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CHEMICAL ANALYSIS OF DNA ALTERATIONS III. ISOLATION AND CHARACTERIZATION OF ADENINE OXIDATION PRODUCTS OBTAINED FROM OLIGO- AND MONODEOXYADENYLIC ACIDS TREATED WITH HYDROXYL RADICALS

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SUMMARY

Oligo- and monodeoxyadenylic acid as well as adenosine and adenine are oxidized by H_2O_2 at concentrations less than 0.1 M and at a pH of 7.4 at the N-7 position of the base moiety leading to adenine-7-*N*-oxide derivatives. At H_2O_2 concentrations higher than 0.1 M and at pH 8.0 two additional products are formed, which have been identified as 8-hydroxy- and 2-hydroxyadenine. The production, isolation and the methods leading to the chemical characterization of these products are described. Adenine-7-*N*-oxide was found to be sensitive to ultraviolet light, yielding adenine and 8-hydroxyadenine. The 7-*N*-oxide of dAMP, whose production and isolation from dAMP is also described, is as stable against 1 M HCl or NaOH as dAMP itself. The oxidation of dAMP to the 7-*N*-oxide and the liberation of adenine-7-*N*-oxide from the deoxynucleotide are two independent reactions, as is shown kinetically. Adenine-7-*N*-oxide was found to be among the reaction products of X-irradiated aqueous solutions of adenine representing the effect of OH radicals. The three oxidation products, being base analogs, were tested for their ability to induce point mutations in the phage T4. All three were found not to be mutagenic base analogs in the test system which, however, does not rule out their significance for the induction of mutations when formed within the DNA of a multiplying organism. 7-methyl- and 7-ethylguanine have also been shown not to induce revertants in phage T4 mutants when added to the growth media, yet they are believed to be responsible for the point mutagenic effect of methyl- and ethylmethanesulfonate, respectively.

INTRODUCTION

OH radicals, which are the simplest oxygen-containing radicals, can be produced either chemically or physically. The purest source for chemically produced OH

Abbreviation: TMSP, 3-trimethylsilo-1-propane-sulfonic acid sodium salt.

radicals is hydrogen peroxide by interaction with transition metals, *e.g.* Fe^{2+}/Fe^{3+} (ref. [1]) or Ti^{3+}/Ti^{4+} (ref. [2]). Since many other chemicals autoxidize *via* intermediate radicals producing H_2O_2, *e.g.* ascorbic acid[3], hydrazines[4] and hydroxylamines[5], they are also sources for the production of OH radicals. These radicals also arise by ultraviolet irradiation of H_2O_2 and by X-irradiation of aqueous solutions. The disadvantage of X-rays, however, is the simultaneous production of other reactive species *e.g.* ·H, ·OOH, H_3O^+ and e^-_{aq} (ref. 6). It is, therefore, difficult to prove that a particular chemical reaction of X-irradiated DNA and its components is caused by a certain one of the reactive species. Nevertheless, similarities of X-ray-induced lesions with those induced by pure OH radicals can be attributed to ·OH contained in X-irradiated solutions. But H_2O_2 — and therefore OH radicals — cannot only be introduced artificially into living cells; it is also produced by redox reactions in all living organisms. Normally, it is destroyed by catalase and peroxidase. Recently, however, SCHÖNEICH[7] has shown that H_2O_2 is able to induce chromatid aberrations in cells in which the catalase concentration is low compared to normal cells. This finding increases the interest in any reaction of DNA with H_2O_2, particularly in view of the induction of somatic mutations and of chromosomal breaks and their possible relation to the induction of cancer.

Agents which produce OH radicals, *e.g.* H_2O_2 and NH_2OH, degrade transforming DNA and oligodeoxyadenylic acid *via* the oxidation of the C-1 carbon of the deoxyribose moiety[8]. In living cells, the consequences of this degradation are chromosomal breaks and extensive chromosomal alterations.

In addition to the degradation of DNA it has been shown that hydrogen peroxide alters all four nucleobases, particularly the pyrimidine bases, to non-ultraviolet-absorbing compounds. The literature is so extensive that reviews must be consulted[9–13].

The only base which forms an additional ultraviolet-absorbing compound is adenine. This product has been discovered and briefly described as altered adenine, A_1, in two previous papers of this series[8,15]. Because of interest in the chemical structure of this compound as well as its influence on the stability of the N-glycosidic linkage of oligo- and monodeoxyadenylic acid and its possible contribution to the mutagenic or lethal effect of radical-producing agents, it was desirable to study this product and its formation more extensively.

This paper is concerned with the reaction of H_2O_2 with deoxyadenylic acid and its components, the chemical characterization of the main reaction product, A_1, as well as two minor products found during this investigation. Some of their reactions are also described. All three substances were tested for their ability to induce reverse transition mutations in rII mutants of the phage T4 (refs. 16, 19). Furthermore, the reaction products of an aqueous X-irradiated solution of adenine were compared to those obtained by treating adenine with H_2O_2.

MATERIALS AND METHODS

Chromatographic amounts of adenine-7-*N*-oxide were obtained when 50 $A_{260\ m\mu}$ units per ml of adenine and, if radioactively labeled material was desired, 0.5 μC/ml of [8-^{14}C] adenine were treated with 0.05 M H_2O_2 in 0.07 M sodium borate buffer at pH 7.4. Chromatography of this mixture in Solvent A (Table II) yielded pure ade-

nine-7-*N*-oxide after elution of the spot with R_F 0.46. The amount of adenine-7-*N*-oxide produced under these conditions varied in different experiments between 8 and 15%. Larger amounts of adenine-7-*N*-oxide were prepared by dissolving 1 g of adenine in 1000 ml of 0.01 M phosphate buffer (pH 7.0), and treating this mixture with 0.1 M H_2O_2 over a period of 5 days at 37°. The reaction was stopped by adding 10 μl of a suspension of 20 mg/ml catalase (Worthington) to the reaction mixture. After 30 min the reaction mixture was evaporated to dryness under reduced pressure. The dry residue was suspended in 100 ml of a mixture of chloroform-ethanol (9 : 1, by vol.) and this slurry was applied to a column (7 cm wide, 10 cm long) filled with silica gel (0.05–0.20 mm, E. Merck, Darmstadt, Germany), which was equilibrated with chloroform–methanol (9 : 1, by vol.). The column was extensively washed with the same solvent until no ultraviolet-absorbing material in the eluent could be detected (2 l). Adenine-7-*N*-oxide was then eluted with chloroform–methanol (1 : 9, by vol.). Because the product was still contaminated with adenine, the eluent was then evaporated to dryness under reduced pressure, applied to a second silica gel column, and eluted under the same conditions used for the first column. The material obtained under these conditions is 96% pure as detected by paper chromatography. Further purification was achieved either by quantitative paper chromatography or chromatography on a cellulose column using Solvent A. Quantities of 50 mg (5 % yield) have been isolated according to this procedure.

Two additional oxidation products of adenine, 8-hydroxyadenine and isoguanine, were produced when adenine was treated with H_2O_2 at concentrations between 0.5 and 2 M in 0.01 M phosphate buffer at pH 8.0 and at pH 8.5. The purification procedure for these two substances was the same as described for adenine-7-*N*-oxide, except that a slightly different final purification on paper or by column chromatography was used. The mixture of adenine-7-*N*-oxide, 8-hydroxyadenine, and isoguanine contaminated with trace amounts of adenine after two purification steps on a silica gel column was applied to a 42-cm-wide paper chromatogram of Schleicher and Schuell 2043b and developed in Solvent A. The ultraviolet-absorbing bands corresponding to the three different substances (see Table II) were cut into long strips and eluted with 10% ammonia. The eluents were freeze-dried and recrystallized 2 to 3 times from water. Adenine-7-*N*-oxide was sensitive to ultraviolet light and was kept in the dark as much as possible during the purification.

Chromatographic quantities of the 7-*N*-oxide of dAMP were synthesized by treating 200 $A_{260\,m\mu}$ units per ml of dAMP with H_2O_2 (0.05 M) in 0.05 M sodium borate buffer (pH 7.4) for 5 days at 37°. Separation of the 7-*N*-oxide of dAMP was achieved by paper chromatography in Solvent B (see Table II). The ultraviolet-absorbing material was eluted with 10% ammonia and then freeze-dried.

The 7-*N*-oxide of dAMP was depurinated according to a method described by TAMM, HODES and CHARGAFF[17].

Kinetic studies of the reaction of dAMP with 0.05 M H_2O_2 in 0.01 M borate buffer at pH 7.4 were carried out in tightly sealed, glass-stoppered centrifuge tubes. Control experiments without H_2O_2 were run in each case. The reaction conditions were the same as those described for the preparation of the 7-*N*-oxide of dAMP. 200-μl aliquots were removed at certain intervals, applied to Schleicher and Schuell 2043b paper and developed in Solvent A. The reaction products were detected by measuring the radioactive distribution in the paper chromatogram with a strip

counter (Baird Atomic Model RSC 372) and by observation with an ultraviolet lamp (Black Light Eastern Corp.). For quantitative measurements the radioactivity-containing spots were eluted with 3 ml of 10 % ammonia. The radioactivity was measured, after placing a sample in BRAY'S solution[18], with a Packard scintillation counter. The absorption was measured at the wavelength of maximal absorption in a Zeiss spectrophotometer PMQ II.

Radiolysis of adenine was performed using a solution of 100 $A_{260\,m\mu}$ units per ml adenine and 10 μC/ml[8-^{14}C]adenine in 0.05 M sodium phosphate buffer at pH 7.0. This solution was irradiated at $4 \cdot 10^6$ rads from a 0.5 kC ^{60}Co source. The irradiation products were analyzed by paper chromatography as described above.

A spectroline lamp R-51 with 90% emission at 253.7 mμ (Black Light Eastern Corp.) was used at a distance of 10 cm for the irradiation of adenine-7-*N*-oxide and also for 8- and 2-hydroxyadenine.

The reducibility of the three monooxides of adenine was tested by dissolving 0.2 mg of each compound in 200 μl of 0.1 M phosphate buffer (pH 7.0), in the presence of 0.4 mg Raney nickel and H_2 at 1 atm. After 6 h of incubation at 37°, the reaction mixtures were extracted with 5×2-ml aliquots of 10 % ammonia. These were pooled, freeze-dried, redissolved in 100 μl 10 % ammonia and chromatographed in Solvent A. The ultraviolet-absorbing spots were eluted with 10 % ammonia and the amount of material in each spot determined spectrophotometrically.

In order to determine the ability of adenine-7-*N*-oxide as well as 2- and 8-hydroxyadenine to induce point mutations, they were tested using the spot test technique and two phage T4 rII mutants[19]. The mutants AP 72, induced by 2-aminopurine, and N 28, induced by 5-bromouracil are known to induce reverse mutations of the transition type in both directions; AP 72, however, preferentially from G–C to A–T and N 28 from A–T to G–C. The plates used in the spot test were prepared as described by FREESE[19]. 1-day-old tryptone-agar plates were overlayed with a mixture of *Escherichia coli* K-12 ($2 \cdot 10^8$/ml), *E. coli* BA ($2 \cdot 10^7$/ml) and either phages AP 72 or N 28 ($2 \cdot 10^7$/ml) in soft agar. One drop of a saturated solution of each of the three adenine-oxides was placed on one side of the plate and a control sample of 2-aminopurine or 5-bromodeoxyuridine on the other. After storage at 4° for 2 h, the plates were incubated for 12–18 h at 37°. Reversion induction by the base analog was detected as an increased number of wild type plaques forming a halo around the location of the base analog.

Ultraviolet absorption spectra were obtained with the Cary Model 15 recording spectrophotometer and infrared absorption spectra with the Perkin–Elmer infracord spectrophotometer. The nuclear magnetic resonance spectra were recorded with a Varian HA 100 and the mass spectra with a LKB gas chromatograph/mass spectrometer.

Deoxyadenosine-5'-monophosphate was purchased from Calbiochem, deoxyadenosine and adenine from Pabst Laboratories, and the ^{14}C-labeled deoxynucleotides, deoxynucleosides and nucleobases from Schwarz BioResearch, Inc. H_2O_2 was bought from Fisher Scientific Co.

RESULTS

Identification of the adenine oxidation products

Adenine-7-N-oxide. When adenine was treated with H_2O_2 (0.05 M) as described

In MATERIALS AND METHODS, one new ultraviolet-absorbing spot with R_F 0.46 in Solvent A was formed (see Table II). Fig. 1 shows the distribution of radioactivity along a chromatogram (developed in Solvent A) when [8-^{14}C]adenine was treated with H_2O_2. Peak I was a non-ultraviolet absorbing degradation product of adenine,

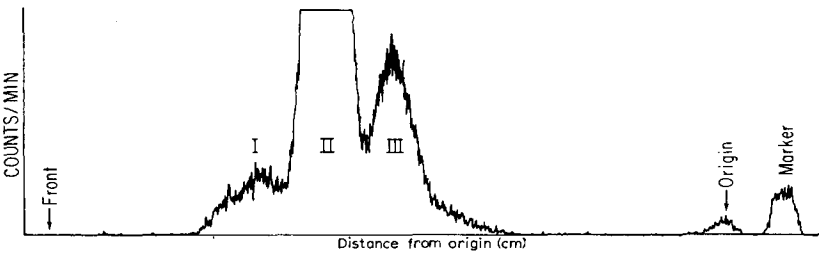


Fig. 1. Radioactive pattern of [8-^{14}C]adenine after reaction with 0.05 M H_2O_2 at pH 7.4 for 5 days at 37°.

which has not been investigated further. Peak II corresponded to the starting material, [8-^{14}C]adenine, and Peak III represented the newly formed ultraviolet-absorbing product with R_F 0.46, which was identified as adenine-7-*N*-oxide as will be seen below. The compound corresponding to Peak III was synthesized in larger quantities and purified as described in MATERIALS AND METHODS. Physicochemical data were obtained from recrystallized samples. The ultraviolet spectrum of this substance showed maxima at 265 mμ (pH 2), 270 mμ (pH 6), and 278 mμ (pH 12), Table I. The resemblance of this compound to adenine indicated that both substances possessed the same chromaphor. Therefore, the compound of Peak III could be expected to be a derivative of adenine. From mass spectroscopical data (Table I), showing an intense peak at m/e 151, it was concluded that this compound was the monooxide of adenine. There are six different possibilities for the monooxidation of adenine (see Fig. 2). Compound I of Fig. 2, which is 6-hydroxylaminopurine, a sample of which was kindly provided by Dr. G. B. BROWN, could be shown to be different in ultraviolet spectrum at acidic and basic pH's and R_F

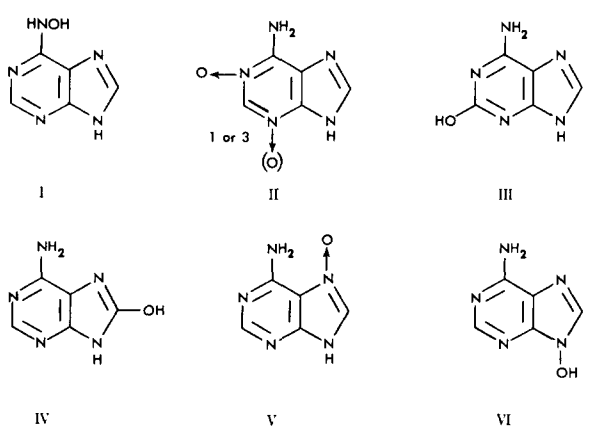


Fig. 2. Possible monooxidation products of adenine.

TABLE I

SPECTRAL DATA FOR SOME ADENINE OXIDATION PRODUCTS

Compound	*Ultraviolet spectra Wavelength (mμ) at different pH*				*Mass spectra m/e for M^+*	*Nuclear magnetic resonance spectra*			
	λ_{max}, *pH* 2	λ_{max}, *pH* 6	λ_{max}, *pH* 12	ε_{max}, *pH* 6		*ppm in* NaO^2H		*ppm in* ^2H_2O	
Adenine	263	265	269	12.3	135	8.20,	8.32	8.05,	8.17
Adenine-7-*N*-oxide	265	270	278		151			8.39,	8.43
8-Hydroxyadenine	270	268	278	12.8 (ref. 20)	151	8.10			
2-Hydroxyadenine	284	286	240 286	8.0 (ref. 20)	151	7.83			

TABLE II

PAPER CHROMATOGRAPHY

Schleicher and Schuell 2043b paper was used for paper chromatography in Solvent A. The same paper, presoaked with 1 % $(NH_4)_2SO_4$ and dried, was used for Solvent B. Solvent A, isopropanol–conc. ammonia– water (7:1:2, by vol.). Solvent B, 1 % $(NH_4)_2SO_4$–isopropanol (1:2, by vol.).

Compound	*R_F values in solvent*	
	A	*B*
Adenine	0.56	0.66
Adenine-1-*N*-oxide	0.46	0.59
Adenine-7-*N*-oxide	0.46	0.59
8-Hydroxyadenine	0.34	0.35
2-Hydroxyadenine	0.29	0.29
6-Hydroxylamine purine*	0.56	0.66
Hypoxanthine	0.52	0.60
8-Hydroxyadenine-1-*N*-oxide*	0.44	0.54
2,8-Dihydroxyadenine*	0.18	
Deoxyadenine	0.64	
dAMP	0.17	0.51
7-*N*-oxide of dAMP	0.17	0.32

* Kindly provided by Dr. G. B. Brown.

in two systems (see Table II). The oxidation of adenine at N-1 or N-3 (Compound II, Fig. 2) would result in a characteristic change of its ultraviolet spectrum, namely the appearance of a second maximum at 231 mμ (pH 7.0) with an extinction coefficient at this wavelength of $41.5 \cdot 10^{-3}$ as shown by BROWN *et al.*[20,39]. This second maximum, however, is missing in the ultraviolet spectrum of the substance of Peak III, Fig. 1 (see ultraviolet spectrum of A_1)[15].

Structures like Compound III and IV (Fig. 2), isoguanine and 8-hydroxyadenine, can also be ruled out since the nuclear magnetic resonance spectrum in ^2H_2O with 3-trimethylsilo-1-propane-sulfonic acid sodium salt (TMSP) as an internal standard showed two signals at 8.39 ppm and 8.43 ppm. These signals correspond to the two aromatic protons at C-2 and C-8 of the purine ring, since the two protons of the amino group are exchanged in ^2H_2O. They are also present under the same conditions in adenine but shifted to 8.05 ppm and 8.17 ppm (see Table I). The oxidation of adenine must have occurred, therefore, at the imidazole ring leading to either Compound V or VI (Fig. 2). The decision in favor of Compound V, the adenine-7-*N*-oxide, was made because dAMP, which is the 9-substituted derivative of adenine (9-ß-deoxyribofuranosyl-5'-phosphate), yielded the same oxidation product as adenine itself (see below) when the altered and purified deoxynucleotide was depurinated. Further confirmation that the altered adenine is adenine-7-*N*-oxide was obtained from a negative ferric chloride test and an m/e 151 *minus* 16 peak (m/e 135) in the mass spectrum. This peak corresponded to the parent compound adenine after the loss of oxygen.

It is possible that compound V (Fig. 2) does not predominantely have the configuration of adenine-7-*N*-oxide but is rather present as the tautomeric 7-*N*-hydroxyadenine. For convenience the compound will be called adenine-7-*N*-oxide until the tautomeric structures are established.

Anal.: Calculated for $C_5H_5N_5O \cdot H_2O$; C, 35.51; H, 4.17; N, 41.40.
Found: C, 35.21; H, 4.32; N, 40.88.

Attempts to further increase the yield of adenine-7-*N*-oxide by reaction with 1–2 M H_2O_2 in 0.05 M phosphate buffer at pH 7.0 resulted in the formation of an additional product with R_F 0.34 (8-hydroxyadenine) in Solvent A (see Table II and Fig. 3a, Peak IV). In addition to 8-hydroxyadenine a third oxidation product of adenine with R_F 0.29 in Solvent A (2-hydroxyadenine) was formed under the same condition as above except at pH 8.0 (see Fig. 3b, peak V). The physicochemical characterization of both substances will be described separately below.

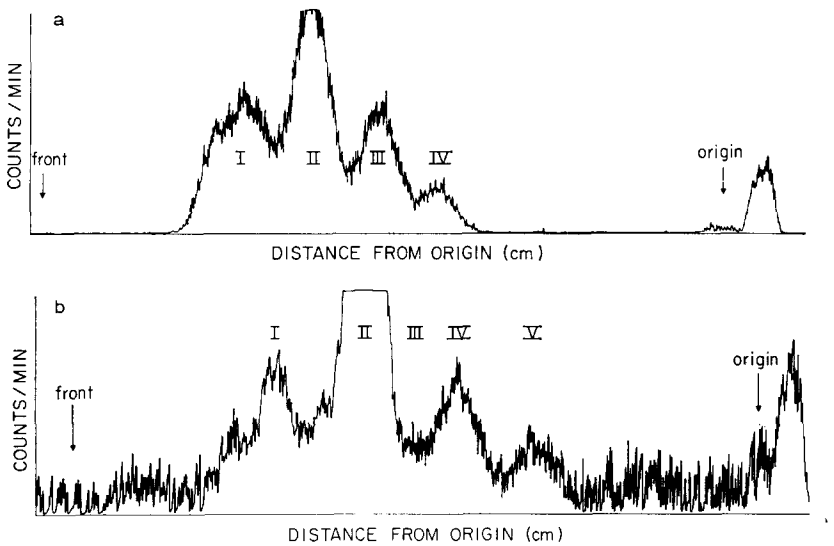
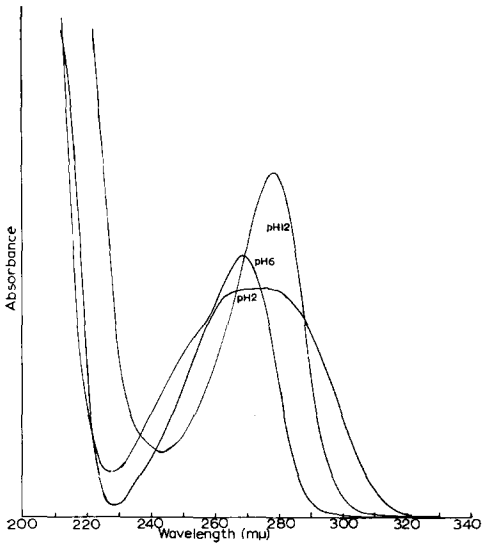


Fig. 3. a, Radioactive pattern of [8-^{14}C]adenine after reaction with 1–2 M H_2O_2 at pH 7.0. b, Radioactive pattern of [8-^{14}C]adenine after reaction with 2 M H_2O_2 at pH 8.0.

8-Hydroxyadenine. This compound was separated from adenine-7-*N*-oxide (R_F 0.46 in Solvent A) and 2-hydroxyadenine (R_F 0.29 in Solvent A, see below), obtained by treating adenine at pH 8.0 (0.01 M phosphate) and 2 M H_2O_2, by preparative paper chromatography and subsequent elution of the band with R_F 0.34 as described in MATERIALS AND METHODS.

Physicochemical data were obtained from 3 times recrystallized samples. The ultraviolet spectrum of this compound (see Fig. 4) at different pH's is in agreement with 8-hydroxyadenine obtained by CAVALIERI AND BENDICH[21] by passing phosgen into an alkaline solution of 4,5,6-triaminopyrimidine sulfate. The mass spectrum with an intense peak at m/e 151 indicated that this substance also was a monooxide of adenine. Of the six possibilities for the monooxidation of adenine (see Fig. 2) Compounds I, II and VI could be ruled out for the same reasons mentioned in connection with the adenine-7-*N*-oxide formation. Since adenine-7-*N*-oxide had an R_F of 0.46, it was concluded that the oxidation occurred at either C-2 or C-8 (Compound II or IV in Fig. 2). This was confirmed by NMR-spectroscopy, showing only one signal at 8.10 ppm in alkaline ^2H_2O instead of the two signals of adenine at 8.20 ppm and 8.32 ppm under the same conditions (see Table I). Compound III, which is isoguanine and commercially available, however, has a different R_F in two solvents and a different ultra-



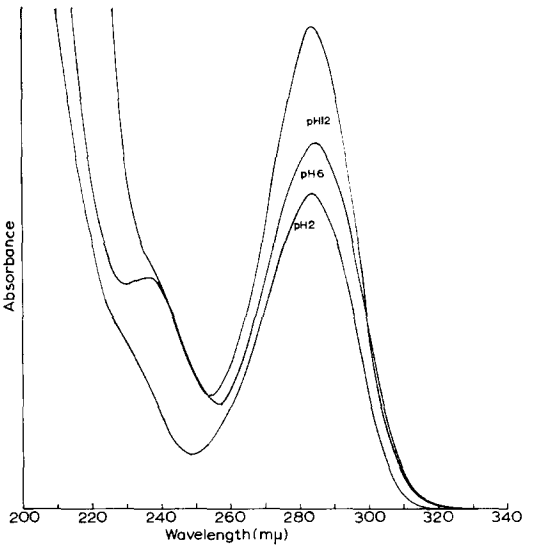


Fig. 4. Ultraviolet spectra of 8-hydroxyadenine at acid, neutral and basic pH.

Fig. 5. Ultraviolet spectra of 2-hydroxyadenine at acid, neutral and basic pH.
(Revised Figs 4 and 5 received July 1st, 1968)

violet spectrum at pH 2.0, 6.0 and 12.0. An infrared absorption band at 1710 cm^{-1} (5.85 μ) confirmed that the compound with R_F 0.34 in Solvent A was 8-hydroxyadenine.

Anal.: Calculated for $C_5H_5N_5O \times H_2O$. C, 35.51; H, 4.17; N, 41.40.

Found.: C, 35.75; H, 4.15; N, 41.26.

2-Hydroxyadenine (isoguanine). This substance was separated from adenine-7-*N*-oxide and 8-hydroxyadenine as described above, except that the band with R_F 0.29 was eluted from the preparative paper chromatogram and recrystallized 3 times from H_2O. Mass spectroscopic measurements revealed that this compound had a molecular weight of 151, which corresponds to a monooxide of adenine. The ultraviolet spectrum is shown in Fig. 5. It is identical to one already attributed to isoguanine in the literature[22]. Since this compound is different from Compounds I and II for the same reasons as mentioned above, and since it is also chromatographically and spectroscopically different from adenine-7-*N*-oxide (Compound V, Fig. 2) and 8-hydroxyadenine (Compound IV, Fig. 2), it was necessary to decide between Structure III and VI only. The NMR spectrum, showing one signal at 7.83 ppm in alkaline ^2H_2O and TMSP as internal standard (see Table I), and the infrared spectrum, which is identical to one obtained from commercially available isoguanine, clearly decided in favor of Structure III, Fig. 2.

Irradiation of adenine and identification of some reaction products

In order to compare the effect of X-irradiation to that of H_2O_2, the reaction products of irradiated adenine were analyzed by paper chromatography.

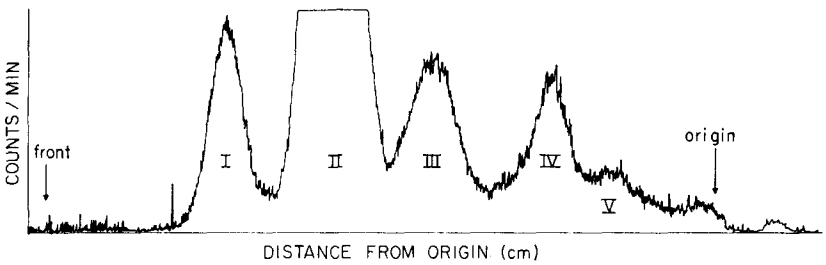


Fig. 6. Radioactive pattern of [8-^{14}C]adenine after irradiation with $5 \cdot 10^6$ rads at pH 7.0.

When adenine and [8-^{14}C] adenine were irradiated with $5 \cdot 10^6$ rads as described in MATERIALS AND METHODS, one minor and three major ultraviolet-absorbing products could be detected on a paper chromatogram. Fig. 6 shows the reaction products obtained by measuring the distribution of radioactivity along the chromatogram with a strip counter. The ultraviolet-absorbing spots designated as Peaks II, III and IV in Fig. 6 were eluted and rechromatographed in two different systems together with known, nonradioactive compounds. The radioactivity of some of the unknown compounds coincided with the ultraviolet absorption of the known compound. Together with the ultraviolet absorption spectra it was possible to identify the ultraviolet-absorbing substances contained in Peak III as adenine-7-*N*-oxide and Peak II as adenine probably contaminated with hypoxanthine, since hypoxanthine has a R_F value similar to adenine in both Solvent A and B. No attempts were made to separate these two substances, since hypoxanthine has already been described as one of the irradiation products of adenine[23]. Peak I and the non-ultraviolet-absorbing Peak V, as well as the smear of radioactivity from the origin to Peak V, was not further characterized. Quantitative measurements revealed that 2.06% of the starting material was converted to the complex breakdown products contained in the smear from the origin to Peak V, 4.41% was converted to the material in Peak V, 8.10% to the material in Peak IV, 13.45% to adenine-7-*N*-oxide (Peak III), 8.42 % to the material in Peak I, and 63.55% remained as adenine, possibly contaminated with an unknown amount of hypoxanthine (Peak II). Comparing these results with those obtained with H_2O_2, it is obvious that the formation of adenine-7-*N*-oxide during irradiation is due to the OH radicals formed during this process.

The oxidation of dAMP

Since adenine-7-*N*-oxide was initially detected only because it was liberated from dAMP after the oxidation of the C-1 carbon of the deoxyribose moiety[8], it was of interest to study the influence of the 7-*N*-oxide formation on the stability of the deoxynucleotide. Any influence weakening the N-glycosidic linkage could contribute to the inactivating effect of OH radicals by depurination. The absence of influence on this linkage could have either a point mutagenic or no effect.

Isolation and characterization of 5'-adenylic acid-7-N-oxide

When [8-^{14}C]dAMP was treated with H_2O_2 (0.05 M) for 5 days at 37°, as described in MATERIALS AND METHODS, several products could be detected after paper

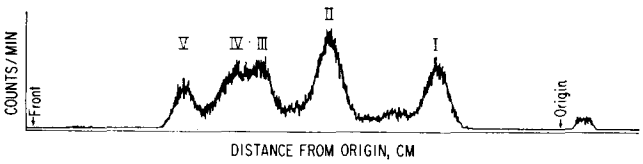


Fig. 7. Radioactive pattern of [8-^{14}C]dAMP after reaction with 0.05 M H_2O_2 at pH 7.4.

chromatography in Solvent B. Fig. 7 shows the distribution of radioactivity along the paper chromatogram. The pattern is similar to one already described in a previous paper[15], except that this time a chromatographic system (Solvent B) was used which separated Peak I (altered dAMP) from Peak II (dAMP). The other Peaks, III, IV and V, corresponded to liberated adenine-7-*N*-oxide, adenine and a non-ultraviolet-absorbing substance, respectively. Peak I was isolated as described in MATERIALS AND METHODS and shown to contain mainly 5'-adenylic acid-7-*N*-oxide and traces of 8- and 2-hydroxydeoxyadenosine-5'-monophosphate (see Table III), since the corresponding bases were found after mild acidic hydrolysis (depurination). Because of the ultraviolet-light sensitivity of adenine-7-*N*-oxide, adenine was also formed during the process of depurination and identification. Traces of an additional, not further characterized and non-ultraviolet-absorbing material with R_F 0.09 in System A could also be detected after depurination.

The 7-*N*-oxide of dAMP is stable in aqueous solution (0.01 M phosphate buffer, pH 7.0) for more than 5 days and also in 1 M NaOH at 37° for at least 5 h. It is depurinated at approximately the same rate as dAMP by 1 M HCl at 37°.

Kinetics of the destruction and alteration of dAMP. In view of the extensive production of 7-*N*-oxide of dAMP, it was of interest to follow the alteration of dAMP quantitatively. When 0.2-ml aliquots (20 $A_{260\,m\mu}$ units) of a reaction mixture containing [8-^{14}C]dAMP and H_2O_2 in concentrations as described in MATERIALS AND METHODS were chromatographed on paper in Solvent B, it was found that the radioactivity of the spot corresponding to dAMP decreased (Curve 2 of Fig. 8). Liberated adenine increased linearly at the same time (Curve 1). The production of 7-*N*-oxide of dAMP (Curve 3) increased within the first 72 h. It later decreased due to the liberation of adenine-7-*N*-oxide, which increased simultaneously with the square of time

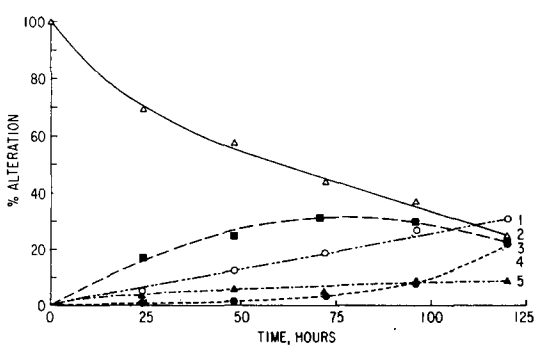


Fig. 8. Kinetics of the reaction of dAMP with 0.05 M H_2O_2.

(Curve 4 of Fig. 6). The non-ultraviolet-absorbing destruction product increased only slightly with time (Curve 5, Fig. 8).

Chemical reactions of the oxidized adenine derivatives

Ultraviolet irradiation. Since adenine-1-*N*-oxide is known to decompose mainly into adenine and 2-hydroxyadenine[24] when irradiated with ultraviolet light at a wavelength of 254 mμ, it was of interest to determine whether or not adenine-7-*N*-oxide shows analogous properties. A solution of [8-^{14}C]adenine-7-*N*-oxide (0.5 $A_{265\,m\mu}$ unit per ml) in 0.01 M phosphate buffer (pH 7.0), was irradiated as described in MATERIALS AND METHODS for 1 h. 3 ml of this solution were freeze-dried, redissolved in 100 μl H_2O and applied to a chromatogram (SS2043b). On the same chromatogram a freeze-dried solution of 3 ml of an unirradiated solution (0.5 $A_{265\,m\mu}$ unit per ml) of adenine-7-*N*-oxide was applied as reference, and the chromatogram was developed in Solvent A. The distribution of radioactivity along the chromatogram was measured with a strip counter. In addition to the undecomposed adenine-7-*N*-oxide (32.6%), two other peaks with R_F 0.38 (48.8%) and R_F 0.57(18.6%) could be detected. The unirradiated control sample showed only a small second peak with R_F 0.38 due to a minor impurity in the original preparation. The peak with R_F 0.38 was shown to be 8-hydroxyadenine and the one with R_F 0.57 adenine by comparing their chromatographic and their ultraviolet absorption characteristics. The mechanism by which adenine-7-*N*-oxide is decomposed into adenine and 8-hydroxyadenine is obviously the same as described by BROWN, LEVIN and MURPHY[24] for adenine-1-*N*-oxide. This behavior is in additional proof that A_1 already described by RHAESE, FREESE and MELZER[15] is adenine-7-*N*-oxide. The other adenine oxidation products, 8-hydroxyadenine and 2-hydroxyadenine, are not affected by ultraviolet irradiation for at least 1 h.

Hydrogenation. To a 1-ml solution (1.0 $A_{270\,m\mu}$ unit per ml) of each [8-^{14}C]adenine-7-*N*-oxide, [8-^{14}C]8-hydroxy- and 2-hydroxyadenine in water, approx. 0.1 mg of Raney nickel was added and the mixture shaken with H_2 at room temperature and atmospheric pressure for 6 h. After removal of the Raney nickel, the reaction mixtures were applied to a chromatogram (SS2043b), which was developed in Solvent A. The corresponding untreated control mixtures were applied adjacent to the reaction mixture. The compounds separated along the chromatogram were detected with a strip counter. It was found that adenine-7-*N*-oxide was reduced to adenine (the R_F of the hydrogenated substance was identical with adenine). Two minor, not further characterized reaction products with R_F 0.11 and R_F 0.90 were also found. The untreated adenine-7-*N*-oxide showed no decomposition. 8- and 2-hydroxyadenine were not affected by H_2 and Raney nickel.

Test for the adenine oxidation products to induce point mutations

Since the adenine oxidation products are base analogs, they might be able to induce point mutations, as 5-bromouracil, for example, induces forward and reverse mutations in the phage T4 rII (refs. 16, 19). In order to test this, it was necessary first to prove that these base analogs can enter the cell and become incorporated into trichloroacetic acid-precipitable material.

When [8-^{14}C]adenine-7-*N*-oxide was added to a growing culture of *Bacillus subtilis* 60015 (at $A_{600\,m\mu}=0.8$) in nutrient broth, 50% of the radioactivity was incorporated into trichloroacetic acid-precipitable material after 30 min of incubation at 37°. The three adenine oxidation products, however, did not induce any revertants in phage T4 rII mutants even though the control samples, 2-aminopurine and 5-bromodeoxyuridine, gave positive results as shown in the spot test described in MATERIALS AND METHODS. Two other substances, 7-methyl- and 7-ethylguanine, whose structures are similar to that of adenine-7-*N*-oxide, were also tested and found to be ineffective.

DISCUSSION

The direct oxidation of adenine to adenine-1-*N*-oxide by H_2O_2 in acetic acid has been reported[25]. The isolation and characterization of adenine-1-*N*-oxide and some of its derivatives has also been described[20,26–28], using H_2O_2 acetic acid mixtures. Perphthalic acid at pH 7 has been used for the synthesis of 1-*N*-oxides of adenine nucleotides[29] and of cytosine nucleotides[30].

In contrast to this almost quantitative conversion of adenine and its derivatives to adenine-1-*N*-oxide by H_2O_2 in acetic acid, there is no detectable amount of this 1-*N*-oxide formed when adenine is treated with H_2O_2 in borate or phosphate buffer at pH 7.0 and above. This difference must be due to the different reactive species in both systems. When adenine-1-*N*-oxide is formed by H_2O_2 in acetic acid solution, it is known that not H_2O_2 itself but rather its reaction product with acetic acid, peroxyacetic acid, reacts with adenine. The reactive species is probably an ^{+}OH, which reacts as an electrophile with adenine at the N-1 nitrogen. A similar reaction mechanism has been suggested for the N-1 oxidation of AMP and CMP by another peroxyacid; namely, monoperphtalic acid[30]. In contrast, H_2O_2 in neutral or slightly alkaline buffer solution, particularly in the presence of trace amounts of transition metals or ultraviolet light, forms OH radicals which react quite differently with adenine. It is known that aromatic molecules exhibit a relatively great stability toward radicals. The stability of the aromatic pyrimidine ring in adenine against oxidation by OH radicals, therefore, can be explained through the delocalization of the π electrons of this ring[31]. The reactivity of the imidazole ring, evidenced by the formation of the 7-*N*-oxide, on the other hand, can be explained with the strong double-bond character of the N-7–C-8 nitrogen-carbon double bond and the presence of high free valences on the atoms forming this bond[32].

The formation of adenine-7-*N*-oxide through OH radicals can, therefore, be described by a reaction mechanism in which the primary attack of an OH radical leads to the quaternization of N-7 with an additional unpaired electron. This unstable product can be stabilized by the removal of a H radical, thus producing a stable adenine-7-*N*-oxide.

At low H_2O_2 concentrations ($<$ 0.1 M and pH 7.0), this reaction mechanism predominates. Increasing H_2O_2 concentrations, however, does not increase the yield of adenine-7-*N*-oxide remarkably, but rather leads to the oxidation of C-8, giving rise to 8-hydroxyadenine. A further increase in H_2O_2 increases only slightly the yield of these products and also the amount of non-ultraviolet-absorbing material (Peak I,

TABLE III

REACTION CONDITIONS AND YIELD OF THE DIFFERENT OXIDATION PRODUCTS

Compound	*Concentration of buffer (M)*	*Buffer*	*pH*	*H_2O_2 Conc. (M)*	*Yield in %*			
					Adenine--7-N-oxide	*8-Hydroxy-adenine*	*2-Hydroxy-adenine*	*7-N-oxide of dAMP*
Adenine	0.01	Phosphate	7.0	0.05	8–15	—	—	—
Adenine	0.01	Phosphate	8.0	2.0	8.0	18.4	13.1	
dAMP	0.05	Borate	7.4	0.05	9.4	—	—	10.8
dAMP	0.05	Borate	8.0	1.0		20.9		20.8

Fig. 1.) but does not result in additional oxidation products. However, when adenine is treated at pH 8 and high concentrations of H_2O_2 ($\geqq$ 1 M), an additional oxidation product, 2-hydroxyadenine, is formed (see Table III). This reaction involves an aromatic substitution by an OH radical. The primary abstraction of a hydrogen from C-2 is either followed by an attack of another OH radical leading to 2-hydroxyadenine in a radical chain termination reaction or by H_2O_2 forming the same product and an additional OH radical (chain propagation reaction).

The oxidation of the adenine moiety at N-7 has no detectable effect on the stability of the dAMP molecule, which has been shown by incubating this product at basic and acidic pH.

Since dAMP is as stable as dAMP-7-*N*-oxide at different pH's, it is concluded that the oxidation of the adenine moiety at N-7 and the liberation of adenine-7-*N*-oxide from the 7-*N*-oxide of dAMP are two independent reactions and that this liberation is due to the oxidation of the C-1 carbon of the deoxyribose moiety as described in a previous paper[14]. The kinetics of the reaction of dAMP with H_2O_2 (see Fig. 8) proves this point, since the formation of adenine and 7-*N*-oxide of dAMP increases linearly with time, at least for the first 50 h, whereas that of adenine-7-*N*-oxide increases with the square of time. The decrease from linearity of the 7-*N*-oxide of dAMP formation (Curve 3, Fig. 8) is due to the liberation of adanine-7-*N*-oxide. After approx. 80 h, the maximal 7-*N*-oxide of dAMP production with 32% of the total dAMP-alteration is reached. 40 h later, however, only 22% is left. Comparing the initial rates of 7-*N*-oxide of dAMP formation and base liberation, it is obvious that adenine alteration is twice as fast as adenine liberation. This might be important for the consideration of the weak mutagenic effect of radical-producing agents, as will be discussed below.

It is known that X-rays which produce, besides others, OH radicals[6], are able to induce mutations in Drosophila[33] as well as plants[34] and other organisms. RÜGER AND KAPLAN[35] and BECKMANN AND KAPLAN[36] observed the induction of mutations by X-irradiation and H_2O_2 in the phage $\varkappa$. As both X-irradiation and H_2O_2 treatment of adenine produce adenine-7-*N*-oxide, it is possible that this compound in fact is responsible for the induction of mutations. A certain similarity of the mutation spectra of ultraviolet light and of H_2O_2 indicated that alterations of adenine might be responsible for the mutation induction[36].

If adenine-7-*N*-oxide is mutagenic, it could induce mutations in phages or bacteria when added to the growth medium only if it can be incorporated into DNA. The incorporation of radioactivity from added [8-^{14}C]adenine-7-*N*-oxide into trichloroacetic acid precipitable material indicates that the purine residue is incorporated into RNA, DNA, or both. Assuming it is incorporated into DNA, the lack of mutation induction could be explained by prior reduction to adenine, or, in the absence of any such modification, the inability of adenine-7-*N*-oxide to produce any mutagenic effect.

There are other base analogs which probably for similar reasons do not induce mutations when added to the growth medium[37]. 7-methyl- and 7-ethylguanine, *e.g.*, which are similar in structure to adenine-7-*N*-oxide, also do not induce mutations in the two transition mutants, as shown in the spot test. Yet they seem to be responsible for the induction of point mutations when formed within DNA (ref. 38).

Since 2- and also 8-hydroxyadenine are only formed at H_2O_2 concentrations

above 1 M, they are unlikely to be responsible for the induction of mutations and therefore excluded from these considerations.

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