, NH), −0.2 (br, 1, NH), 269 (s, 1, imidazole-H), 5.8 (overlapping qqq, 6, OCH_2's and NCH_2), 8.65, 8.66, and 8.69 (overlapping ttt, 9, CH_3's): $\lambda_{max}^{95\%\ EtOH}$ 237 nm (ϵ 10,300), 262 (11,200), 300 (15,700); (0.1 N HCl) 304 (5,600); (0.1 N NaOH) 246 sh (11,600), 324 (18,400); mass spectrum (70 eV) m/e: 297 (M^+), 251 (M^+–EtOH), 223 (251^+–C_2H_4), 208 (251^+–C_2H_5N), 205 (251^+–EtOH), metastable at 211 (297 → 251). Anal. Calcd. for $C_{12}H_{19}N_5O_4$: C, 48.47; H, 6.44; N, 23.56. Found: C, 48.69 H, 6.45; N, 23.75.

Conversion of II to isoguanine (IX): A solution of 1.0 g (3.7 mmol) of the adenine–DEP product in 75 ml of concentrated NH_4OH was heated in a sealed flask at 120°C for 3 hr. Removal of the solvent and two recrystallizations from water gave a $C_5H_5N_5O$ product, mp > 320°C, yield 0.39 g (71%), identical in UV (λ_{max} 283 in aq. HCl, 285 in aq. NaOH), mass spectrum, and two paper chromatographic systems with an authentic sample of isoguanine.

TLC of carbethoxylated adenines: Compounds I, II, V, and VIII were examined by TLC on Silica Gel F-254 with indicator (E. Merck) in water-saturated butanol. It was found that compound II, the dicarbethoxylated product from adenine, moved appreciably faster in the organic solvent than the monocarbethoxylated compounds (V and VIII) or adenine (I). As shown in Figure 1, the derivatives were chromatographically pure.



Results and Discussion. The reaction of adenine with DEP was first carried out under the conditions usually employed for the inactivation of enzymes, in dilute aqueous solution, buffered at pH 5 or 7. It was then found that the same product could be obtained in favorable yield using more concentrated aqueous solutions and a mole ratio of approximately 2.5:1 of DEP to adenine. The precipitated product, isolated by filtration after cessation of CO_2 evolution, was a colorless, blue-fluorescing solid, mp > 320°C, of molecular formula $C_{10}H_{15}N_5O_4$, as shown by microanalytical and mass spectral determinations. The elemental composition indicated the gain of two carbethoxyl groups and the loss of one carbon atom from adenine, by necessity either carbon-2 or carbon-8. The nmr spectrum confirmed the presence of two different ethoxyl groups, indicated one proton on a heteroaromatic ring at τ 2.51 (DMSO-d_6), suggestive of an imidazole 2-hydrogen, and detected two N-H's which could be exchanged in deuterium oxide. Structure II, 5(4)-carbethoxyaminoimidazole-4(5)-*N'*-carbethoxycarboxamidine (one tautomeric form is shown), was favored by the ultraviolet spectra, which showed diminished absorbance in acidic solution, consistent with protonation of the amidine moiety, and a shift of the maximum by approximately 25 nm to longer wave length in basic solution, consistent with extended conjugation in the anion.

Model compounds were made to assess their reactivity and compare their spectroscopic properties with those of the adenine-DEP product. 6-*N*-carbethoxyaminopurine (V), which had been prepared previously by a rather circuitous route,[16] was readily available through mediation by 1-ethoxyethyl (EOE) protection. 9-(1-Ethoxyethyl)adenine (III) (9-EOE-adenine) was obtained by heating a suspension of adenine in acetal at the reflux temperature. The compound was prepared in better yield by the reaction of 6-chloropurine with acetal to give 6-chloro-9-(1-ethoxyethyl)purine (VI) followed by reaction with ethanolic ammonia in a sealed tube. The ease with which the reaction of adenine, and especially 6-chloropurine, occurs with acetal makes the EOE group an easy one to attach.[17] Moreover, the EOE group possesses the advantage of making a more distinctive contribution to the nmr spectrum than does the tetrahydropyranyl group. It was possible to replace one of the N^6-hydrogens in III by a carbethoxyl group using NaH and diethyl carbonate in dimethoxyethane. The product, 6-*N*-carbethoxyamino-9-(1-ethoxyethyl)purine (IV), was hydrolyzed to 6-*N*-carbethoxyaminopurine (V) in 50%-aqueous acetic acid for an overall yield of 71% from III. The isomeric 9-carbethoxyadenine (VIII) was prepared from adenine by the method of Dyer, Reitz, and Farris.[18]

Neither of these carbethoxyadenines (V, VIII) reacted further with DEP under the conditions that converted adenine to the dicarbethoxylated product II. By contrast, 6-ethylaminopurine (VII) also reacted with DEP to yield a product, $C_{12}H_{19}N_5O_4$, with ultraviolet spectral properties similar to those of the $C_{10}H_{15}N_5O_4$ product obtained from adenine. The nmr spectra also showed similarities consistent with the formulation of the 6-ethylaminopurine-DEP product as 5(4)-*N*-carbethoxyaminoimidazole-4(5)-*N'*-carbethoxy-*N''*-ethylcarboxamidine. The observations of the lack of reaction of V and VIII, and the similarity of reaction of I and its N^6-ethyl derivative with DEP, are consistent with structure II for the adenine-DEP product. Also consistent with the assigned structure was the efficient conversion of the adenine–DEP product to isoguanine (IX) by treatment with ammonia at 120°C.

The reaction between DEP and adenine in aqueous solution is probably initiated by nucleophilic attack of *N*-1[19–23] or *N*-3[24–28] of the pyrimidine moiety on the pyrocarbonate carbonyl, with liberation of CO_2 and ethanol. A formal sequence of ring opening, removal of the C-2 by hydrolysis as formate,[21] and reaction of the freed amino group with a second molecule of DEP would account satisfactorily for the production of II. A diminishing of the nucleophilicity by the carbethoxyl group in V and VIII would explain why these compounds do not react with ethyl pyrocarbonate under the stated conditions, while the noninterference of an N^6-ethyl group is consistent with the pyrimidine ring as the locus of the initial attack.

Finally, the reactivity of adenine with DEP, as demonstrated in this preliminary study, suggests the possibility of modifications in biologically active nucleic acids. It would be desirable to exercise caution in the use of this reagent on the tacit assumption that the nucleic acid components are left intact. On the other hand, modifications effected by this reagent may be useful in the study of certain biological functions and specificities of the nucleic acids.

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Abbreviations: DEP, diethyl pyrocarbonate; EOE, 1-ethoxyethyl; TLC, thin-layer chromatography.

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