The Deamination of Cytidine and Cytosine by Acidic Buffer Solutions. Mutagenic Implications*

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ABSTRACT: Cytidine and cytosine are deaminated at 95° by a variety of aqueous buffers of pH <6.0. Adenosine and guanosine are not deaminated under these conditions.

The rate of the reaction with cytidine has been studied and appears to be dependent upon the concentration of buffer anion and of protonated cytidine. In 0.5 M sodium citrate buffer, a rate maximum occurs at about pH 3.5. Two alternative mechanisms for the reaction are discussed. One involves direct hydrolysis of protonated cytidine, while the other invokes an intermediate addition to the 5,6 double bond of cytidine. It is suggested that deamination of cytosine, rather than depurination, is responsible for the known mutagenic effect on bacteriophage of warm, acidic carboxylate buffers.

Nitrous acid, a potent chemical mutagen, exerts its effect by the deamination of the amino groups of the adenine, cytosine, and guanine residues of the nucleic acids (Schuster and Schramm, 1958). The fact that three bases are attacked has made it difficult, however, to correlate a mutational event with a particular chemical change. We have been studying alternative chemical methods of deamination of the nucleic acid components in order to develop potential mutagenic agents of greater specificity. It is known that adenine, cytosine, and guanine are deaminated during the hydrolysis of nucleic acids under strongly acidic conditions (Jordan, 1960). We have now found that cytidine and cytosine can be deaminated, in acidic buffer solutions of pH up to 5.0, under conditions which do not deaminate adenosine or guanosine. In order to find the optimal conditions for this reaction, and to obtain information about the mechanism, we have studied the dependence of the rate of deamination upon the pH, and the type and concentration of the buffer.

Experimental Section

Methods and Materials. Bases and nucleosides were purchased from Schwarz Bioresearch, Inc., Orangeburg, N. Y., and were found to be chromatographically homogeneous in the solvent systems described below. Buffer solutions were prepared by dissolving the appropriate amount of buffer acid in water and adjusting the solution to the desired pH (as measured on a Beckman Zeromatic pH meter) by adding sodium or potassium hydroxide. Reactions were run by immersing the solutions in a thermostat or heating bath at 95 or 97°. All reactions were run with an initial cytidine concentration of 0.05 M. At the conclusion of each reaction, the pH was rechecked and was found to have changed by <0.1 unit. Ultraviolet absorbances were measured, for kinetic purposes, on a Beckman DU spectrophotometer. Ultraviolet spectra were obtained on a Perkin-Elmer 202 spectrophotometer.

Paper chromatography was carried out by the descending technique on Whatman No. 40 paper. Thin layer chromatography was carried out on 1-mm thick layers of Avicel microcrystalline cellulose (American Viscose Co., Marcus Hook, Pa.). After development of the chromatograms, ultraviolet-absorbing materials were located with the aid of an ultraviolet lamp equipped with a short wavelength filter. The solvent systems employed were: I, 1-butanol–water (86:14); II, isobutyric acid–ammonia–water (66:4.7:29.3); III, 2-propanol–ammonia–water (7:1:2); IV, saturated aqueous ammonium bicarbonate; V, methanol–HCl–water (7:1:2). The R_F values, for thin layer chromatography, are given in Table I.

KINETIC DETERMINATIONS. In those cases where the buffer absorbed appreciably at wavelengths >255 mμ

TABLE I: R_F Values.

Compd	Solvent I	II	III	IV	V
Cytosine	0.22	0.79	0.67		
Uracil	0.36	0.66	0.59	0.73	
Cytidine	0.16	0.78	0.56		0.46
Uridine	0.25	0.65	0.61		0.61
Adenosine		0.76	0.55	0.55	
Guanosine		0.61	0.32	0.46	

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in the ultraviolet (mercapto compounds and pyridine) an aliquot of the reaction mixture was withdrawn, streaked on Whatman 40 paper, and run in solvent system I. It was sometimes necessary to rerun the paper in the same solvent system to separate cytidine and uridine from each other and from the ultraviolet absorbing materials of the buffer. A blank paper, containing buffer only, was also run. The spots representing cytidine and uridine, or cytosine and uracil, were cut out and eluted into water. The relative amount of each substance was determined from its ultraviolet absorbance at its maximum at pH 1. The 250/260 and 280/260 ratios were also obtained routinely as a check of the identity of each substance. The absorbances were read *vs.* a suitable blank cut out from the paper that contained buffer only.

When the buffer did not absorb appreciably in the ultraviolet, a direct spectrophotometric method was used. The details of this method have been described (Loring, 1955). The wavelengths utilized by us were 260 and 280 mμ at pH 7. The formulas used to compute the relative amounts of cytidine and uridine were as follows

$$\text{cytidine} = (2.83A_{280} - A_{260})/12.6$$

$$\text{uridine} = (A_{260} - 1.08A_{280})/6.10$$

In the above formulas, A_{260} and A_{280} represent the measured absorbance of the solution at 260 and 280 mμ, respectively. For this method to be valid it is necessary that the cytidine is converted only to uridine and that no other ultraviolet-absorbing materials be produced. To check this, the final reaction mixture in each run was examined by paper chromatography in solvent I. No substances absorbing appreciably in the ultraviolet, other than cytidine or uridine, were observed. In one case, the absorption at the isosbestic point of cytidine and uridine was followed during the reaction and was found not to change, within experimental error.

In general, the direct method was more convenient and seemed more precise. The data were plotted as ln of 100/% cytidine *vs.* time and straight lines were readily fitted through the points so obtained. The pseudo-first-order rate constants were obtained from the slope. In one instance, a duplicate determination of the rate constant was run and the values obtained agreed to within 1% of each other. When the paper chromatography method was used, rate constants were generally not determined, but only the per cent of cytidine hydrolyzed in a given amount of time. The numerical values so obtained on duplicate runs usually fell within 3 units of each other and of the value derived by the first method.

Results

The product formed by the reaction of cytosine and mercaptoacetate buffer was isolated by paper chromatography in solvent system I. It was identified as uracil by the identity of its R_F in solvent systems I–V, and of its ultraviolet spectra at pH 4, 11, and 14, with authentic uracil. In other reactions, the identity was routinely confirmed by its R_F in solvent I and its 250/260 and 280/260 ratios in the ultraviolet at pH 1. The product formed by reaction of cytidine with citrate buffers was identical with uridine in its R_F in solvent systems I–IV. In one reaction, the final mixture was adsorbed onto Norit A charcoal, and eluted with aqueous ethanolic ammonia, to remove salts. The resulting solution was evaporated to dryness, dissolved in water, and passed through a short column of Amberlite CG 120 resin, H^+ form. Unreacted cytidine was retained on the column. The aqueous eluate from the column was evaporated and crystallized from isopropyl alcohol–petroleum ether (bp 30–60°) to give a product which was identical with authentic uridine in its infrared and ultraviolet spectrum and melting point. A mixture melting point with authentic uridine was not depressed.

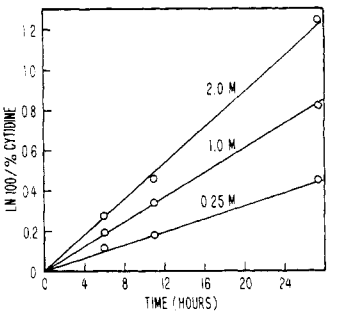


FIGURE 1: Plot of first-order kinetics for the deamination of cytidine in citric acid–sodium citrate buffers, pH 4.0, 95°.

Kinetic studies were run for the deamination of cytidine to uridine. The reactions in all cases were found to be first order in cytidine. Typical plots, showing the kinetics of deamination of cytidine in citrate buffers of various strength at pH 4.0, are shown in Figure 1.

A comparison was first made of the effectiveness of various 0.5 M carboxylate buffers at pH 4.0, 97°. The rate constants ($\times 10^{-2}$/hr) for these are as follows: acetate, 1.3; oxalate, 2.8; lactate, 1.3; succinate, 2.0; formate, 1.4; citrate, 3.4. The variation of the rate of deamination of cytidine with the concentration of a particular buffer, citrate, at 95°, pH 4.0, was then studied. The rate constants determined are plotted *vs.* concentration in Figure 2. The increase in rate over the concentration range is roughly proportional to the increase in buffer concentration over the concentration range 0.1–2.0 M. An extrapolation to a hypothetical unbuffered solution maintained at pH 4.0 suggests that there would still be deamination of cytidine under those conditions. In another experiment, the per cent of deamination of cytidine in various concentrations of acetate buffer at pH 4.5, 97°, over 21 hr was measured. The amount of reaction again was found to increase with buffer concentration, being 10% in 0.5 M acetate,

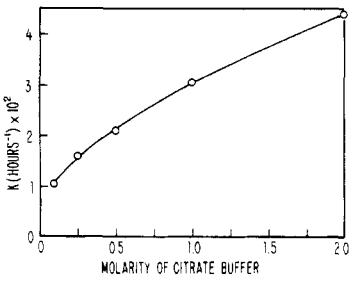


FIGURE 2: The variation of the rate of deamination of cytidine in citric acid–sodium citrate buffer with buffer concentration, pH 4.0, 95°.

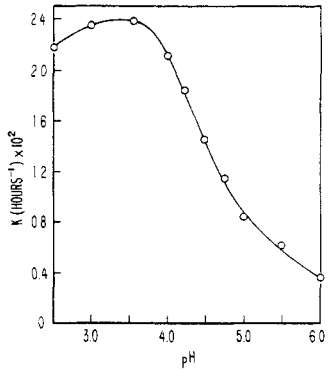


FIGURE 3: The variation of the rate of deamination of cytidine in 0.5 M citric acid–sodium citrate buffer with pH at 95°.

14.5% in 1.0 M acetate, and 24% in 2.0 M acetate. No attempt was made to maintain constant ionic strength during these runs. However, one run was conducted with 0.5 M citrate, at pH 4.0 and 95°, with added 1 M sodium chloride. The rate was found to be 13% slower than in the absence of the sodium chloride. This indicates a small negative salt effect. The increase in the rate of deamination with increasing concentration of citrate buffer would thus be slightly larger than that shown in Figure 2, if constant ionic strength were maintained.

These results indicate general acid or base catalysis. The former seems less likely on the basis of experiments discussed below. The greater effectiveness of polycarboxylic acids in the deamination reaction is then due to the fact that they furnish a higher concentration of carboxylate ions. It is interesting that buffers derived from acetic acid (pK_a = 4.75), lactic acid (pK_a = 3.86), and formic acid (pK_a = 3.75) were approximately equally effective. Presumably the greater concentration of buffer anions furnished at pH 4.0 by the last two acids is compensated by their lesser catalytic ability, compared to acetate, according to the Brønsted catalysis law.

The deamination of cytidine proceeded also in non-carboxylate buffers. Pyridine–HCl buffer, 0.5 M, pH 5.0, 95°, deaminated 7% in 13 hr. Citrate, under those conditions, converted 11% of cytidine to uridine.

Mercapto acids were found to be unusually effective in this reaction. Because of their ultraviolet absorbance, the paper chromatography method was used. The per cent of cytidine deaminated by these buffers (0.5 M) in 21 hr at 95°, pH 4.0, is as follows: mercaptoacetate, 32.5%; mercaptosuccinate, 29.5%; β-mercaptopropionate, 22.5% (*cf.* acetate, 16%; citrate, 34%). A mixture containing acetate and added thioethanol was not found to be better than acetate alone. It thus appears necessary for the mercapto and acid groups to be proximate in the same molecule for best results. The ability of mercaptoacetic acid to deaminate cytosine has been previously noted (Simon and Meneghini, 1963).

The variation of the rate constant for 0.5 M citrate buffer over the pH range 2.5–6.0 was measured and the results are indicated in Figure 3. A definite maximum occurs at about pH 3.5. The decline in rate over the range 3.5–6.0 may be due to the involvement of protonated cytidine (pK_a = 4.2) in the rate-limiting step of the reaction. This would be compensated to some extent by an increase in the concentration of carboxylate anions at higher pH values. At pH values <3.5, cytidine is largely protonated, and the slow decrease in rate observed from pH 3.5 to 2.5 may be due to the decreasing concentration of carboxylate anions. The rate constant for deamination by dilute HCl at pH 2.3, 95°, was found to be 1.7 × 10^{-2}/hr, only 19% less than 0.5 M citrate at that pH and temperature. Under those conditions, water is presumably the principal base catalyzing the reaction.

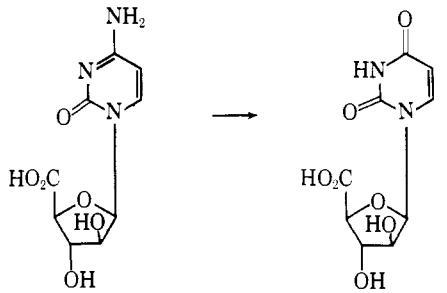
If, as assumed above, the rate of deamination were assumed to depend upon the concentration of protonated cytidine, and general base catalysis were involved, then one would expect this rate to be the same in all aqueous solutions of pH <3.0 in the absence of any effective base but water. The amount of cytidine deaminated at 97° in 21 hr, as determined by the paper chromatography method, is as follows: 0.5 M acetic acid, pH 2.8, 34%; 1.0 M HCl, pH 0.1, 36%; 0.1 M $HClO_4$, 35%. The rate of deamination of cytidine in refluxing 0.4 N H_2SO_4 has been reported (Loring and Ploeser, 1949) and seems consistent with these data.

The following observations, relevant to the discussion on mutagenesis below, were also made. A small amount of deamination (2%) was observed when cytidine was refluxed in distilled water or in 0.5 M sodium acetate solution (pH 7.2) for 21 hr. When cytosine was warmed at 50° in acetate or mercaptoacetate buffer, a definite spot corresponding in R_F to uracil (solvent I) could be observed after 19 hr.

Discussion

Two possible mechanisms for the reaction, consistent with the data above, are outlined in Figure 4. The path II → V → VI is analogous to the hydrolysis of an amide and has the advantage of simplicity. However, little direct evidence has been reported to support its occurrence in known reactions involving nucleophilic displacement of the amino group of cytosine. The

longer pathway II → III → IV → VI has some analogy and allows a unique role to be played by the buffer anion B^-. It involves a partial saturation of the cytosine ring by nucleophilic addition of buffer anion, deamination of the dihydrocytosine intermediate, and loss of the buffer anion to give a uracil derivative. This route is similar to that established (Brown and Phillips, 1965) for the reaction of the potent mutagen, hydroxylamine, with cytosine derivatives. It has been shown (Johns *et al.*, 1965), that the deamination of dihydrocytosine derivatives resembling intermediate III is a rapid process in aqueous solution, even at 37°. The ability of a particular nucleophile (an SH group) to add reversibly and intramolecularly to the 5,6 double bond of a uracil ring has been demonstrated (Chambers and Kurkov, 1963). It is of interest in this connection that the cytosine derivative VII was reported to be largely deaminated to the uracil derivative VIII, merely on recrystallization from water (Imai and Honjo, 1965). This facile reaction is best explained in terms of the addition–elimination mechanism. The proximity of the carboxyl group to the C=C double bond of the cytosine ring



VII VIII

would be expected to greatly facilitate its addition to it. In the cases that we have studied, however, we have not been able to detect any indication of intermediates corresponding to III and IV. If they do occur, their concentration must be low compared to those of cytidine and uridine.

The hydrolytic deamination of cytidine to uridine is a slow reaction, with 16 hr being required for half-reaction in 2.0 M citrate buffer at pH 4.0 and 95°. A search for more rapid conditions is continuing. This, however, does not preclude the potential usefulness of this reaction at a lower temperature for mutagenic purposes, because the change of a single base or base pair on a nucleic acid chain is believed sufficient to produce a mutation (Freese, 1963). It should be noted, in fact, that mutations are indeed produced on incubation of T_4 phage in acetate or succinate buffers at temperatures from 37 to 54° and pH values from 4.2 to 5.0 (Freese, 1959; Strack *et al.*, 1964). These were ascribed to depurination, followed by the random insertion of another base into the resulting gap. It was reported, however (Freese, 1959), that 77% of the mutations so produced could be caused to revert by base analogs, which indicated that they were transition mutations. These results are more readily accommodated by the deamination of cytosine residues than by depurination. We have

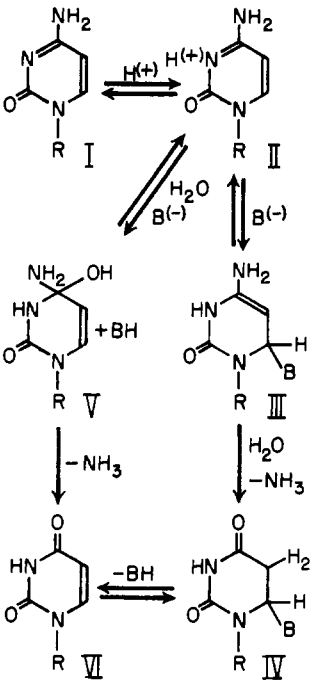


FIGURE 4: Mechanisms for deamination of cytosine and cytidine. R = H or β-D-ribofuranosyl; B^- = buffer anion.

observed that when cytosine is heated in acetate or mercaptoacetate buffer at 50°, pH 4.5, for 19 hr, a spot corresponding to uridine appears on chromatography. It may further be possible that deamination is responsible, in part, for the mutations produced upon heating *Escherichia coli* in neutral solution (Zamenhof and Greer, 1958). While the deamination of cytidine proceeds most rapidly under the acidic conditions described above, a small amount could be detected after heating cytidine in distilled water, or sodium acetate solution, pH 7.2.

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A Procedure for the Measurement of Molecular Weights by the Archibald Method. I. Theoretical Analysis*

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ABSTRACT: The Archibald method of measuring molecular weights relies at present on an empirical extrapolation of the gradient curve of $\partial c/\partial r$ *vs.* r to the ends of the solution column. In this first paper an extrapolation procedure is examined by means of computer solutions to the differential equations, and also by study of the Fujita–MacCosham equation [Fujita, H., and MacCosham, V. J. (1959), *J. Chem. Phys. 30*, 291]. A linear extrapolation of $\partial c/\partial r$ *vs.* r gives satisfactory accuracy, provided certain conditions are met. It is also shown that the error can be large when these conditions are not met.

The method introduced by Archibald (1947) is one of the most common methods for measuring molecular weights with the analytical ultracentrifuge. Its popularity stems from the fact that the method permits relatively rapid measurements, as opposed to other centrifugal methods. (For a detailed discussion see Schachman, 1959.) More recently the theory for this method has been extended for use with nonideal, heterogeneous systems (Kegeles *et al.*, 1957). In spite of this and other developments, no thoroughly sound theoretical guide exists for the practical evaluation of data by extrapolation to the ends of the solution column as required by the method. The need for extrapolation arises because optical phenomena cause deterioration of schlieren patterns at both ends of the solution column and thereby prevent direct reading at the ends; nor does the Rayleigh optical system circumvent this difficulty to permit unambiguous measurements at the ends (LaBar and Baldwin, 1962).

Peterson and Mazo (1961) used a digital computer to study the extrapolation to the meniscus, but they were unable to find general conditions for the extrapolation and recommended that the extrapolation be carried out with a computer by successive iteration using constants from the formula of Fujita and MacCosham (1959). The purpose of this study is to establish and to test a procedure for carrying out such extrapolations in routine experimental work.

Fundamental Equations and Methods for Extrapolation. The Archibald condition, based upon the boundary condition that the flow of each component is zero at the ends of the solution column at all times, is given for general application by (Kegeles *et al.*, 1957; Fujita *et al.*, 1962; Kotaka and Inagaki, 1964)

$$(1/rc)(\partial c/\partial r) = M^*(t)\omega^2(1 - \bar{v}\rho)/RT \quad (\text{at } r = r_a \text{ and } r_b) \quad (1)$$

$$M^*(t) = M(t)[1 - M(t)Bc + 0(c^2)] \quad (2)$$

where r, r_a, and r_b are the radial distances to any given point, to the meniscus, and to the base, respectively; t is time; c is the concentration at r and t in grams per unit volume; $\partial c/\partial r$ is the concentration gradient at r and t in grams per volume per distance; $M^*(t)$ and $M(t)$ are the apparent Archibald molecular weight and Archibald molecular weight, respectively, at r and t; B is the

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