Reactions of Cytosine Derivatives with Acidic Buffer Solutions. II. Studies on Transamination, Deamination, and Deuterium Exchange*

Robert Shapiro and Robert S. Klein†

ABSTRACT: Cytidine reacts with certain aromatic amines in aqueous solution at pH 4 to give transamination to N_4-arylcytidines and deamination to uridine. The transamination products of cytidine with aniline, *o*-aminophenol, and β-naphthylamine have been isolated and characterized. Aniline reacts more slowly than *p*-aminophenol, and α-naphthylamine gives only trace amounts of transamination products. These results are discussed with reference to the known carcinogenic properties of aromatic amines. Studies have been continued on the previously reported deamination of cytidine by carboxylate buffers (Shapiro, R., and Klein, R. S. (1966), *Biochemistry 5*, 2358). The use of a 0.5 M solution of mellitic acid, titrated with pyridine to pH 5, is recommended as suitable for synthetic or mutagenic purposes. It has been noted that H_5 of cytidine exchanges for deuterium when cytidine is heated in D_2O in acidic citrate buffers. Deuterium exchange is more rapid than deamination. This is interpreted in terms of a common addition–elimination mechanism for both deuterium exchange and deamination.

In our previous paper (Shapiro and Klein, 1966), we reported that cytidine and cytosine are slowly deaminated to uridine and uracil, respectively, by hot carboxylate or pyridine buffers of pH less than 6. We suggested that this reaction was responsible for the mutations observed upon incubation of T_1 phage in carboxylate buffers (Freese, 1959; Strack *et al.*, 1964). Two possible mechanisms for the reaction were discussed. One involved a direct hydrolysis of the amino group of a protonated cytosine, while the other resembled the mechanism established for the reaction of the potent mutagen, hydroxylamine, with cytosine derivatives (Brown and Phillips, 1965). This involved the reversible addition of a nucleophile to the 5,6 double bond of protonated cytosine, prior to the replacement of the amino group.

We have now examined the reaction of cytidine with primary aromatic amine buffers. Both deamination and transamination reactions, similar to that with hydroxylamine, have been observed. The transamination reaction has been studied as a possible model for the biological action of carcinogenic aromatic amines. Studies have also continued on the deamination reaction in carboxylate buffers, to seek out conditions that maximize the reaction rate. Finally, we have observed that the C-5 hydrogen of cytidine is readily exchanged for deuterium in hot acidic D_2O buffers, and the characteristics of this reaction have been examined.

Experimental Section

Methods and Materials. The source of nucleosides, preparation of carboxylate buffers, instruments for determining ultraviolet spectra and pH, and procedures for paper and thin layer chromatography have already been described (Shapiro and Klein, 1966). Except where it is stated otherwise, thin layer chromatography was conducted on Avicel microcrystalline cellulose (American Viscose Co., Marcus Hook, Pa.) in the solvent 1-butanol–water (86:14). The R_F values in this system for the cytosine derivatives prepared are N_4-phenylcytosine (0.85), N_4-phenylcytidine (0.80), N_4-β-naphthylcytidine (0.78), and *o*-hydroxyphenylcytidine (0.57). The first three compounds appeared as fluorescent spots and the last one as a dark spot. Whatman Column Chromedia CF 11 was used for column chromatography on cellulose. The ultraviolet absorbance of the eluate at 265 mμ was monitored by a Gilson absorption meter and recorded using a Texas Instruments Co. rectilinear recorder. Infrared spectra were obtained with a Perkin-Elmer Infracord spectrophotometer. Only prominent bands in the areas 2.8–4.0, 5.5–6.5, and 12–15 μ are reported. Nuclear magnetic resonance spectra were determined using a Varian A-60 spectrophotometer and are reported on the τ scale with tetramethylsilane (τ 10.00) as standard. The solvent was hexadeuteriodimethyl sulfoxide. Microanalyses were obtained on an automatic CHN analyzer (F & M Scientific Co. Model 185). Melting points were mea-

* From the Department of Chemistry, New York University, New York, New York 10003. *Received July 20, 1967.* This research was supported by grants from the U. S. Public Health Service (GM-11437-03 and -04).

† National Institutes of Health Predoctoral Fellow (1965–1967).

sured using a Thomas-Hoover melting point apparatus and are uncorrected.

Preparation of N_4-Phenylcytosine (II, R = H; Ar = C_6H_5). A solution of 7.28 ml (80 mmoles) of aniline (distilled over zinc dust) titrated to pH 4.0 with HCl and 0.444 g (4 mmoles) of cytosine in 80 ml of H_2O was prepared. It was heated at 95° in a thermostat for 32 hr. The solution was cooled and the pH was adjusted to approximately 8 by addition of Na_3PO_4. The solution was shaken with 40 ml of benzene. The benzene layer, on separation, contained the product of reaction as a fine suspension. This was separated by centrifugation. The extraction process was repeated using the aqueous layer and a fresh 40-ml portion of benzene, to give more material. A further crop was obtained by cooling the aqueous layer. The crops were combined, recrystallized from ethanol–water, and dried under vacuum at 78° overnight to give 98 mg (13%) of N_4-phenylcytosine: mp 274–276°, lit. (Whitehead and Traverso, 1960) mp 266°. The ultraviolet spectra in acid and alkali also agreed with those reported. An analytical sample was prepared by a second recrystallization: infrared absorption (KBr) at 3–4 (br), 5.88, 6.10, 6.28, 6.40, 12.85, 13.35, and 14.40 μ.

Anal. Calcd for $C_{10}H_9N_3O$: C, 64.16; H, 4.85; N, 22.45. Found: C, 64.15; H, 5.07; N, 22.62.

Preparation of N_4-Phenylcytidine (II, R = β-D-ribofuranosyl; Ar = C_6H_5). A solution of 3.64 ml (40 mmoles) of aniline (distilled over zinc dust) titrated to pH 4.0 with HCl and 0.486 g of cytidine (2 mmoles) in 40 ml of water was prepared. It was heated at 95° in a thermostat for 72 hr. The solution was cooled, adjusted to pH 7.5 by addition of ammonium bicarbonate, and extracted three times with benzene. The aqueous layer was concentrated and worked up by partition chromatography on a cellulose column (5 × 40 cm, wet) using ethyl acetate saturated with water as the eluent. The first major peak to be eluted contained the product and gave no evaporation, 378 mg (56%) of N_4-phenylcytidine as a slightly yellowish solid. This was purified by recrystallization from ethyl acetate–CH_2Cl_2 (4:1) with the use of a small amount of charcoal to give a white solid which decomposed while melting when heated above 200°: infrared absorption (KBr) at 2.99, 6.09, 6.26, 6.39, 12.75, 13.30, and 14.50 μ; ultraviolet maximum (H_2O, pH 1) at 294 mμ (Σ 16,900), shoulder at 225 mμ, minimum at 249 mμ; maximum (H_2O, pH 7) at 292 mμ (Σ 18,500), shoulder at 235 mμ, minimum at 248 mμ; pK_a = 3.2 (spectrophotometric determination); nuclear magnetic resonance spectrum, τ 0.34, broad (NH); 1.76, doublet (H_6); 1.94–2.85, multiplet (5-phenyl CH); 3.73, doublet (H_5); 3.99 (H_1'); 4.78, broad (2′,3′,5′-OH); and 5.74–6.27 broad doublet (H_2O plus sugar CH). A sample was dried under vacuum at 65° for 18 hr for analysis.

Anal. Calcd for $C_{15}H_{17}N_3O_5 \cdot H_2O$: C, 53.41; H, 5.63; N, 12.46. Found: C, 53.60; H, 5.63; N, 12.31.

Preparation of N_4-o-Hydroxyphenylcytidine (II, R = β-D-ribofuranosyl; Ar = o-HOC_6H_4). A solution was prepared in 1-butanol–H_2O (86:14) containing 8.73 g (80 mmoles) of *o*-aminophenol (Fisher Scientific Co., purified by treatment with zinc dust in acidic solution), 0.972 g (4 mmoles) of cytidine, and sufficient HCl to bring the solution to pH 4. The total volume was 80 ml. The solution was heated at 95° for 73 hr in a thermostat, cooled, and evaporated to dryness under vacuum at room temperature. Ether and ammonium bicarbonate solution (pH 7.5) were added to the solids. The ether layer was removed and the aqueous layer was extracted with several further portions of ether. The aqueous solution was evaporated to dryness in the presence of Whatman Column Chromedia CF 11 cellulose to adsorb the solids, and the cellulose was packed onto a column (6 × 60 cm) of cellulose. This was eluted in the same manner as in the preparation of N_4-phenylcytidine, and the first major peak, on evaporation, yielded the product. This was purified by dissolving in ethanol and reprecipitating with ether to give 579 mg (41%) of N_4-*o*-hydroxyphenylcytidine as a white solid which melted with gas evolution over the range 160–185° when heated: infrared absorption at 2.99, 6.09, 6.14 (sh), 6.40 (sh), 12.80, and 13.30 μ; ultraviolet maximum (H_2O, pH 1) at 290 mμ (Σ 15,250), minimum at 247 mμ; maximum (H_2O, pH 6.6) at 285 mμ (Σ 13,900), shoulder at 236 mμ, minimum at 251 mμ; maxima (H_2O, pH 12) at 234 (Σ 15,000) and 276 mμ (Σ 11,700); shoulder at 325 mμ, minima at 225 and 256 mμ; pK_a = 3.4, 9.0 (spectrophotometric determination); nuclear magnetic resonance spectrum, τ 0.33, broad (NH); 1.81, doublet (H_6); 2.27, doublet (1-benzene CH); 2.95 (3-benzene CH); 3.68, doublet, (H_5); 4.05 (H_1'), 4.30–5.05, broad (2′,3′,5′-OH), 5.75–6.65, broad doublet (H_2O plus sugar CH); the phenolic hydroxyl was not observed. A sample was dried under vacuum at 78° for analysis.

Anal. Calcd for $C_{15}H_{17}N_3O_6 \cdot H_2O$: C, 50.99; H, 5.42; N, 11.89. Found: C, 51.10; H, 5.18; N, 11.88.

Kinetics of Reaction of Cytidine with Aniline and o-Aminophenol. A solution containing 0.486 g (2 mmoles) of cytidine and 2.18 g (20 mmoles) of *o*-aminophenol (purified as described above) in H_2O was titrated to pH 4.0 by addition of HCl. The total volume was 40 ml. A similar solution, in which the *o*-aminophenol was replaced by an equivalent amount of aniline, was also prepared, as were two controls, in which the cytidine was omitted. The reaction mixtures were heated at 95° in a thermostat, and 5-ml aliquots were withdrawn after 19, 39, and 63 hr. Each aliquot was neutralized with NH_4HCO_3 and extracted with five 10-ml portions of ether. The aqueous layer was readjusted to 5 ml, and a sample of 0.05 ml was withdrawn and worked up by paper chromatography in butanol–water (86:14). Spots corresponding to cytidine, uridine, and N_4-arylcytidine were cut out and eluted into 25 ml of water. Their ultraviolet spectra were taken, using the eluate from this corresponding areas of chromatography of the control reactions as blanks. The concentration of each substance was determined from the ultraviolet maximum. The ether washings were found by chromatography to contain no nucleoside products.

Kinetics of Deamination of N_4-Phenylcytidine and

N_4-o-Hydroxyphenylcytidine. Solutions containing 0.2 mmole of N_4-phenylcytidine or N_4-*o*-hydroxyphenylcytidine in 4 ml of 0.5 N sodium citrate buffer (pH 4.0) were heated in a thermostat at 95°. At intervals, aliquots of 0.3 ml were withdrawn. The N_4-arylcytidine and uridine were separated and their concentrations were determined by the paper chromatographic–ultraviolet spectrophotometric method described immediately above.

Preparation of N_4-β-Naphthylcytidine (II, R = β-D-ribofuranosyl; Ar = β-$C_{10}H_7$). The reaction was conducted in the same manner as that with *o*-aminophenol, but on one-eighth the scale and for 67.5 hr. The β-naphthylamine was recrystallized from methanol–water before use. After evaporation of the solvent from the reaction mixture, dilute ammonia was added. The suspension was shaken three times with benzene and three times with ether, and the organic layer was removed after each treatment. The aqueous suspension was heated, and the solids were dissolved. The solution was stored in the refrigerator, and the resulting precipitate was filtered to give 135 mg (70%) of N_4-β-naphthylcytidine as a white solid: infrared absorption (KBr) at 2.99, 6.09, 6.19, 6.28, and 6.45 (sh) μ, with a number of weak bands between 12 and 14 μ; ultraviolet maximum (H_2O, pH 1) at 288 mμ (Σ 27,700), minimum at 253 mμ; maxima (pH 7) at 281 (Σ 29,100) and 310 mμ (Σ 24,300), shoulder at 272 mμ; minima at 257 and 291 mμ; nuclear magnetic resonance spectrum, τ −0.23, broad (NH); 1.34, singlet (1-naphthalene CH); 1.72, doublet (H_6); 1.90–2.70, multiplet (6-naphthalene CH); 3.70, doublet (H_5); 3.92 (H_1'); 4.42–4.95, broad doublet (2′,3′,5′-OH), and 5.65–6.50, broad triplet (H_2O plus sugar CH). A sample was recrystallized from water and dried for several days under vacuum at room temperature. On heating, it sintered at 137° and melted with decomposition at 158–162°.

Anal. Calcd for $C_{19}H_{19}N_2O_5 \cdot 2H_2O$: C, 56.29; H, 5.72; N, 10.36. Found: C, 56.3; H, 5.31; N, 10.9.

Another analytical sample was dried under vacuum at 78° for 3 days.

Anal. Calcd for $C_{19}H_{19}N_3O_5$: C, 61.78; H, 5.18; N, 11.38. Found: C, 61.13; H, 5.00; N, 11.16.

Reaction of Cytidine with α-Naphthylamine. The conditions used with β-naphthylamine were employed, using α-naphthylamine purified by steam distillation. After 42.5 hr of heating, a 2-ml aliquot was removed and the solvent was evaporated. Dilute ammonia solution and benzene–hexane (1:1) were added. The organic layer was separated and two more extractions with benzene–hexane were performed. The organic and aqueous layers were examined by thin layer chromatography in butanol–water (86:14) on both cellulose and silica gel (Merck silica gel G). A β-naphthylamine–cytidine reaction was also run for comparison. In the α-naphthylamine reaction, the aqueous layer contained only cytidine, uridine, and a faint spot of the same R_F as N_4-β-naphthylcytidine. This was best detected by periodate–benzidine spray (Cifonelli and Smith, 1954) on the silica gel plate. The organic layer contained no nucleoside product. A portion of both reactions was worked up by preparative paper chromatography and the proportions of products were determined by the ultraviolet spectrophotometric method. The presumed N_4-α-naphthylcytidine showed a maximum (H_2O, pH 1.5) at 284 mμ, and a maximum (H_2O, pH 9) at 287 mμ.

Kinetics of Deamination of Cytidine by Carboxylate Buffers. The general methods described in our previous paper (Shapiro and Klein, 1966) were employed. The direct spectrophotometric method was used when the buffers did not absorb appreciably in the ultraviolet region. When the buffers had absorption, cytidine and uridine were first separated by paper or thin layer chromatography in 1-butanol–water (86:14). In those reactions involving pyridine or imidazole, it was found advisible to neutralize each aliquot with ammonia or NH_4HCO_3 before the separation. In the 3,5-dimethylpyrazole reaction, this compound was extracted with ether from the neutralized solution before chromatography. In a control reaction which was run with guanosine in pyridinium mellitate buffer, it was necessary to perform thin layer chromatography in two directions to resolve all possible products. The plate was run first in 1-butanol–water (86:14), and then at right angles in isobutyric acid–ammonia–water (66:4.7:29.3).

Exchange of H_5 of Cytidine in Citrate Buffer in D_2O. The citric acid to be used was first dissolved in D_2O (99.8%, Bio-Rad Laboratory, Richmond, Calif.) and the solution was evaporated to dryness. The cytidine to be used was separately dissolved in D_2O, and the D_2O was distilled as an azeotrope with benzene. This was done to exchange hydrogen bound to oxygen or nitrogen for deuterium. A solution, 1 M in cytidine and of the molarity desired in citric acid, was prepared in 5 ml of D_2O and the pD was adjusted with DCl or NaOD to the desired value. This was measured using a pH meter and adding 0.4 to the observed value (Glasoe and Long, 1960). The reaction mixture was heated in a thermostat at 95° and aliquots of 0.6 ml were withdrawn at intervals. These were cooled, acidified with DCl to pD 1.5, and examined on a Varian A-60 nuclear magnetic resonance machine, using an expanded scale to scan the region from τ 0.8 to 5. The positions of the peaks observed were: cytidine H_6, τ 1.76; uridine H_6, τ 2.05; cytidine H_5, τ 3.54; and uridine H_5 + H_1' of cytidine and uridine, τ 4.10. The amount of exchange of cytidine was calculated from the intensities of the H_5 and H_6 peaks. The average of five integrations of each peak was used for this purpose. As the exchange proceeded, the doublet peak of H_6 of cytidine disappeared and was replaced by a singlet.

Results

Cytidine (I, R = β-D-ribofuranosyl) and cytosine (I, R = H) are deaminated when heated at 95° in aqueous carboxylate or pyridine buffers of pH <6 (Shapiro and Klein, 1966). The use of primary aromatic amine buffers resulted in transamination to N_4-arylcytosine

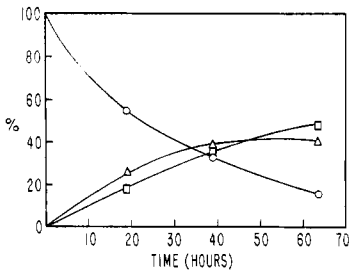


FIGURE 1: Plot of per cent of cytidine (○), uridine (□), and N_4-phenylcytidine (△) *vs.* time in the reaction of cytidine (0.05 M) with 0.5 M aniline hydrochloride buffer (pH 4.0, 95°).

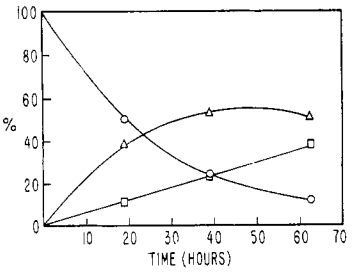
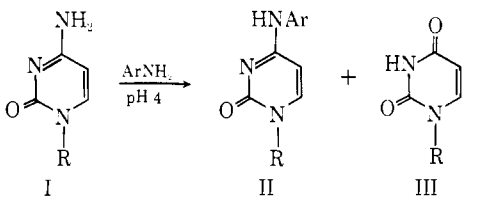


FIGURE 2: Plot of per cent of cytidine (○), uridine (□), and N_4-*o*-hydroxyphenylcytidine (△) *vs.* time in the reaction of cytidine (0.05 M) with 0.5 M *o*-aminophenol hydrochloride buffer (pH 4.0, 95°).

derivatives, as well as deamination. The properties of the transamination product of cytosine and aniline, N_4-phenylcytosine (II, R = H; Ar = C_6H_5) agreed with those reported in the literature (Whitehead and Traverso, 1960). New compounds were prepared by reaction of cytidine with buffered solutions of aniline,



o-aminophenol, and β-naphthylamine. The analyses and spectral properties of these compounds indicated N_4-arylcytidine structures for them (II, R = β-D-ribofuranosyl; Ar = C_6H_5, *o*-HOC_6H_4, and β-$C_{10}H_7$). Owing to the limited solubility of β-naphthylamine and its salts in aqueous solution (pH 4), it was desirable for preparative purposes to run the reaction with cytidine in a mixed organic–aqueous solvent. When the reaction was run in aqueous solution (pH 4) with a much lower concentration of β-naphthylamine, the formation of transamination product could still be demonstrated chromatographically, but the yield was less. Surprisingly, the transamination reaction failed with α-naphthylamine. Under conditions where cytidine reacted with β-naphthylamine to give a mixture containing 53% of product II, 16% of uridine (III, R = β-D-ribofuranosyl), and 31% of unreacted cytidine, α-naphthylamine yielded less than 2% of transamination product, 35% of uridine, and 63% of unreacted cytidine. The failure of the α-naphthylamine reaction may be due to a steric effect, but additional studies will be necessary to establish this point. Another amine that failed to give the transmination reaction was the mutagen, 9-aminoacridine. The same steric factors would presumably be at work here and, additionally, this compound (pK_a = 10) would exist entirely in a less nucleophilic protonated form in acidic solution. Imidazole buffer (at pH 5.5) and 2,4-dimethylpyrazole buffer (at pH 3.5) also gave no transamination products with cytidine, nor any unusual rate of deamination.

A study was made of the course of the reaction of cytidine with 0.5 M aniline and *o*-aminophenol buffers at pH 4, 95°. The results are summarized in Figures 1 and 2. It can be seen that the transamination reaction for both amines proceeds more rapidly than deamination. However, as the transamination product (II) itself is slowly hydrolyzed to uridine (see below), the latter eventually becomes the major product. The curves also indicate that the transamination reaction proceeds more rapidly with *o*-aminophenol than with aniline, and *o*-aminophenol gives a higher maximum yield of arylcytidine. A plot of ln (100/% cytidine) gave a straight line for both reactions, indicating that the disappearance of cytidine is first order in cytidine. The rate constants (presumably the sum of first-order constants for transamination and deamination) are 3.5×10^{-2}/hr for the *o*-aminophenol reaction and 2.9×10^{-2}/hr for that with aniline. The rate constant for deamination of cytidine by 0.5 M citrate buffer (pH 4.0, 95°) is 2.1×10^{-2}/hr. The hydrolyses to uridine of N_4-phenylcytidine and N_4-*o*-hydroxyphenylcytidine were studied separately, by heating these compounds separately in 0.5 M citrate buffer (pH 4.0). The hydrolyses were found to be first order in arylcytidine, with rate constants of 1.6×10^{-2}/hr for N_4-*o*-hydroxyphenylcytidine and 1.0×10^{-2}/hr for N_4-phenylcytidine. The order of ease of hydrolysis to uridine is thus cytidine (pK_a = 4.2) > *o*-hydroxyphenylcytidine (pK_a = 3.4) > phenylcytidine (pK_a = 3.2). This agrees with the hypothesis that it is the protonated form of the molecule that reacts, though other factors are undoubtedly also influencing the rates.

Additional studies were made on the deamination reaction in carboxylate buffers. These were done in order to define the conditions most suitable for the mutagenic or synthetic use of the reaction. In our earlier paper (Shapiro and Klein, 1964), we reported that polycarboxylate buffers were more suitable than monocarboxylate ones, and that increasing buffer concentration increased the rate. We have found now that 0.5 M mellitic acid (benzenehexacarboxylic acid) is

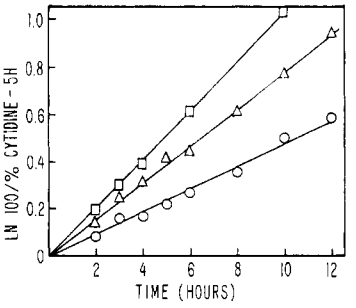


FIGURE 3: Plot of first-order kinetics for the exchange of H-5 of cytidine for D in 0.5 M citric acid–sodium citrate buffers in D_2O 95°, at pD 3.4 (○), 4.4 (□), and 5.4 (△).

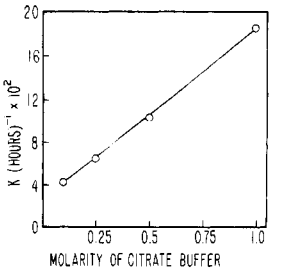


FIGURE 4: The variation of the first-order rate constants for the exchange of H-5 of cytidine for D with buffer concentration, citric acid–sodium citrate buffers, D_2O, pD 4.4, 95°.

superior to 2.0 M citric acid, the best buffer previously reported. Solubility considerations defined the maximal concentration of buffer used in each case. The rate constants determined for several citrate and mellitate buffers are summarized in Table I. It was thought that it would be most useful for mutagenic purposes to keep the pH as close to neutral as possible, as side reactions giving lethal results might be favored by acid. The deamination rates in both buffers fell off considerably when the pH was raised from 4 to 5. This drop could be largely made up, however, by replacing the alkali metal used in the buffer by pyridinium ion. Pyridine buffers have a deaminating effect of their own at pH 5, and enhance the total rate of deamination. Thus, a buffer composed of 0.5 M mellitic acid, titrated with pyridine to pH 5 (3.6 M in pyridine), appears to be the most suitable one encountered thus far for the specific deamination of cytidine. Under conditions (19.5 hr, 95°) which converted 58% of cytidine to uridine, adenosine and guanosine showed no deamination and only a slight amount of cleavage of the *N*-glycosidic bond.

Two possible mechanisms for the deamination reaction were discussed in our earlier paper (Shapiro and Klein, 1966). The simpler one involved a direct nucleophilic attack by water at C-4 of protonated cytidine. The other postulated the addition of buffer anion, initially, to the 5-6 double bond of protonated cytidine to give a 5,6-dihydrocytidine intermediate. This deaminated, and then eliminated the buffer anion to give uridine. This mechanism implied that if the deamination reaction were conducted in D_2O, the deamination would be accompanied by exchange of H-5 for deuterium. This reaction was conducted in citrate buffers and the exchange was followed by nuclear magnetic resonance spectrophotometry. It was found that exchange was more rapid than deamination and, except in the study at pD 2, was essentially complete before appreciable deamination had occurred. The deuterium-exchange reaction was found to be first order in cytidine. Typical plots of data, showing the kinetics of exchange in 0.5 M citrate buffers of differing pD, are given in Figure 3. The variation of the rate constant for exchange with citrate buffer concentration at pD 4.4 is shown in Figure 4. As was the case with deamination, the rate increases with increasing concentration of the buffer anion. The variation of the rate constant for exchange with pD, in 0.5 M citrate buffers, is summarized in Figure 5. A rather sharp maximum at about pD 4.6 is indicated. The deamination reaction had previously been shown to have a maximum at pH 3.5, but to fall off less rapidly with increasing acidity (Shapiro and Klein, 1966). The rate constants for exchange and deamination are not comparable, as the

TABLE I: Deamination Rates for Cytidine.[a]

Buffer (M)	pH 4.0	pH 5.0
Citrate (0.5) (sodium)	2.1	0.84
Citrate (2.0) (sodium)	4.4	
Citrate (2.0) (pyridinium)	3.9	
Mellitate (0.5) (lithium)	5.0	2.0
Mellitate (0.5) (pyridinium)		4.6

[a] First-order rate constants in $hr^{-1} \times 10^2$ for carboxylate buffers, 95°, 0.05 M cytidine.

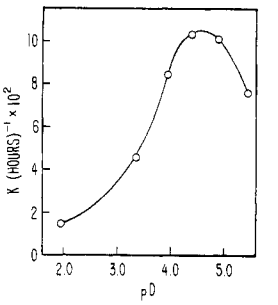


FIGURE 5: The variation of the first-order rate constants for the exchange of H-5 of cytidine for D with pD, 0.5 M citric acid–sodium citrate buffers, D_2O, 95°.

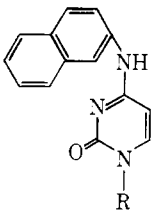
former was conducted in D_2O, and the latter in H_2O. To obtain a point of comparison, deamination was studied by the ultraviolet spectrophotometric method at 95°, pD 4.4, 0.5 M citrate. The deamination rate constant was found to be 1.1×10^{-2}/hr as compared to 10.3×10^{-2}/hr for exchange. As the deamination rate in H_2O under these conditions had previously been found to be 1.6×10^{-2}/hr, the solvent isotope effect, K_{H_2O}/K_{D_2O} for deamination was thus 1.5.

Discussion

The deamination reaction in acidic buffers appears to be one example of a general reaction of cytosine derivatives with nucleophiles. Thus reactions also occur with hydroxylamine (Brown and Phillips, 1965), semicarbazide (Hayatsu *et al.*, 1966), and Girard-P reagent (Kikugawa *et al.*, 1967) in mildly acidic solution, and these substances have proved useful as mutagens and as reagents for the modification of nucleic acids. The ultimate products of the reactions have been the transaminated ones, N_4-substituted cytosine derivatives. We have now found that when cytidine reacts with certain acidic primary aromatic amine buffers, both deamination and transamination occur, with the transamination reaction being the predominant initial one (see Figures 1 and 2). The formation of a transamination product from the reaction of cytosine with aniline hydrochloride at 165° had previously been reported (Whitehead and Traverso, 1960) as had a reaction of deoxycytidine and *n*-butylamine in a sealed tube at 105° (Miller and Fox, 1964).

The prominent carcinogenic properties of a number of aromatic amines have long been recognized. Oxidation products of the amines, rather than the amines themselves, have been implicated as the actual carcinogens, and hydroxylamino and *o*-hydroxy metabolites of the aromatic amines have been suggested for this role (Clayson, 1962). In recent years, the hypothesis has been put forward that the reaction of a chemical carcinogen with a nucleic acid is important in the induction of cancer by these compounds, and the reactions of carcinogens with nucleic acids and their components have been explored (Magee and Farber, 1962; Troll *et al.*, 1963). It has been demonstrated that the carcinogen *N*-acetoxy-2-acetamidofluorene, a possible aromatic amine metabolite, reacts readily with guanosine and with the guanine residues of nucleic acids (Kriek *et al.*, 1967). It has been pointed out, however, that more than one metabolite may be involved in aromatic amine carcinogenesis, and that *o*-aminophenols cannot be excluded as possible carcinogens (Boyland and Manson, 1966). Treatment of DNA with 2-amino-1-naphthol has been shown to have a profound effect on its thermal stability and on its priming activity with RNA polymerase (Troll *et al.*, 1963; Belman *et al.*, 1964). The transamination reaction is thus another possible model for the reaction of a carcinogenic amine with a nucleic acid. It is noteworthy in this respect that the introduction of an *o*-hydroxy group in aniline promotes the rate of its transamination reaction with cytidine. It is further of interest, and somewhat surprising, that the potent carcinogen β-naphthylamine forms the transamination product (II, R = β-D-ribofuranosyl; Ar = β-naphthyl) in 53% yield under conditions in which the much less carcinogenic α-naphthylamine reacts to less than 2%. It must be pointed out that the conditions selected by us for preparation of the transamination product in significant quantities (pH 4, 95°) are far from physiological ones. However, the degree of modification of a nucleic acid needed for a physiological effect may be quite small. Treatment of TMV-RNA with aniline (pH 5 and 25°) causes a limited loss of infectivity which has been attributed to a very slow random reaction with a base, perhaps cytosine (Steinschneider and Fraenkel-Conrat, 1966). The rate of reaction of our amines with cytidine is considerably slower than the rate observed with semicarbazide (Hayatsu *et al.*, 1966). Further studies are needed to determine whether suitably substituted aromatic amines will show an enhanced rate of reaction, and whether this correlates with their carcinogenic activity.

We have examined a model of a DNA segment to assess the effect on it of the replacement of a cytosine amino group by a β-naphthylamino residue. The conformation of the amino group appears to be a key factor. If the amino group is oriented as in II, then normal hydrogen bonding by the Watson–Crick scheme can take place, and the naphthalene ring remains on the outside of the double helix. Another conformation, as shown in IV, can be produced by a rotation of the amino group of 180°, however. The naphthalene

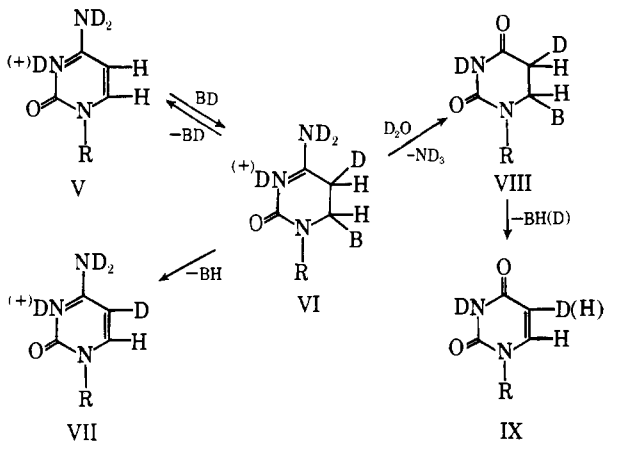


IV

moiety then fits quite well into the assembly of stacked bases of DNA. The guanine residue opposite the modified cytosine is displaced. Its hydrogen bonds are broken, but it retains π-electron interactions with one neighboring base and with the naphthalene moiety. Intercalation mechanisms have received prominent attention with respect to the method of interaction of acridine mutagens (Lerman, 1964; Pritchard *et al.*, 1966) and of polycyclic aromatic hydrocarbons (Boyland and Green, 1962; Liquori *et al.*, 1962) with DNA. The bonding discussed here can be considered a covalent intercalation, in which the intercalating agent is attached to a suitable position on the DNA molecule by a covalent bond.

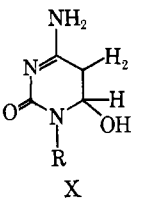
In continuing our studies on the deamination of cytidine in carboxylate buffers, we have tried to define conditions as close to neutrality as possible, that would still give an adequate rate of deamination. This would minimize side reactions in the synthetic or mutagenic

SCHEME I



use of the reaction. Our best present conditions are the use of a 0.5 M solution of mellitic acid (benzenehexacarboxylic acid), titrated with pyridine to pH 5. Both the cationic and anionic species are effective buffers at that pH, and both participate in the deamination reaction.

The discovery that the 5-hydrogen of cytidine is readily exchanged for deuterium in 0.5 M citrate buffer lends further support to the addition–elimination mechanism for the deamination reaction. Both the deamination and deuterium-exchange reactions in D_2O can be accommodated by Scheme I, where R = β-D-ribofuranosyl and B = the buffer anion. The cytosine cation (V) is attacked by the buffer anion, with subsequent addition of a deuteron to C-5, to give intermediate VI. This can eliminate the buffer anion and revert to V or to the cation of cytidine-D_5 (VII). Cytidine-D_5 can of course also undergo addition and subsequent deamination. The intermediate VI is also attacked by D_2O with loss of ND_3 to give VIII, which subsequently loses the buffer anion and hydrogen or deuterium to give uridine (IX). This reaction pathway is slower than the formation of VII. Both exchange and deamination reactions show a maximum in 0.5 M citrate in the vicinity of pH or pD 4. Since the rate of exchange diminishes more rapidly with increasing acid concentration, it can be predicted that the ratio of the rate of exchange to deamination would be greater at pD 4 than at pD 1–2. This is reminiscent of the situation described by Johns and co-workers (Johns *et al.*, 1965). They prepared, by a photochemical reaction, the hydrate of cytidine 3′-phosphate (X, R = β-D-ribofuranosyl 3′-phosphate) and measured the



rates of its deamination to uridine 3′-phosphate and dehydration to cytidine 3′-phosphate. The rate of dehydration here showed a maximum at about pH 5, and the ratio of dehydration to deamination was greater at pH 4 or 5 than at more acidic pH.

Acknowledgments

We wish to thank Drs. William J. Wechter and Robert C. Kelly of the Upjohn Co., Kalamazoo, Mich., for stimulating discussions on the mechanisms of deamination and exchange. The nuclear magnetic resonance spectra of N_4-arylcytidines were determined by Mr. Robert Servis, and the infrared spectra by Mr. Frank Litterio.

References

Belman S., Huang, T., Levine, E., and Troll, W. (1964), *Biochem. Biophys. Res. Commun. 14*, 463.

Boyland E., and Green, B. (1962), *Brit. J. Cancer 16*, 347.

Boyland, E., and Manson, D. (1966), *Biochem. J. 101*, 84.

Brown, D. M., and Phillips, J. H. (1965), *J. Mol. Biol. 11*, 663.

Cifonelli, J., and Smith, F. (1954), *Anal. Chem. 26*, 1132.

Clayson, D. B. (1962), Chemical Carcinogenesis, Boston, Mass., Little, Brown, Chapter 9.

Freese, E. (1959), *Brookhaven Symp. Biol. 12*, 63.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Hayatsu, H., Takeishi, K., and Ukita, T. (1966), *Biochim. Biophys. Acta 123*, 445.

Johns, H. E., Le Blanc, J. C., and Freeman, K. B. (1965), *J. Mol. Biol. 13*, 849.

Kikugawa, K., Hayatsu, H., and Ukita, T. (1967), *Biochim. Biophys. Acta 134*, 221.

Kriek, E., Miller, J. A., Juhl, U., and Miller, E. C. (1967), *Biochemistry 6*, 177.

Lerman, L. S. (1964), *J. Cellular Comp. Physiol. 64, Suppl. 1*, 1.

Liquori, A. M., DeLerma, B., Ascoli, F., Botre, C., and Trasciatti, M. (1962), *J. Mol. Biol. 5*, 521.

Magee, P. N., and Farber, E. (1962), *Biochem. J. 83*, 114.

Miller, N., and Fox, J. J. (1964), *J. Org. Chem. 29*, 1772.

Pritchard, N. J., Blake, A., and Peacocke, A. R. (1966), *Nature 212*, 1360.

Shapiro, R., and Klein, R. S. (1966), *Biochemistry 5*, 2358.

Steinschneider, A., and Fraenkel-Conrat, H. (1966), *Biochemistry 5*, 2735.

Strack, H. B., Freese, E. B., and Freese, E. (1964), *Mutation Res. 1*, 10.

Troll, W., Belman, S., and Levine, E. (1963), *Cancer Res. 23*, 841.

Whitehead, C. W., and Traverso, J. J. (1960), *J. Am. Chem. Soc. 82*, 3971.