The Reaction of Ribonucleosides with Nitrous Acid. Side Products and Kinetics*

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ABSTRACT: The reactions of adenosine, cytidine, and guanosine with nitrous acid have been reinvestigated. The chemical properties of the principal side product of reaction of guanosine, 2-nitroinosine, have been studied. It was converted to guanosine by reducing agents, hydrolyzed in alkali to xanthosine, and hydrolyzed in acid first to 2-nitrohypoxanthine and then to xanthine. 2-Nitrohypoxanthine was also formed in the reaction of guanine with nitrous acid. The reaction of guanine, xanthine, or xanthosine with nitrous acid produced another side product, 8-nitroxanthine, in small yield. No side products of reaction were detected in the reactions with adenosine and cytidine. The kinetics of deamination of adenosine, cytidine, and guanosine by nitrous acid have been followed from 0 to 50°, pH 3.75–5.0, by a spectrophotometric method. The reactions were pseudo-first order in nucleoside and the reactivity, under a variety of conditions, was found to be guanosine > adenosine > cytidine.

Other workers have studied the reactivity of the corresponding bases within a variety of nucleic acids. A comparison is made between the rates observed within nucleic acids and the present ones with nucleosides, and the differences noted are discussed.

Nitrous acid reacts with those common heterocyclic components of nucleic acids that contain a primary amino group: adenine, cytosine, and guanine. The principal products of the reactions were established many years ago as hypoxanthine, uracil, and xanthine, respectively (Strecker, 1861; Kossel, 1865; Kossel and Steudel, 1903). The products contain a carbonyl group in place of the amino group, and the reaction is generally known as deamination. The same changes occur when the corresponding ribonucleosides, adenosine, cytidine, and guanosine, react with nitrous acid (Levene and Jacobs, 1910). It has been demonstrated that the reactions outlined above also occur within a nucleic acid when it is treated with nitrous acid (Schuster and Schramm, 1958; Schuster, 1960). Since the discovery that nitrous acid produces mutations when applied to TMV or to its RNA (Gierer and Mundry, 1958), it has come into wide use as a chemical mutagen with a variety of simple organisms (Orgel, 1965). There has been considerable interest in determining which of the above reactions is responsible for the mutagenic effect of nitrous acid. There is now general agreement that the deaminations of cytosine and adenine are both mutagenic (Freese, 1963; Orgel, 1965). The situation with respect to the deamination of guanine to xanthine within a nucleic acid is less clear. On the basis of studies of the kinetics of deamination, killing, and induction of mutations by nitrous acid in T_2 phage, it was concluded that deamination of guanine to xanthine is not mutagenic and may be lethal (Vielmutter and Schuster, 1960). It has been shown that synthetic polyribonucleotides containing uracil and guanine gain no new coding properties when treated with nitrous acid (Ochoa, 1963). On the other hand, it was found that nitrous acid strongly promotes the mutagenic replacement of guanine by adenine in single-stranded DNA phages (Tessman *et al.*, 1964). Polyriboxanthylic acid complexes well with a number of synthetic polynucleotides, and the suggestion has been made that xanthine could function in place of adenine in a nucleic acid, thereby causing a mutation (Michelson and Monny, 1966).

Recently, a number of additional observations have been made which are not explained by the above scheme. It has been found that nitrous acid has a cross-linking effect on DNA (Geiduschek, 1961; Horn and Herriot, 1962; Becker *et al.*, 1964). A dAT copolymer was progressively inactivated as a template by nitrous acid, and this was ascribed to reactions other than deamination (Kotaka and Baldwin, 1964). It has been shown that nitrous acid produces deletion mutations in T_2 phage (Tessman, 1962) and causes a weak mutagenic transformation of thymine to cytosine in single-stranded DNA phages (Tessman *et al.*, 1964). The guanine of TMV-RNA was not all converted to xanthine by extensive nitrous acid treatment but some was consumed in an unknown reaction (Schuster and Wilhelm, 1963).

In addition to its use for mutagenic purposes, the nitrous acid reaction of nucleic acids has been utilized to infer information about the secondary structure of nucleic acids. This was primarily done by measuring the relative rates of deamination of adenine, cytosine, and guanine within a variety of nucleic acids (Schuster, 1960; Litman, 1962; Lochmann and Stein, 1963; Schuster and Wilhelm, 1963). Differences in reactivity

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observed were attributed to the involvement of certain amino groups in hydrogen bonding.

We wish to report here the results of a reinvestigation of the reactions of the ribonucleosides, adenosine, cytidine, and guanosine with nitrous acid. This was done for two reasons. It was hoped that information might be obtained about side reactions which would provide greater understanding of some of the peculiar effects of nitrous acid in biological systems. In particular, more information was desired on the formation and properties of 2-nitroinosine, the side product of reaction of guanosine with nitrous acid that was previously discovered in these laboratories (Shapiro, 1964). We also wished to obtain reliable data on the relative rates of deamination of the amino groups of adenine, cytosine, and guanine, when they were not in the environment of a polynucleotide chain. This would permit an assessment of the validity of conclusions drawn from relative rates of deamination within a nucleic acid. It was felt that the ribonucleosides were suitable for this purpose. The free bases offer greater solubility problems and are subject to a N_7–N_9 tautomeric equilibrium which does not occur with nucleosides or nucleic acids. The deoxynucleosides are less suitable for kinetic purposes because of the complication caused by the greater lability to acid of their *N*-glycoside bonds, particularly that of deoxyxanthosine. The formation of additional ultraviolet-absorbing products from the liberated deoxyribose provides further difficulties (Garrett *et al.*, 1966).

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. Ultraviolet absorbances were measured, for kinetic purposes, on a Beckman DU spectrophotometer. Ultraviolet spectra were obtained on a Perkin-Elmer 202 spectrophotometer. The ultraviolet absorption of the eluate from ion-exchange columns was followed at 265 mμ with a Gilson absorption meter and recorded with a Texas Instruments Co. rectilinear recorder. Determinations of pH were made on a Beckman Zeromatic pH meter. Infrared spectra were obtained with a Perkin-Elmer Infracord spectrophotometer. Paper chromatography was carried out by the descending technique on Whatman No. 40 paper. Thin-layer chromatography was carried out on Avicel microcrystalline cellulose (American Viscose Co., Marcus Hook, Pa.). The layers were 0.75 mm thick for ordinary work, and 2.25 mm thick for preparative work. After development of the chromatograms, ultraviolet-absorbing materials were located with the aid of an ultraviolet lamp equipped with a short-wavelength filter. The principal solvent systems employed and new R_F values obtained are given in Table I. Microanalyses were performed in duplicate by Mr. George I. Robertson, Jr., Florham Park, N. J., and on an automatic CHN analyzer (F & M Scientific Corp. Model 185) and averaged.

TABLE I: R_F Values.[a]

Compound	Solvent System[b] A	B	C
2-Nitroinosine (NH_4^+ salt)	0.89	0.34	0.49[c]
2-Nitrohypoxanthine (NH_4^+ salt)	0.82	0.26	0.58
8-Nitroxanthine	0.76[c]	0.14[c]	0.50[c]
2-Sulfonamidoinosine (Na^+ salt)	0.90	0.15	

[a] All data are for paper chromatography unless otherwise noted. [b] Solvent systems are: (A) water, (B) isobutyric acid–ammonia–water (66:4.7:29.3) (all proportions v/v), and (C) 2-propanol–water (7:3). [c] This value is for thin-layer chromatography on cellulose.

Preparation of 2-Nitroinosine (I, R = β-D-Ribofuranosyl) as Ammonium Salt. A suspension of 4.52 g (15 mmoles) of guanosine monohydrate in 600 ml of 3.6 N acetic acid–sodium acetate buffer (pH 3.75) was cooled to 0° in a three-necked flask, fitted with mechanical stirrer and dropping funnel. The funnel was used to introduce 100 ml of 8 N $NaNO_2$ solution. The suspension was stirred for 10 hr at 0° and then allowed to warm up to room temperature, with stirring, over 12 hr. The resulting clear yellow solution was applied directly to a column (21 cm high, 5-cm diameter) containing 410 ml of Amberlite CG 400 (Mallinckrodt) ion-exchange resin, acetate form. The column was eluted with water until the ultraviolet absorption of the eluate at 265 mμ began to fall, and then with 1 N NaCl solution. A new peak was eluted (xanthosine) and the ultraviolet absorption of the elute fell to a constant low value. The eluting solvent was changed to 0.1 N HCl in 1 N NaCl solution, and the peak that emerged was collected. This was adsorbed onto 1.2 g of Norit A charcoal, and the charcoal was filtered using a Millipore filter apparatus. The charcoal was washed with water until the washings gave a negative test for chloride ion and then eluted with 6% NH_3–44% ethanol–50% H_2O solution. The eluate was evaporated under vacuum at room temperature to give 178 mg (3.6%) of the ammonium salt of 2-nitroinosine. The preparation contained traces of xanthosine and of the ammonium salt of 2-nitrohypoxanthine. It could be further purified by grinding it with absolute ethanol and filtering (the impurities are removed in the filtrate).

Yield Studies on the Formation of 2-Nitroinosine. Solutions were prepared containing 13 mg of guanosine in 5 ml of 3.75 N acetic acid–sodium acetate buffers which varied in pH from 3.0 to 5.0. To each solution was added 2.5 ml of 4 N $NaNO_2$ and the solutions were allowed to stand at 21°. At intervals, aliquots of 1 ml were withdrawn. Each aliquot was brought to pH 1 with HCl, 25 mg of urea was added, and the solution was heated for 10 min at 50°. (This was done to destroy

nitrite ion. A control showed that less than 5% of the 2-nitroinosine was destroyed by this treatment.) The solution was brought to a pH of 7 by the addition of sodium phosphate buffer and diluted to 5 ml. The absorption in the ultraviolet region at 350 mμ was measured and the yield was calculated from the known extenction coefficient of 2-nitroinosine (ε 3600) at that wavelength. The yield was found to vary regularly from 6% at pH 3.0 to 3% at pH 5.0. A yield of 7% was indicated when glacial acetic acid was used as the solvent.

A similar study was performed at pH 3.75 with the temperature varying. The indicated yields were 7% at 0°, 6% at 21°, and 5% at 50°.

In another study at pH 3.75, 21°, the concentration of the added nitrite solution was allowed to vary from 1 to 8 N. The yield varied accordingly from 4 to 8.5%. An experiment in which buffer strength varied from 1.5 to 12 N showed little variation in the yield.

Reduction of 2-Nitroinosine. A. WITH SODIUM BOROHYDRIDE AND PALLADIUM ON CHARCOAL. 2-Nitroinosine (2 mg), ammonium salt, 75 mg of $NaBH_4$ (Metal Hydrides, Inc., Beverly, Mass.), and 45 mg of 5% palladium on carbon (Engelhard Industries, Inc., Newark, N. J.) were combined in 15 ml of water and the reaction mixture was allowed to stand under nitrogen with magnetic stirring at room temperature for 1 hr. The reaction mixture was treated with 1 ml of glacial acetic acid and filtered after several minutes. The solids in the funnel were washed with 10 ml of 6 N NH_3 solution. The solutions and washings were combined and concentrated under vacuum to 1 ml. The solution was examined by thin-layer chromatography. Only one spot, with an R_F equal to that of guanosine was seen in solvents A, B, and in isopropyl alcohol–NH_3–H_2O (7:1:2). The ultraviolet spectra of the solution, at pH 4.5 and 10.5, was identical with that of guanosine

B. WITH SODIUM HYDROSULFITE. To a solution of 10 mg of the ammonium salt of 2-nitroinosine (I, R = β-D-ribofuranosyl) in 3 ml of water, was added 12 mg of sodium hydrosulfite in small portions. Small samples were withdrawn after each addition for analysis by thin-layer chromatography in solvents A and B. After the addition of 6 mg of sodium hydrosulfite, the yellow solution had decolorized and spots of R_F 0.61, 0.67, 0.90, and 0.97 were observed on chromatography in solvent A. After 12 mg of sodium hydrosulfite had been added, the spots of R_F 0.61 and 0.90 were the only significant ones observed. A portion of the solution was worked up by preparative paper chromatography. The material of R_F 0.61 coincided with guanosine in its ultraviolet spectra at pH 1, 7, and 11, R_F in solvents A and B, and electrophoretic mobility in potassium borate buffer (pH 9.1). The material of R_F 0.90 showed in its ultraviolet spectrum (H_2O) a maximum at 255 mμ and a shoulder at 269 mμ. Upon electrophoresis in 0.1 N sodium acetate–acetic acid buffer (pH 4.5) (21 v/cm, 80 min) it moved 5.5 cm as an anion. To a portion of the solution containing this material HCl was added to bring its concentration to 0.1 N. The solution was heated at 95° for 90 sec. The solution was examined by means of thin-layer chromatography in solvents A and B. Only one spot, corresponding in R_F to guanosine, was seen.

Alkaline Hydrolysis of 2-Nitroinosine. A solution of 1 mg of the ammonium salt of 2-nitroinosine (I, R = β-D-ribofuranosyl) in 2 ml of 2 N sodium hydroxide was kept at 21° for 90 min. An identical solution was heated at 100° for 90 min. Each solution was neutralized with HCl and treated with Norit A charcoal. The charcoal was filtered using a Millipore filter apparatus, washed, and eluted with concentrated NH_3–ethanol–H_2O (1:2:1). The eluate was concentrated to a small volume and examined by means of thin-layer chromatography in solvents A and B. The sample from the reaction run at room temperature showed spots corresponding in R_F to starting material and xanthosine. Only a spot corresponding to xanthosine resulted from the sample that had been heated. The ultraviolet spectrum of this solution corresponded to that of xanthosine at pH 2 and 10.

Acidic Hydrolysis of 2-Nitroinosine. A solution of 12 mg of the ammonium salt of 2-nitroinosine (I, R = β-D-ribofuranosyl) in 2 ml of 0.1 N HCl was heated at 90°. Samples of 0.1 ml were withdrawn at intervals, neutralized with sodium phosphate buffer, and examined by paper chromatography in solvents A and B. After 20 min, the starting material had almost entirely disappeared and a new yellow material, which gave a negative response to periodate–benzidine spray (Cifonelli and Smith, 1954) was observed. After 90 min, this in turn had largely disappeared and a new spot, with the same R_F as xanthine, had appeared. After 170 min of heating, the solution was cooled to room temperature and allowed to stand for 2 days. A pale yellow precipitate appeared. It was filtered, dissolved in ammonia solution, and worked up by means of preperative paper chromatography in solvent A. The principal band (R_F 0.49) was cut out and eluted into water. It was shown to be identical with xanthine in its R_F in solvents A and B, butanol–H_2O (86:14), and isopropyl alcohol–NH_3–H_2O (7:1:2). Its ultraviolet spectrum at pH 2, 5.5, and 10.3 coincided with those of xanthine.

Preparation of 2-Nitrohypoxanthine (I, R = H) by Hydrolysis of 2-Nitroinosine, Ammonium Salt. A solution of 100 mg of the ammonium salt of 2-nitroinosine (I, R = β-D-ribofuranosyl) in 5 ml of 0.2 N HCl was kept at 50° for 16 hr. The solution was evaporated to dryness under vacuum at room temperature, dissolved in 0.5 ml of dilute ammonia solution, and worked up by means of preparative thin-layer chromatography in solvent A. The yellow band of R_F 0.83 was cut out, eluted into water, and purified by adsorption onto Norit A charcoal and elution with NH_3–ethanol–H_2O (1:2:1). The solution was evaporated to 0.25 ml, acidified with a drop of concentrated HCl, and allowed to stand in the refrigerator overnight. The yellow precipitate (7 mg) of 2-nitrohypoxanthine was collected by centrifugation; ultraviolet maxima (H_2O): pH 1, 331 mμ; pH 7, 237 and 344 mμ; pH 13, 392 mμ. Spectrophotometric titration revealed pK_a values of approximately 3 and 10.

Anal. Calcd for $C_5H_3N_5O_2 \cdot H_2O$: C, 30.2; H, 2.5; N, 35.2. Found: C, 30.6; H, 1.9; N, 35.1.

On another run, the ammoniacal solution from elution of the charcoal was evaporated directly to give the

ammonium salt of 2-nitrohypoxanthine. Its infrared spectrum (KBr) showed bands in the carbonyl area at 6.19 and 6.45 μ.

Preparation of 2-Nitrohypoxanthine (I, R = H) and 8-Nitroxanthine (III) by Reaction of Guanine with Nitrous Acid. A solution of 2.42 g (0.016 mole) of guanine in 500 ml of 4 N HCl was cooled to 0° and 100 g (14.5 moles) of sodium nitrite was added. The reaction was allowed to proceed with stirring overnight. Portions of Norit A charcoal were added and separated by filtration (Millipore filter apparatus) and this was repeated until the solution had a constant low absorption above 300 mμ in the ultraviolet region. The charcoal was washed with water and eluted with NH_3–ethanol–H_2O (1:2:1). The eluate was evaporated, dissolved in dilute HCl, and clarified by filtration. The filtrate was applied to a column (32 × 6 cm) of Rexyn 50 (Fisher Scientific Co.) cation-exchange resin in the acid form. The column was eluted with water and the absorption at 350 mμ of successive fractions was measured. A peak was rapidly eluted, and this solution was concentrated to 2 ml under vacuum and worked up by preparative paper chromatography in solvent B. The yellow band of faster R_F was eluted into water, and further purified by preparative chromatography in solvent A, adsorption onto Norit A charcoal, and elution. The resulting solution was concentrated and crystallized as described above to give 7 mg of 2- nitrohypoxanthine. Its ultraviolet spectrum and R_F values were the same as those of the material isolated from the acidic hydrolysis of 2-nitroinosine.

The slower yellow band from chromatography in solvent B was purified in a similar manner and yielded 14.5 mg of a yellow solid. This corresponded in its infrared spectrum, ultraviolet spectrum, and R_F in solvents A, B, C, and 1-butanol–H_2O (86:14) to a sample of 8-nitroxanthine prepared by an alternative procedure (Jones and Robins, 1960).

Another reaction of guanine with nitrous acid was conducted using 600 ml of 3.6 N acetic acid–sodium acetate buffer of pH 3.75 and 100 ml of 8 N $NaNO_2$ solution. The mixture was stirred for 2 weeks at room temperature. The precipitate was filtered and washed with 6 N NH_3. The combined filtrate and washings were worked up by an anion-exchange procedure identical with that used for 2-nitroinosine. The product was 37 mg of yellow solid. A 2.5-mg sample of this was applied to a 4-ml column of Amberlite CG 120 (Mallinckrodt) resin, acidic form, and the column was eluted with water. Two ultraviolet-absorbing peaks appeared. The earlier one, with 5% of the material (by spectrophotometric analysis), was 8-nitroxanthine, and the later one, with 95% of the material, was 2-nitrohypoxanthine.

Preparation of 8-Nitroxanthine from Xanthosine. To 1 g (3.5 mmoles) of xanthosine in a three-necked flask were added 200 ml of glacial acetic acid and 75 g (1.1 moles) of sodium nitrite. The solution was heated to 90°, the flask was stoppered, and the reaction mixture was stirred for 3 days at that temperature. Four 20-g (0.29 mole) portions of sodium nitrite were added to the solution during this time, as well as enough HCl to keep the pH below 3. The reaction was then allowed to cool, the white precipitate was filtered, and ultraviolet-absorbing materials were removed from the filtrate by adsorption onto Norit A charcoal. The charcoal was filtered using a Millipore apparatus, washed, and eluted with concentrated NH_3–ethanol (1:1). The eluate was concentrated to a small volume, brought to pH 3.5 with HCl, and applied to a column (6 × 40 cm) of Amberlite CG 120 (Mallinckrodt) cation-exchange resin in the acid form. The column was eluted with water. Those early fractions (1.5), which had considerable absorption above 300 mμ in the ultraviolet region, were combined and evaporated to dryness. A total of 37 mg of yellow solid which had the infrared spectrum of 8-nitroxanthine were obtained (5.3%).

Kinetic Determinations. A direct spectrophotometric method was used. The general details of this method have been described (Loring, 1955).

DEAMINATION OF CYTIDINE TO URIDINE. To 3.0 ml of 3.6 N acetic acid–sodium acetate buffer (pH 3.75) (or 4.5 or 5.0) was added 0.1 mmole of cytidine. The solution was immersed in a contant-temperature bath at 0, 21.5, 37.5, or 50° and 0.5 ml of 4 N $NaNO_2$ solution was added. At intervals, 0.12-ml portions were withdrawn, added to 2 ml of K_2HPO_4 buffer (pH 7.4), and the solution was diluted to 50 ml. The absorbance was read at 260, 280, and 360 mμ against an appropriate blank containing only buffer. The reading at 360 mμ was used to calculate the concentration of nitrite present (ϵ_{360} 22) and this value used to correct the readings at 260 and 280 mμ for absorption by nitrite. This value was also used to guide the occasional addition of portions of $NaNO_2$ to the original reaction mixture so as to keep the total nitrite concentration approximately constant. At the end of the reaction, the pH was found to have held constant to within 0.1 unit. The formulas used to compute the relative amounts of cytidine and uridine have been given elsewhere (Shapiro and Klein, 1966).

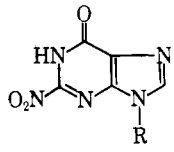
DEAMINATION OF ADENOSINE TO INOSINE. The procedure outlined above for cytidine was followed. Readings were taken at 254, 265, and 360 mμ. The formulas used to calculate the relative amounts of adenosine and inosine were: adenosine = $(1.97A_{265} - A_{254})/12.8$ and inosine = $(A_{254} - 1.01A_{265})/5.06$. In these formulas, A_{265} and A_{254} represent the absorbances of the solution at 265 and 254 mμ, respectively, after correction for absorption by nitrite.

CONVERSION OF GUANOSINE TO XANTHOSINE AND 2-NITROINOSINE. The procedure outlined above for cytidine was followed, except that the volumes of acetate buffer and sodium nitrite solution used were doubled and 0.24-ml aliquots of reaction solution were withdrawn. They were added to 4 ml of K_2HPO_4 buffer (pH 7.4) and the volume of the solution was diluted to 5 ml with water. The absorbance of this solution was measured at 325 and 360 mμ and the concentrations of 2-nitroinosine and nitrite were determined from the following equations: nitrite = $(A_{360} - A_{325})/9.2$ and nitroinosine = $(A_{325} - 0.59A_{360})/1.2 \times 10^3$, where A_{325} and A_{360} represent the measured absorbances of the solution at 325 and 360 mμ. The solution was then

further diluted by a factor of 1:10 and the absorbances at 260 and 285 mμ were measured. These were corrected for absorbance by nitrite and 2-nitroinosine at those wavelengths and the corrected absorbances at 260 and 285 mμ were used to compute the concentrations of guanosine and xanthosine as follows: guanosine = $(A_{260} - 1.08A_{285})/5.35$ and xanthosine = $(2A_{285} - A_{260})6.5$.

Results

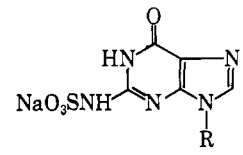
As we reported earlier (Shapiro, 1964), the reaction of guanosine with nitrous acid in acetate buffer produced 2-nitroinosine (I, R = β-D-ribofuranosyl) in



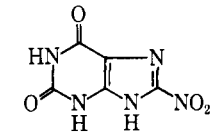
I

addition to xanthosine. Because of the absorption of 2-nitroinosine above 300 mμ in the ultraviolet region, it was possible to readily follow the variation of the amount of 2-nitroinosine formed with temperature, pH, and nitrite and buffer concentration. The formation of 2-nitroinosine was favored by increasing the nitrite concentration from 1 to 8 N. This was expected, as 2-nitroinosine was presumably formed by reaction of nitrite ion with the intermediate diazonium ion derived from guanosine and nitrous acid. The yield of 2-nitroinosine also increased as the pH was lowered from 5.0 to 3.0. A temperature of 0° produced slightly better yields than 21 or 50°, while buffer concentration mattered little. In a number of cases, the amount formed reached a maximum and slowly declined, indicating that the product was not stable under the conditions of the reaction. The highest yield observed was 8.5%. The best preparative conditions appear to be in glacial acetic acid, at 0°, with a large excess of nitrite.

No other 2-nitropurine has been reported in the literature, nor has a pyrimidine with a nitro group in the corresponding position. It seemed of interest to explore the chemical properties of the compound. Guanosine was the only product observed upon reduction by $NaBH_4$ and 5% palladized charcoal (Neilson *et al.*, 1962). Sodium hydrosulfite reduction, on the other hand, gave another major product in addition to guanosine. This had an ultraviolet spectrum resembling that of guanosine, gave a positive response to periodate–benzidine spray, migrated as an anion upon electrophoresis at pH 4.3, and was completely converted to guanosine by treatment with 0.1 N HCl at 95° for 90 sec. On the basis of these properties it was tentatively identified as the sodium salt of 2-sulfamidoinosine (II, R = β-D-ribofuranosyl). The formation of sulfamidic acids in the reduction of aromatic nitro compounds by sodium hydrosulfite has been observed by several workers (Schröter and Möller, 1957).



II



III

2-Nitroinosine was readily attacked by hot alkali or acids, with hydrolysis of the nitro group to a keto group. This ready reactivity of a nitro group to nucleophilic displacement has been observed with other nitroheterocycles (Shepherd and Fedrick, 1965). Sodium hydroxide converted 2-nitroinosine to xanthosine, slowly at room temperature and within 90 min at 100°. Acidic hydrolysis took place in two stages. This initial product was a yellow substance whose analysis and negative response to periodate–benzidine spray indicated loss of the sugar. Its infrared spectrum and ultraviolet spectra in acidic and neutral solution resembled those of 2-nitroinosine. An additional dissociation took place at pH 10. The compound is considered to be the nitrobase, 2-nitrohypoxanthine (I, R = H). Acidic hydrolysis of this compound converted it to xanthine. It was also possible to isolate this compound, although in quite low yield, from the reaction of guanine with nitrous acid. A second yellow side product was also isolated from this latter reaction. When the reaction was run in acetate buffer, only tiny amounts of this substance were present, but its amount exceeded that of 2-nitrohypoxanthine when the reaction was run in HCl. This product was identified as 8-nitroxanthine (III) by comparison with a sample synthesized by another method (Jones and Robins 1960). The formation of 8-nitroxanthine could also be detected chromatographically when a suspension of xanthine was stirred with nitrous acid. The 8-nitro compound was presumably formed *via* an intermediate 8-nitroso compound, which was further oxidized. This is of interest because a direct nitration of unmethylated purine derivatives has not previously succeeded (Jones and Robins, 1960). When xanthosine was allowed to react with nitrous acid in acetic acid under vigorous conditions (90°, 3 days), the product obtained, in 5% yield, was again 8-nitroxanthine. The glycosidic bond was labile under the conditions used and cleaved either prior to, or subsequent to, the introduction of the nitro group. No other side products were observed to be formed in more than trace amounts in the reactions of the ribonucleosides studied with nitrous acid.

The kinetics of the reaction of adenosine, cytidine, and guanosine with nitrous acid were followed by a direct spectrophotometric method. This proved more accurate than a method in which the nucleoside and its deamination product were first separated by a paper chromatographic procedure (R. Shapiro and R. F. Dods, unpublished data). Another worker has found difficulty in obtaining reproducible results in separating deamination products of nucleic acids by the use of a paper chromatographic procedure (Carbon, 1965). In following the deamination of adenosine and cytidine by our spectrophotometric method, readings were

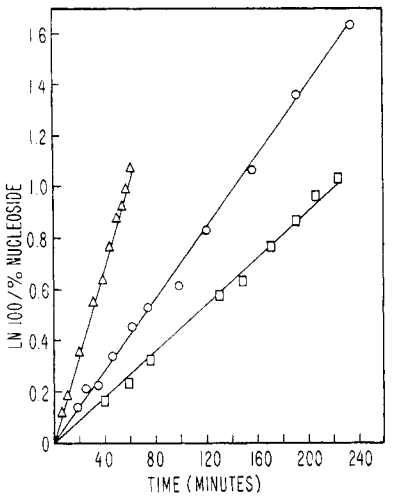


FIGURE 1: Plot of pseudo-first-order kinetics for the reaction of adenosine (○), cytidine (□), and guanosine (△) with 0.57 N $NaNO_2$ in 3.1 N acetic acid–sodium acetate buffer, pH 3.75, 37.5°.

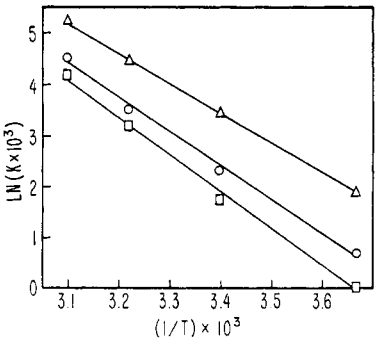


FIGURE 2: Dependence of rate constants, K (min^{-1}), upon absolute temperature for the reaction of adenosine (○), cytidine (□), and guanosine (△) with 0.57 N $NaNO_2$ in 3.1 N acetic acid–sodium acetate buffer (pH 3.75).

taken at three wavelengths (see the Experimental Section). These were used to follow the concentrations of the nucleosides, its deamination product, and nitrite. In the deamination of guanosine, readings at four wavelengths were necessary in order to determine the amounts of guanosine, xanthosine, 2-nitroinosine, and nitrite. There was a slow loss of nitrite during the reactions due to the decomposition of nitrous acid as well as to the reaction with nucleoside. It was necessary to add portions of sodium nitrite from time to time to keep the nitrite concentration approximately constant. This was found to be the greatest source of error, as the average nitrite concentration in the adenosine and cytidine reactions varied by up to 5% from run to run. In the guanosine reaction (where the determination of nitrite concentration was less accurate due to absorption of 2-nitroinosine above 300 mμ), the average nitrite concentration varied from run to run by up to 10%. The accuracy of the rate constants is limited to the same values. It was possible to keep the pH constant, despite the loss of nitrous acid, by the use of a moderately concentrated buffer solution.

The data from each run, when plotted, gave smooth curves corresponding to a first-order reaction in nucleoside. Kinetic studies were run at four temperatures at pH 3.75 and 4.5, and one temperature at pH 5.0. Typical plots of data are seen in Figure 1. The rate constants determined are summarized in Table II. These show that under all conditions studied, the relative reactivity of nitrous acid is guanosine > adenosine > cytidine. Increasing pH produces a slight increase in selectivity for guanosine and adenosine with respect to cytidine, as relative rates of reaction at 37.5° are 3.6:1.4:1 at pH 3.75, 3.9:1.6:1 at pH 4.5, and 6.0:2.6:1 at pH 5.0.

A plot of the logarithms of the rate constants against the reciprocals of the absolute temperatures gives approximate straight lines, in accord with the Arrhenius equation. This is illustrated for pH 3.75 in Figure 2. It can be seen from this that decreasing the temperature increases the relative reactivity of guanosine and adenosine with respect to cytidine and of guanosine with respect to adenosine. A similar situation prevails at pH 4.5, except that the temperature effect on the relative reactivities of adenosine and guanosine is less than the experimental error of the measurements.

The relative selectivity for guanosine that can be achieved for guanosine may be of use for synthetic purposes, and a controlled deamination of a dinucleotide containing guanine and cytosine has already been reported (Whitfeld and Witzel, 1963). For mutagenic purposes, however, a deamination method which selects

TABLE II: Rate Constants (× 10^3).[a]

	Temperature (°C)			
pH	0	21.5	37.5	50
	Guanosine			
3.75	6.7	31	85	180
4.5	1.3	9.7	18	58
5.0			3.7	
	Adenosine			
3.75	2.0	9.9	33	90
4.5	0.52	3.3	7.0	23
5.0			1.6	
	Cytidine			
3.75	1.0	5.5	24	65
4.5	0.20	1.5	4.4	16
5.0			0.62	

[a] Pseudo-first-order rate constants in min^{-1}, for 3.1 N acetic acid–sodium acetate buffer, sodium nitrite concentration (0.57 N), nucleoside concentration = 0.029 N for adenosine and cytidine, and 0.014 N for guanosine.

in favor of cytosine or adenine would seem more valuable, and other approaches to this problem are being studied (Shapiro and Klein, 1966). It is difficult to rationalize the order of reactivity of the nucleosides in terms of the chemical structures involved. The basicity of the nucleosides follows the reverse order. However, the site of protonation in these compounds differs from the site of attack by nitrous acid. It is of interest that the relative reactivity (to nitrous acid) of guanosine to cytidine is greater at pH 5.0 than at pH 3.75. At pH 3.75, cytidine ($pK_a = 4.2$) is largely protonated whereas guanosine ($pK_a = 1.6$) is not. At pH 5.0, both are unprotonated. An attempt has been made to predict the ease of deamination by nitrous acid of the three bases by means of molecular orbital calculations (Pullman and Pullman, 1963). However, neither the order predicted from the net positive charges on the carbon atoms carrying the amino groups, nor that derived from the free valences of the same carbon atoms, is in accord with that determined experimentally by us for the ribonucleosides.

TABLE III: Relative Reactivities to Nitrous Acid.[a]

	Base		
	Guanine	Adenine	Cytosine
Ribonucleoside[b]	6.5	2.2	1.0
tRNA (*E. coli*)[c]	2.1	1.4	1.0
TMV RNA[d]	2.1	1.9	1.0
TMV (intact)[e]	0.01	0.53	1.0
Thymus DNA[f]	2.2	0.44	1.0
Thymus DNA, heat denatured[f]	1.3	0.63	1.0

[a] The rate for cytosine, in each case, is arbitrarily set at 1.0. [b] pH 4.5, 21.5°, 3.1 N sodium acetate buffer–0.57 N $NaNO_2$. [c] pH 4.3, 0.25 N acetate buffer–1.0 N $NaNO_2$ (Carbon, 1965). [d] pH 4.3, 21.5°, 0.25 N acetate buffer–1.0 N $NaNO_2$ (Schuster and Schramm, 1958). [e] pH 4.2, 21° N acetate buffer–1.0 N $NaNO_2$ (Schuster and Wilhelm, 1963). [f] pH 4.2, 20°, 0.25 N acetate buffer–1 N $NaNO_2$ (Schuster, 1960).

Discussion

The formation of 2-nitroinosine as a product of the reaction of guanosine with nitrous acid occurred under all of the conditions studied. It seems likely that it occurs to some extent when a microorganism is treated with nitrous acid. This may be responsible for some of the unusual biological effects of such treatment. 2-Nitroinosine exists as an anion at pH 7 (Shapiro, 1964) and should not hydrogen bond with the other naturally occurring bases. It should be noted that at a recent conference, it was reported that dilute nitrous acid treatment of yeast generally induces base substitution mutations whereas more concentrated nitrous acid causes addition–deletion mutations (von Borstel, 1966). This could be due to the increased rate of formation of the 2-nitro compound in the presence of greater nitrite concentration. The formation of this compound does not explain, however, the loss of guanine observed in the treatment of TMV–RNA with nitrous acid (Schuster and Wilhelm, 1963). Under the acidic conditions used in that work for analysis of the nucleic acid, any 2-nitrohypoxanthine formed would be hydrolyzed to xanthine and thus scored as a normal deamination of guanine. While we also observed the formation of 8-nitroxanthine in reactions of guanine, guanosine, and xanthosine with nitrous acid, this is probably of little biological significance because of its very slight formation under conditions resembling those employed for mutagenic purposes.

In Table III, our relative rates of deamination of adenosine, cytidine, and guanosine at pH 4.5, 37°, are compared with those of other workers in intact nucleic acids or whole viruses. They are expressed as a ratio, with the reactivity of cytosine arbitrarly set as 1. It should be kept in mind that the conditions used in the various studies were not identical, but varied slightly with respect to temperature and pH, and more in buffer strength. The determination of base ratios in the TMV and thymus DNA cases involved the less reliable paper chromatographic method. The actual first-order rate constants were only determined in the present work. In the others the per cent decrease of each base was plotted against time. Despite these differences, the same general order of reactivity is observed in the first three cases. No special biological effects need to be invoked to explain the reactivity of the bases. The reactivity in intact TMV is, however, dramatically different, and reinforces the author's contention that steric hindrance or hydrogen bonds to protein may be responsible for this (Schuster and Wilhelm, 1963). Another set of workers have studied reactivities of the bases alone and in ribonucleic acids (Lochmann and Stein, 1963). In view of their very large experimental error, due perhaps to a failure to keep nitrite concentration and pH constant, their results have been omitted from this table.

The results of some studies of deamination rates in DNA are also summarized in Table III. In comparing these rates with ours, the considerations discussed for the RNA studies should be kept in mind, as well as the fact that the sugar moiety differs from that in the present work. The greater reactivity of guanine relative to adenine and cytosine in thymus DNA was attributed to the fact that the amino groups of adenine and cytosine were involved in hydrogen bonding at the pH studied, while that of guanine was not. The lesser relative reactivity of guanine in heat-denatured thymus DNA was seen to support this fact (Schuster, 1960). In light of our data with the ribonucleosides, we feel that this explanation is not valid, as the faster deamination of guanine can be explained on the basis of its greater innate reactivity to nitrous acid. The most striking fact in these results is rather the relative inertness to nitrous acid of the adenine in DNA. A similar order in reactivities was ob-

served by the same worker with DNA from other sources and by another worker with pneumococcal DNA (Litman, 1962). In this last work, other information about the structure of the DNA was inferred from the rate curves. A plot of first-order kinetics for the guanine and cytosine reactions yielded bent lines. It was suggested on the basis of this that the guanine in pneumococcal DNA was heterogeneous, and that a cytosine moiety was not deaminated until the guanine opposite it was deaminated. It seems to us that the observations could also be ascribed to the uncompensated loss of nitrous acid from the reactions and the unreliability of the paper chromatographic method of analysis for this purpose.

In future studies on the chemical modification of nucleic acids, we would suggest tht reaction rates within nucleic acids always be discussed with reference to the rates exhibited by suitable model compounds, that rate constants be determined where feasible, and that variables such as pH and the concentration of reactive species be as closely controlled as possible.

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