RADIOLYSIS OF AQUEOUS SOLUTIONS OF ADENINE

BY

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RADIOLYSIS OF AQUEOUS SOLUTIONS

OF ADENINE

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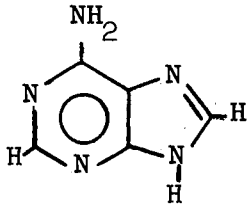
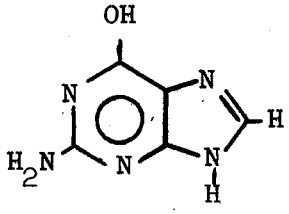
CHAPTER I

INTRODUCTION

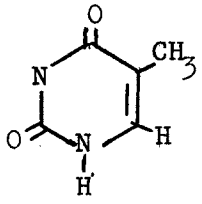
The harmful effects of ionizing radiation on living cells are believed to originate partially, if not wholly, from alteration of the DNA. Since DNA is a complicated polymeric substance, it is reasonable to study the radiolysis of its components as a first step. The DNA bases (Figure 1) provide a good beginning. The arrangement of these bases in DNA constitutes the genetic code; hence, if they are damaged or destroyed, the organism may die or be mutated.



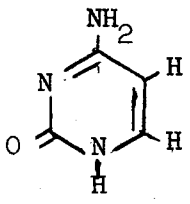
Adenine



Guanine



Thymine



Cytosine

Figure 1. The Four DNA Bases

Adenine was chosen for study because relatively little was known about its radiolysis. The remainder of this Chapter contains a selected literature survey on the irradiation of adenine in aqueous solution. Chapter II contains two papers reporting the principal results of this investigation in a form suitable for publication in Radiation Research. These papers omit descriptions of several experimental details, which are covered in Chapter III.

LITERATURE SURVEY

In one of the earliest investigations of the irradiation of adenine in aqueous solution, E. S. G. Barron, et al. (1) determined the effect of x-irradiation on the absorption spectrum of adenine and other DNA bases. Adenine has an intense absorption maximum at 260 nm. For an oxygen-containing 4×10^{-5} M solution of adenine, the absorbance decreased as the adenine was decomposed. Assuming no absorbance at 260 nm by the products of irradiation, the decomposition yield was calculated to be 0.676 molecules/100 ev (1). This value is lower than might be expected in view of the amount of reactive species produced by irradiation of water (G for production of hydroxyl radical ($OH\cdot$) and hydrated electron (e^-_{aq}) is approximately 3 (2,3,4)), and the high reactivity of these species with adenine (5,6,7). This low value was later affirmed by Scholes, Weiss, and Ward (8). They attributed the low decomposition yield of adenine to the occurrence of reconstitution.

A summary of reported values of G(-adenine), as determined by both ultraviolet and chromatographic methods, is shown in Table I for the radiolysis both in the presence and absence of oxygen. The variety of conditions and methods used in their determinations precludes

TABLE I

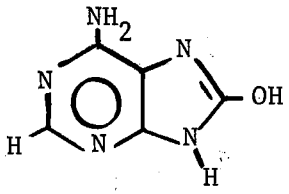
TYPICAL LITERATURE VALUES OF G(-ADENINE)

G(-Adenine) O_2 Present		G(-Adenine) O_2 Absent	Concentration		Dose	
UV	Chrom	Chrom	Moles Liter^{-1}	Conditions	(Rads)	Ref.
0.676			4.0×10^{-5}	pH 7 Buffer	Extrapolated	1
1.09	1.13		2.0×10^{-4}	pH 5.1	Extrapolated	8
	0.85		1.0×10^{-3}	H_2O	3×10^5	9
	0.54		7.4×10^{-3}	pH 7 Buffer	5×10^6	13
	1.2		1.2×10^{-4}	H_2O	---	10
		0.96	7.4×10^{-3}	pH 7 Buffer	1×10^6	12
		0.35	1.0×10^{-3}	H_2O	4.4×10^6	9
		0.6	1.0×10^{-4}	H_2O	---	10

comparison. However, the trend seems to be toward slightly higher values of G(-adenine) with oxygen present.

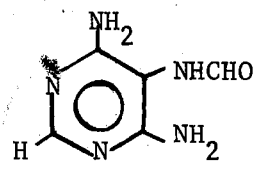
Conlay (9) demonstrated the presence of 8-hydroxyadenine and 4,6-diamino-5-formamidopyrimidine (Compounds I and II, Figure 2) in the radiolyzed solutions of adenine both with oxygen present and absent. Van Hemmen and Bliechrodt (10) identified I, II, and III (6-amino-8-hydroxy-7,8-dihydropurine) in the absence of oxygen. They found I and small amounts of II in the presence of oxygen. In addition to I and II, Ponnamperums, et al. (11,12) reported that hypoxanthine and 4-amino-5-formamido-6-hydroxypyrimidine were formed in small yields when adenine was irradiated in the absence of oxygen.

Rhaese (13) reported adenine 7-N-oxide (IV) formed in the presence of oxygen.



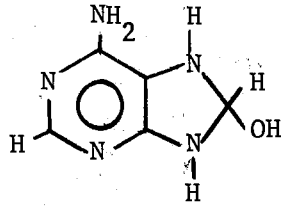
8-Hydroxyadenine

I



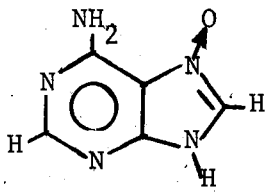
4,6 Diamino-5-formamidopyrimidine

II



6-Amino-8-hydroxy-7,8-dihydropurine

III



Adenine-7-N-oxide

IV

Figure 2. Major Ultraviolet Absorbing Compounds Produced in the Radiolysis of Adenine

The compounds in Figure 2 have substantial absorptions at 260 nm. Therefore, the decomposition yield of adenine calculated on the basis that the products do not absorb is low. To determine the amount of adenine actually decomposed, various investigators used chromatographic methods to analyze the radiolyzed solutions (8,9,10,12,13). The accuracy of these methods is, however, not high. Furthermore, the yield decreases with dose, and most values reported by investigators were determined at high doses. An accurate determination of the initial destruction yield of adenine is reported in this study. We find that our G(-adenine) values are higher than the previously reported values.

From the number of products formed in the radiolysis, it may be inferred that the decomposition mechanisms are complex. The reactive species produced by interaction of γ-rays with water play a major role in the radiolysis of an organic substrate. Water is decomposed mainly as shown in Equation 1 (14). In the absence of an organic solute, the hydrated electron ($(H_2O\cdot)^-$) can react with the hydronium ion (H_3O^+) (Equation 2) (15) with a rate constant equal to 2×10^{10} liters/mole-sec (16).

$$3\ H_2O \longrightarrow (H_2O\cdot)^- + OH\cdot + H_3O^+ \qquad (1)$$

$$H_3O^+ + (H_2O\cdot)^- \longrightarrow H\cdot + 2\ H_2O \qquad (2)$$

In the presence of molecular oxygen, $(H_2O\cdot)^-$ is scavenged (15) as shown in Equation 3, with a rate constant equal to 2×10^{10} liters/mole-sec (16).

$$(H_2O\cdot)^- + O_2 \longrightarrow HO_2\cdot + OH^- \qquad (3)$$

Reaction schemes for the radiolysis of adenine have been proposed by many investigators (9,10,17,18,19). Figure 3 shows the mechanisms proposed by Van Hemmen and Bleichrodt (10) for formation of some major products. They proposed that 8-hydroxyadenine (I) was formed via disproportionation of hydroxyl radicals (reaction a) in accordance with Keck (19) or by reaction of the hydroxyl radical adduct of adenine with adenine (reaction b). In contrast to mechanisms proposed by other investigators (9,20), they show that the amount of 4,6 diamino-5-formamidopyrimidine (II) formed was not affected by adding N_2O of sufficient concentration to completely scavenge e^-_{aq}. They concluded that e^-_{aq} was not involved in formation of this compound (reaction c).

The mechanisms of product formation are undoubtedly more complex than Figure 3 suggests. Oxygen in the radiolysis solution plays a major role in the determination of reaction pathways since it reacts with the hydrated electron and the reactive species derived from adenine. Quantum-chemical calculations (21,22) suggest that radicals attack adenine mainly at the N_7-C_8 double bond. Indeed, the radiation products which have been found in neutral solutions (9,10,11,12,13) provide evidence for OH· attack at the N_7-C_8 double bond. The products formed are also pH dependent. Holian and Garrison (17) concluded that in radiolysis of acid solutions, the C_4-C_5 double bond is the major locus of attack. In neutral solutions, these same authors (18) found no evidence for this locus of attack.

In order to further clarify and add new knowledge to the information on the radiolysis of adenine, we have studied the radiolysis with emphasis on finding the initial destruction yield of adenine under various conditions and identification of the products by radiolysis.

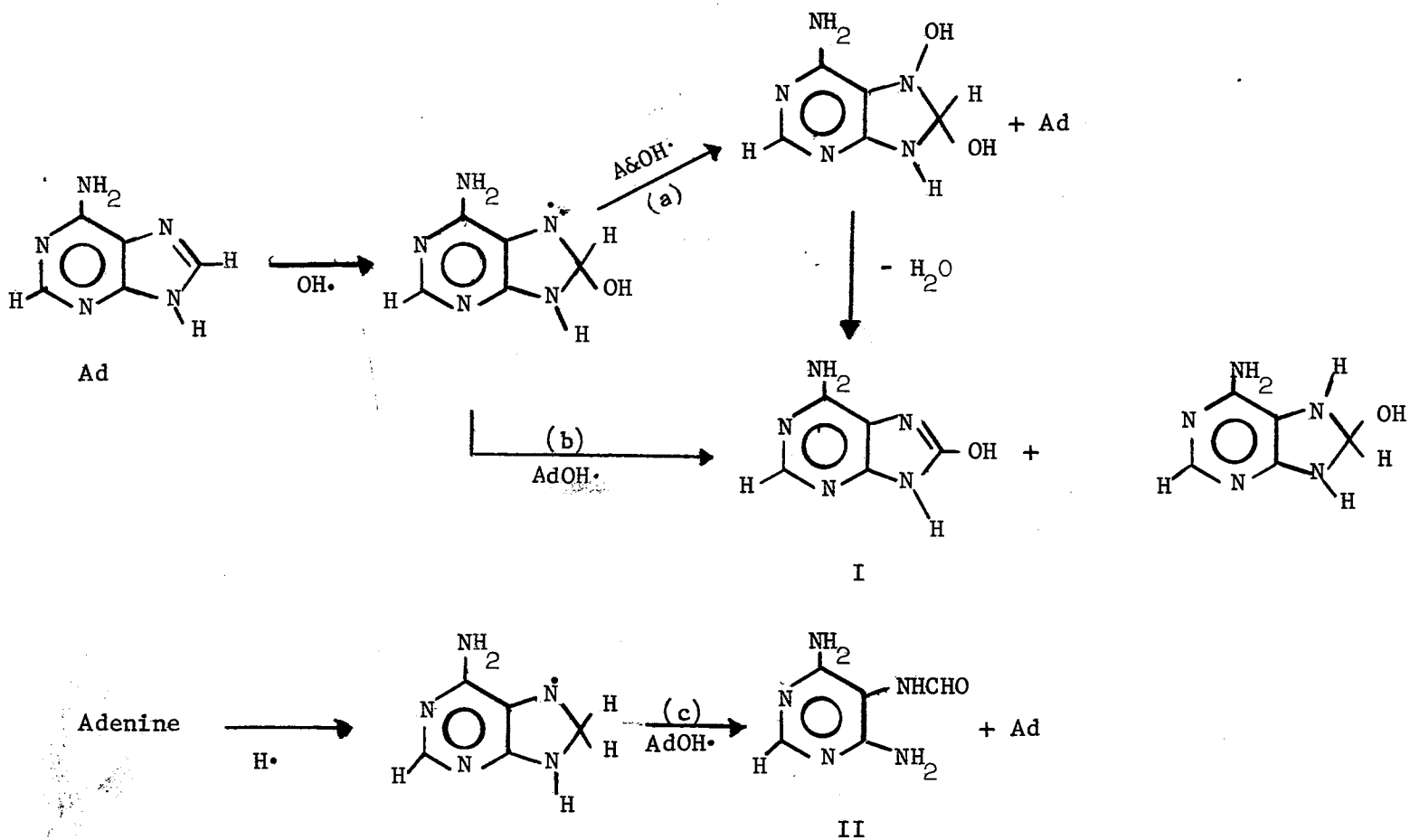


Figure 3. Suggested Mechanisms for the Decomposition of Adenine During Radiolysis

CHAPTER II

RADIOLYSIS OF AQUEOUS SOLUTIONS OF ADENINE -- I

NEW DECOMPOSITION YIELD STUDIES

C. A. Mannan, G. Gorin, S. E. Scheppele, and C. Lehman*

Mannan, C. A., Gorin, G., Scheppele, S. E., and Lehman, C. The Radiolysis of Aqueous Solutions of Adenine -- I, New Decomposition Yield Studies. To be submitted for publication in Radiation Research.

Aqueous solutions of adenine were irradiated by ^{60}Co γ-rays. The rate of decomposition was determined by ultraviolet spectroscopy and a specific colorimetric test for adenine. The decomposition yield of a 2×10^{-4} M solution adenine equilibrated with air was found to be 1.83 molecules/100 ev and 1.96 molecules/100 ev for an oxygen-saturated solution of the same concentration. Increasing the concentration of adenine to 2×10^{-3} M gave a G-value of 2.10 for air-equilibrated solutions and 3.10 for oxygen-saturated solutions.

Under anoxic conditions, the G-value for adenine decomposition is 1.10 for a 2×10^{-4} M solution and 1.05 for a 2×10^{-3} M solution. The rather low yield in these cases is attributed to reconstitution of the adenine.

INTRODUCTION

The yield of water radicals ($(H_2O\cdot)^-$, OH·) produced by ionizing radiation is about 3 radicals/100 ev (2,3,4). Pulse radiolysis (5) and competition experiments (5,6,7) indicate that adenine reacts very

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*Undergraduate NSF Summer Research Participant.

rapidly with these radicals ($k = 10^{10}$ liters/mole-sec). In contrast, the decomposition yield of adenine in neutral aqueous solution has been reported to range from 0.35 to 1.2 molecules/100 ev, depending upon the concentrations of adenine and oxygen in the solution (1,8,9,10,12). It is difficult to evaluate these values because they were determined using a variety of conditions and methods. The yields are lower than might be anticipated in light of the above information. This has been ascribed to the occurrence of reconstitution reactions (8,10). Since the radiosensitivity of the bases is of prime importance for an understanding of the effects of radiation on DNA, the purpose of the present study was to determine the initial destruction yields of adenine under a variety of conditions. We present data in this paper which show that the initial destruction yields of adenine are substantially higher than previously reported.

MATERIALS AND METHODS

Adenine was obtained from Sigma Chemical Company. The water was purified by: 1) distillation, 2) passage through deionizing resins, 3) distillation from alkaline permanganate, and 4) redistillation. Phosphate buffer (0.05 M, pH 7.0) was prepared from 2.76 g NaH_2PO_4 and 4.26 Na_2HPO_4 per liter.

All irradiations were performed in a Gammacell 200 Cobalt 60 Unit (Atomic Energy of Canada Ltd.) (23). Dose rates were determined by Fricke dosimetry (24) using $G(Fe^{3+}) = 15.5$. The dose rate was between 2800 and 3300 rads/min.

Quantitative absorption measurements were performed either with a Beckman DU Spectrophotometer equipped with a Gilford Model 22 photometer

and light source stabilizer or with a Cary 14 recording spectrometer. One centimeter cells were used.

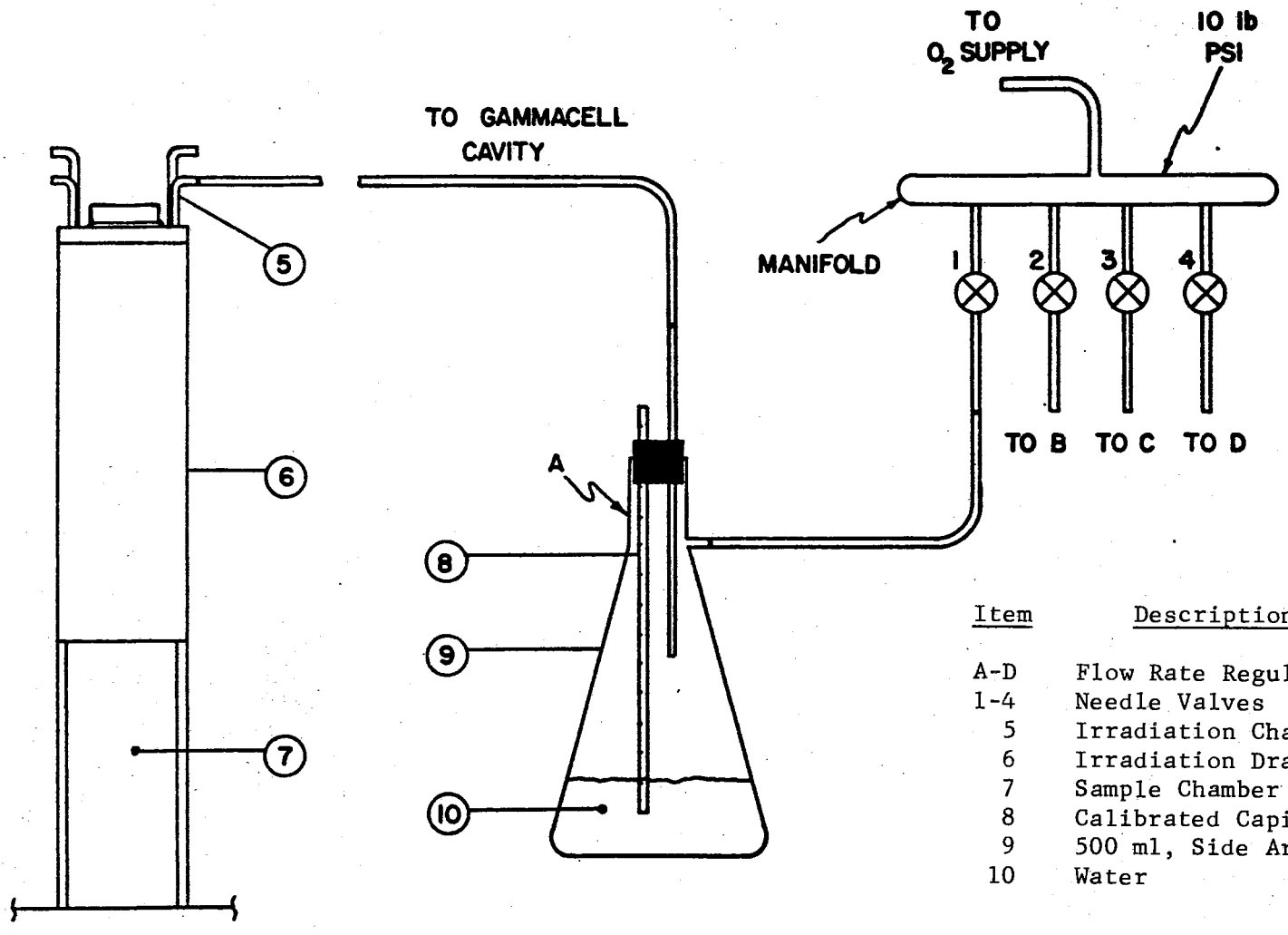
Irradiations in the presence of air or oxygen were performed in 3-dram pyrex vials with 5 ml of adenine solution. Seven vials could be accomodated in the cell holder provided for the Gammacell (23).

Solutions were saturated with oxygen by bubbling the gas directly into the solution while in the Gammacell by means of a specially designed inlet system to the irradiation chamber (23) at a flow rate of 1-2 ml sec^{-1}. The flow rate was regulated by means of the system shown in Figure 4. (Oxygen is approximately 1.25×10^{-3} M in saturated solutions at 20°C. (25)) Equilibriation with air was accomplished by shaking the solutions thoroughly before and at several points in the irradiations. Oxygen concentration is approximately 2×10^{-4} M in air-equilibrated solutions at 20°C (25).

Solutions were degassed on a high vacuum line by a freeze-thaw technique in 6 cm by 2 cm tubes equipped with a 10/30 joint. After degassing, the cells were sealed by allowing the heated glass to collapse while the system was exposed to vacuum. The sealed samples then irradiated.

The G-values for radiolysis were determined by the specific colorimetric test for adenine described by Davis and Morris (26). All determinations were made at least three times, and the average values are reported in this paper.

8-Hydroxyadenine and 4,6-diamino-5-formamidopyrimidine were prepared by the method of Cavalieri, et al. (27,28).



Item	Description
A-D	Flow Rate Regulators
1-4	Needle Valves
5	Irradiation Chamber Inlet
6	Irradiation Drawer
7	Sample Chamber
8	Calibrated Capillary
9	500 ml, Side Arm Flask
10	Water

Figure 4. Oxygen Flow Rate Regulator for Gammacell 200 (not drawn to scale)

RESULTS AND DISCUSSION

Radiolysis of Anoxic Solutions of Adenine

Figure 5 shows the typical graph for the decrease in absorbance of a degassed 2 x 10^{-4} M solution of adenine in pH 7 phosphate buffer given a total dose of 87 krads in 29 krad doses. Since products which absorb in the region of 260 nm are formed in the radiolysis (9,10,12), i.e., 8-hydroxyadenine, hypoxanthine, 4,6-diamino-5-formamidopyrimidine, and 6 amino-8-hydroxy-7,8-dihydropurine, the true decomposition yield of adenine cannot be determined from the decrease in absorbance.

Applying the colorimetric test developed by Davis and Morris (26), the amount of adenine left in the radiolyzed solution may be determined if no products interfere with the test. In addition to the compounds tested by these investigators, tests of 8-hydroxyadenine, 4,6-diamino-5-formamidopyrimidine and isoquanine were negative. Assuming the test to be specific for adenine, the color test gives true G(-adenine) values. The initial destruction yield determined by the color test for the 2 x 10^{-4} M solution was 1.1 molecules/100 ev compared to 0.505 molecules/100 ev calculated from the decrease in absorbance (Figure 5). The true G-values and apparent G-values at a number of doses are compared in Figure 6 for this irradiated solution.

Increasing the concentration of adenine from 2 x 10^{-4} M to 2 x 10^{-3} M resulted in a G-adenine value of 1.05 molecules/100 ev determined by the color test. Figure 7 shows the true G(-adenine) at several doses and the decrease in absorbance of the radiolyzed solutions. The true G(-adenine) values for both solutions tested compare very well with the results of Ponnamperuma, et al. (12) who

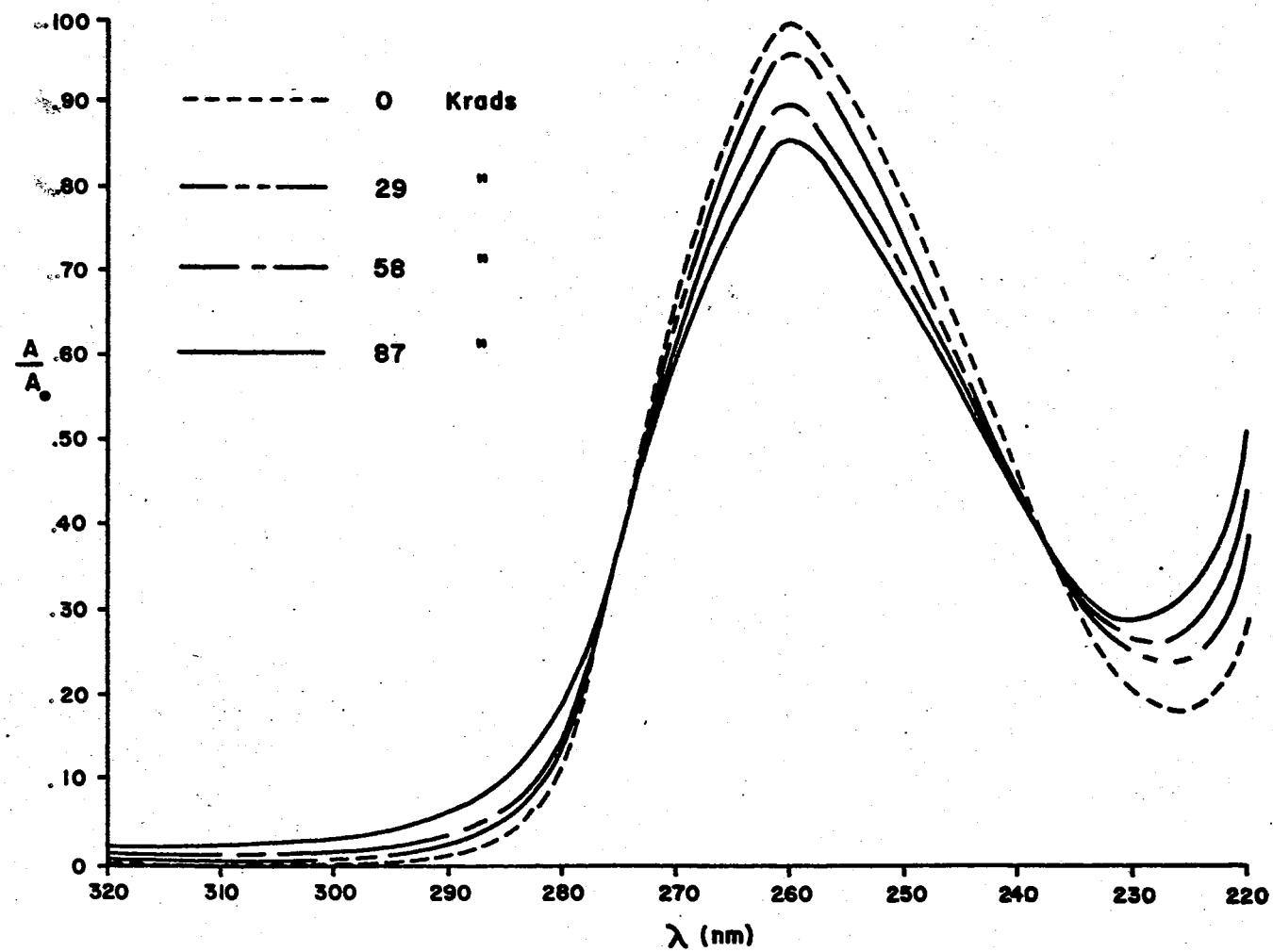


Figure 5. Ultraviolet Spectra of Radiolyzed Adenine Solutions (2×10^{-4} M, Degassed)

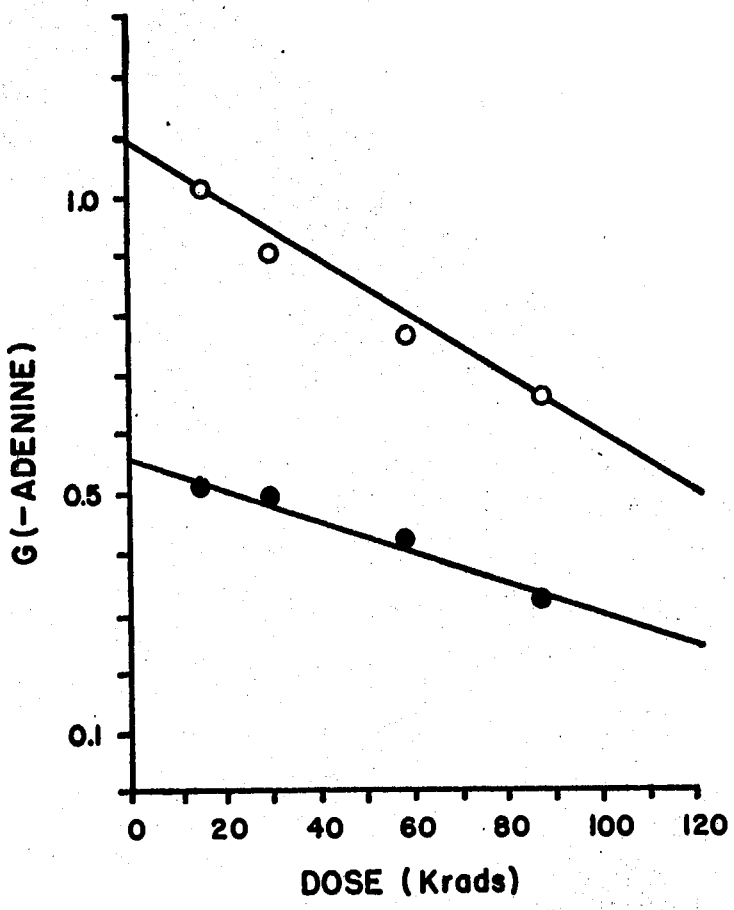


Figure 6. Comparison of G(-Adenine) Determined From the Decrease in Absorbance (●) to the True Value Determined by the Colorimetric Test (○) for a 2×10^{-4} M Degassed Solution

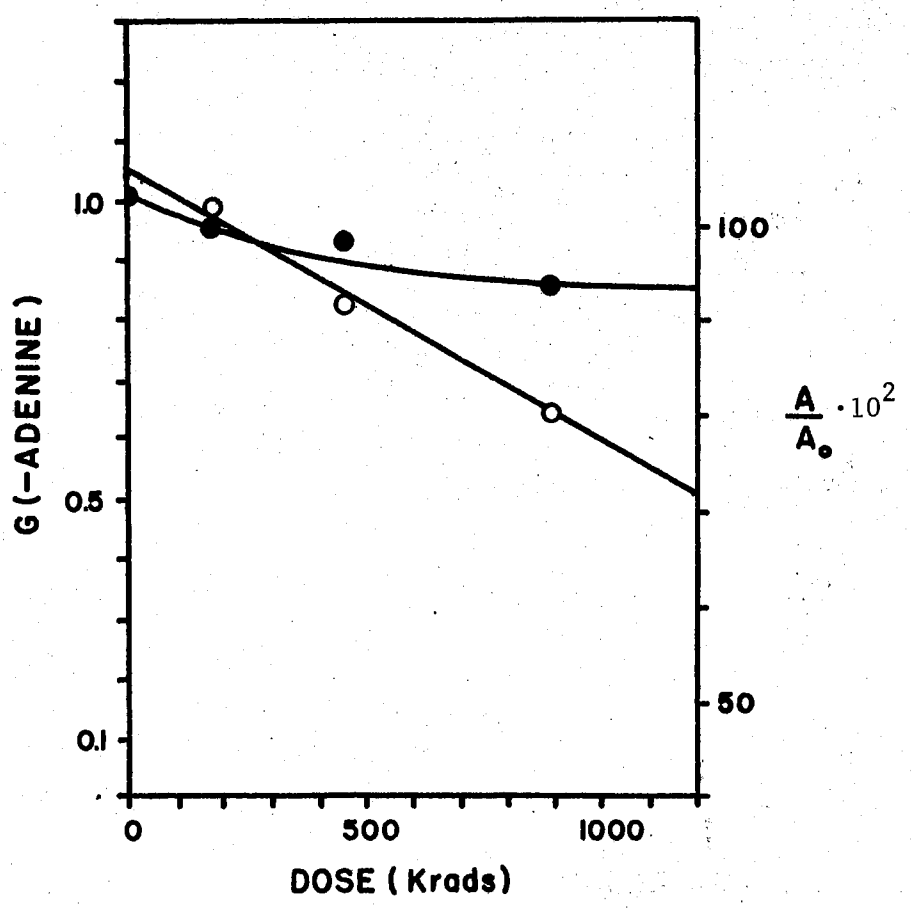


Figure 7. True G(-Adenine) Values (○) and the Decrease in Absorbance at 260 nm (●) for a 2×10^{-3} M Degassed Solution

reported a G(-adenine) value of 0.96 at 10^6 rads for a 7.4×10^{-3} M solution.

Apparently, the rather low destruction yield of the degassed solution may be attributed to, among others, electron transfer reactions of the following type (29,30):

$$AdH\cdot + (H_2O\cdot)^- \longrightarrow Ad + H_2 + OH^- \quad (1)$$

$$AdOH\cdot + (Ad\cdot)^- + H_3O^+ \longrightarrow Ad + AdHOH \quad (2)$$

$$2\ AdOH\cdot \longrightarrow Ad + AdOH + H_2O \quad (3)$$

In the degassed solutions, these types of reactions could occur much more than when oxygen is present since oxygen may react with the adenine radical adducts at many stages in the reactions.

Radiolysis of Oxygen-Containing Solutions of Adenine

The presence of oxygen in the radiolysis solutions of adenine introduces the possibility of new reaction pathways involving it. It competes with adenine for the water radicals (14) and also reacts with the adenine radical anion and other radical species. Therefore, if the oxygen is not replenished, it is quickly used up. This effect in irradiated systems has been demonstrated by Boag (31). We observed that in systems where oxygen is not replenished, the mechanisms of radiolysis change. The data presented in Table II clearly shows that if oxygen is not replenished, the absorbance at 260 nm is higher as the radiolysis progresses than for an oxygen-replenished solution, indicating a change in the radiolysis mechanisms as oxygen is used up. Therefore, for systems to be given large doses, as concentrated solutions must be, oxygen must be replenished.

TABLE II

COMPARISON OF THE ABSORPTION AT 260 NM OF OXYGEN-REPLENISHED AND NON-REPLENISHED IRRADIATED 1 x 10^{-3} M ADENINE SOLUTIONS

Dose (krads)	$A/A_o \cdot 10^2$ (replenished)	$A/A_o \cdot 10^2$ (non-replenished)
75	92.6	92.6
150	84.6	88.0
225	81.0	85.0
330	77.0	82.3

Figure 8 shows the decrease in absorbance for a 2 x 10^{-4} M air-equilibrated solution of adenine in pH 7 buffer given a total dose of 93 krads in 15.5 krad intervals. The spectra show two remarkable features: 1) a significant and rapid decrease in the absorbance at the maximum, in contrast to that of the degassed solution, and 2) the existence of "isobestic" points at 238 nm and 280 nm. At these wavelengths, products are formed in constant proportions to the adenine decomposed.

Absorbing products such as 8-hydroxyadenine (8,11), 4,6-diamino-5-formamidopyrimidine (8), and adenine-7-N oxide (10) which absorb at 260 nm have been identified in oxygen-containing solutions. Therefore, the decomposition yield based upon the decrease in absorbance of adenine at 260 nm will give only a minimum value of G(-adenine), i.e., G(-adenine = 1.01 for the 2 x 10^{-4} M adenine solution (Figure 9). In contrast, the yield calculated from the colorimetric test is 1.83 molecules/100 ev. Figure 9 shows the "true" G(-adenine) values and the apparent G(-adenine) values versus dose for the 2 x 10^{-4} M solution.

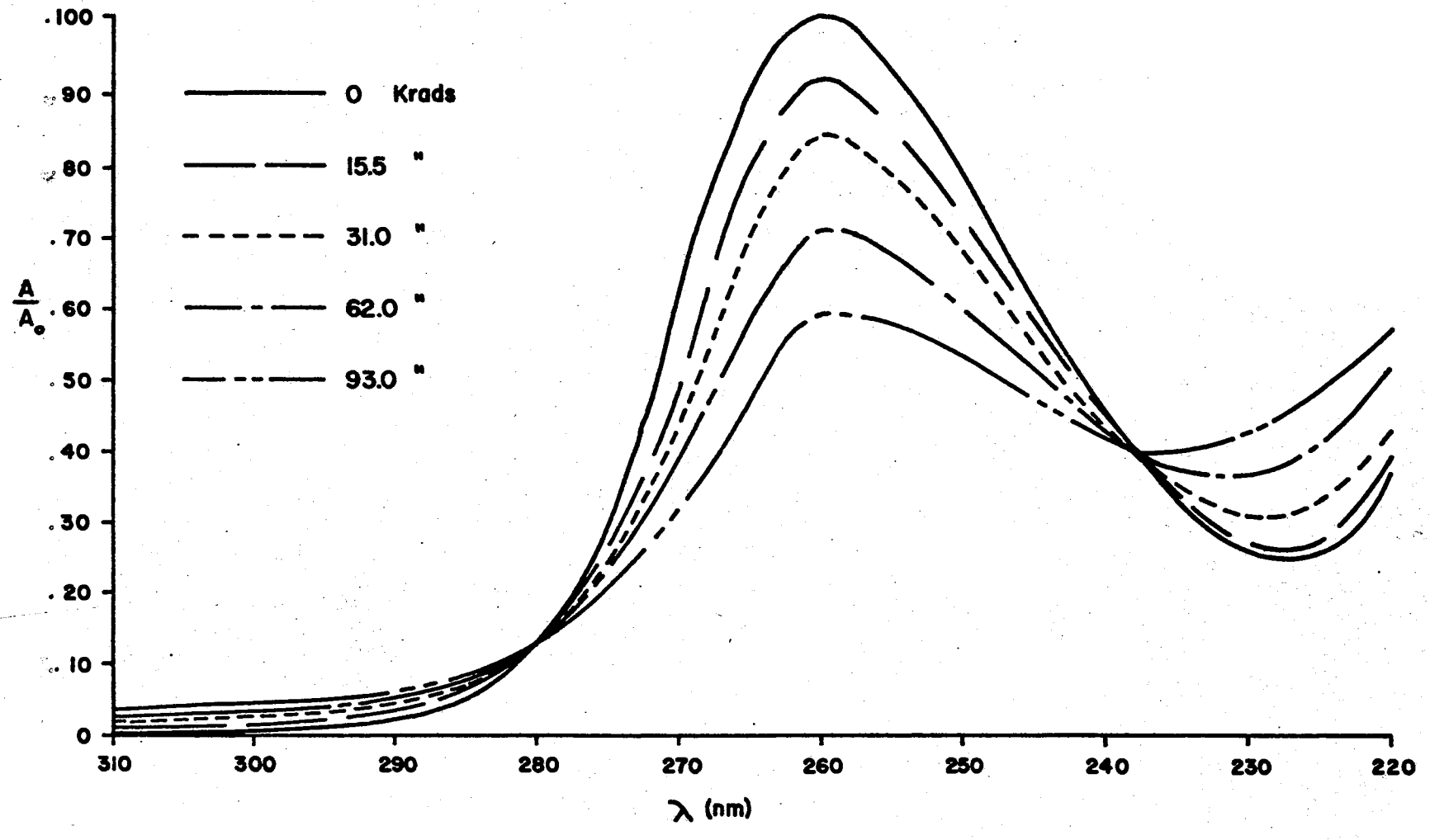


Figure 8. Ultraviolet Spectra of Radiolyzed Adenine Solutions (2×10^{-4} M, Air-Equilibrated)

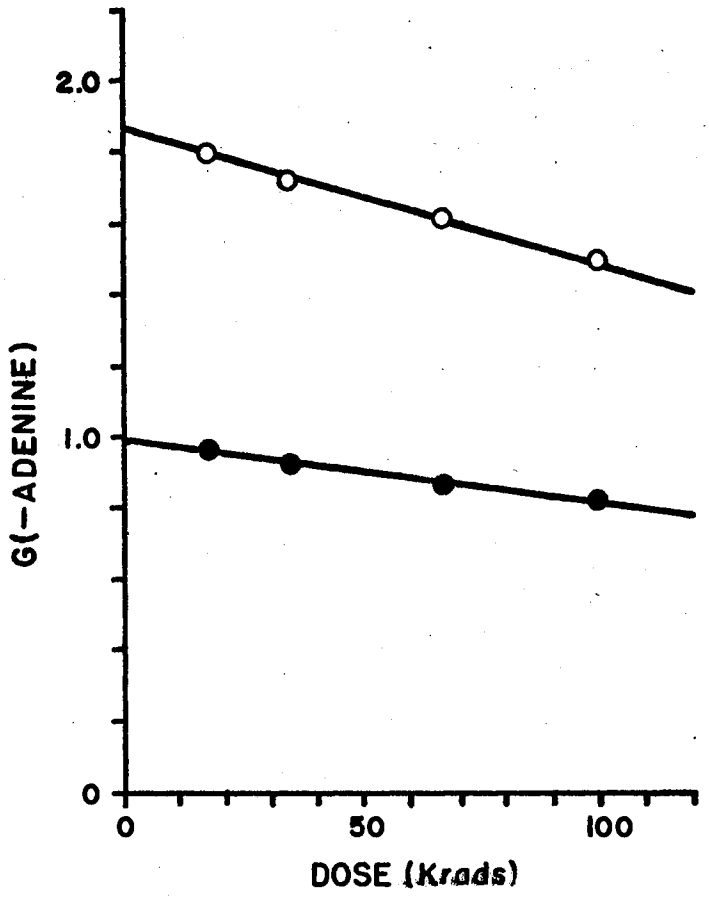


Figure 9. True and Apparent G(-Adenine) Versus Dose (2×10^{-4} M Air-Equilibrated Solutions)
(○) - true, (●) - apparent

In order to test the effect of changing adenine and oxygen concentration, solutions of oxygen-saturated 2 x 10^{-4} M adenine, air-equilibrated 2 x 10^{-3} M adenine, and oxygen-saturated 2 x 10^{-3} M adenine were radiolyzed. Table III presents the "true" G(-adenine) values and the decrease in absorbance at increasing doses for these solutions. Except for the 2 x 10^{-3} M oxygen-saturated solution, the initial G(-adenine) values are around 2 molecules/100 ev. Although higher, the general trend in G-values seems to be in reasonable agreement with Van Hemmen and Bleichrodt (10).

TABLE III

COMPARISON OF EFFECT OF CHANGING ADENINE AND OXYGEN CONCENTRATION

Dose (Rads)	Solution G(-Adenine)		
X 10^4 (2 x 10^{-3} M) X 10^3 (2 x 10^{-4} M)	2 x 10^{-4} M O_2 Saturated	2 x 10^{-3} M Air-Saturated	2 x 10^{-3} M O_2 Saturated
0 (extrapolated)	1.96	2.10	3.10
13	1.87	2.00	2.92
26	1.74	1.82	2.66
52	1.65	1.65	2.34
78	1.47	1.52	1.88

The radiolysis mechanisms are undoubtedly complex. Competition reactions occur between adenine, oxygen, $H_2PO_4^-$, and $HPO_4^=$ for e^-_{aq} and adenine and oxygen for the hydrogen atom (H·). Oxygen also interferes in various steps in the reaction pathways. Therefore, variations in concentration of the reactants and the rates of reaction of the reactants with each other play an important role in determination of

the radiolysis pathways. At this time, we are unable to elucidate the mechanisms for the various solutions we have studied.

ACKNOWLEDGEMENT

This work was supported by the Research Foundation of Oklahoma State University. Also, we wish to thank John Richie for his valuable assistance.

RADIOLYSIS OF AQUEOUS SOLUTIONS OF ADENINE -- II

IDENTIFICATION OF PRODUCTS AND PRODUCT YIELDS

IN THE PRESENCE OF MOLECULAR OXYGEN

C. A. Mannan, S. E. Scheppele, and G. Gorin

Mannan, C. A., Scheppele, S. E., and Gorin, G., Radiolysis of Aqueous Solutions of Adenine -- II, Identification of Products and Product Yields in the Presence of Molecular Oxygen. To be submitted for publication in Radiation Research.

After irradiation by ^{60}Co γ-rays, neutral aqueous oxygen-containing solutions of adenine yield many products, both ultraviolet absorbing and non-ultraviolet absorbing. The non-ultraviolet absorbing products outnumber the ultraviolet absorbing products because of the tendency of the adenine adducts to break up after reaction with oxygen. A number of products still containing the 8-position carbon atom have been separated by tlc and their yields calculated from liquid scintillation data. These include urea, 8-hydroxyadenine, and 4,6 diamino-5-formamidopyrimidine. Mass spectrometric analysis also confirms the presence of many fragments in the radiolyzed solutions.

INTRODUCTION

Many previous investigations of the radiolysis of adenine (I), Figure 10, in aqueous solution have been carried out in the absence of molecular oxygen. This is not unreasonable since oxygen is expected to complicate the radiolysis. For example, we have found the initial yield of adenine in non-oxygen containing solutions to be about 1.1 molecules/100 ev (32). In contrast, reported values of the initial yield in oxygen containing solutions vary from 1.8 to 3.4 molecules/100 ev, depending on concentration of the reactants (32). Therefore,

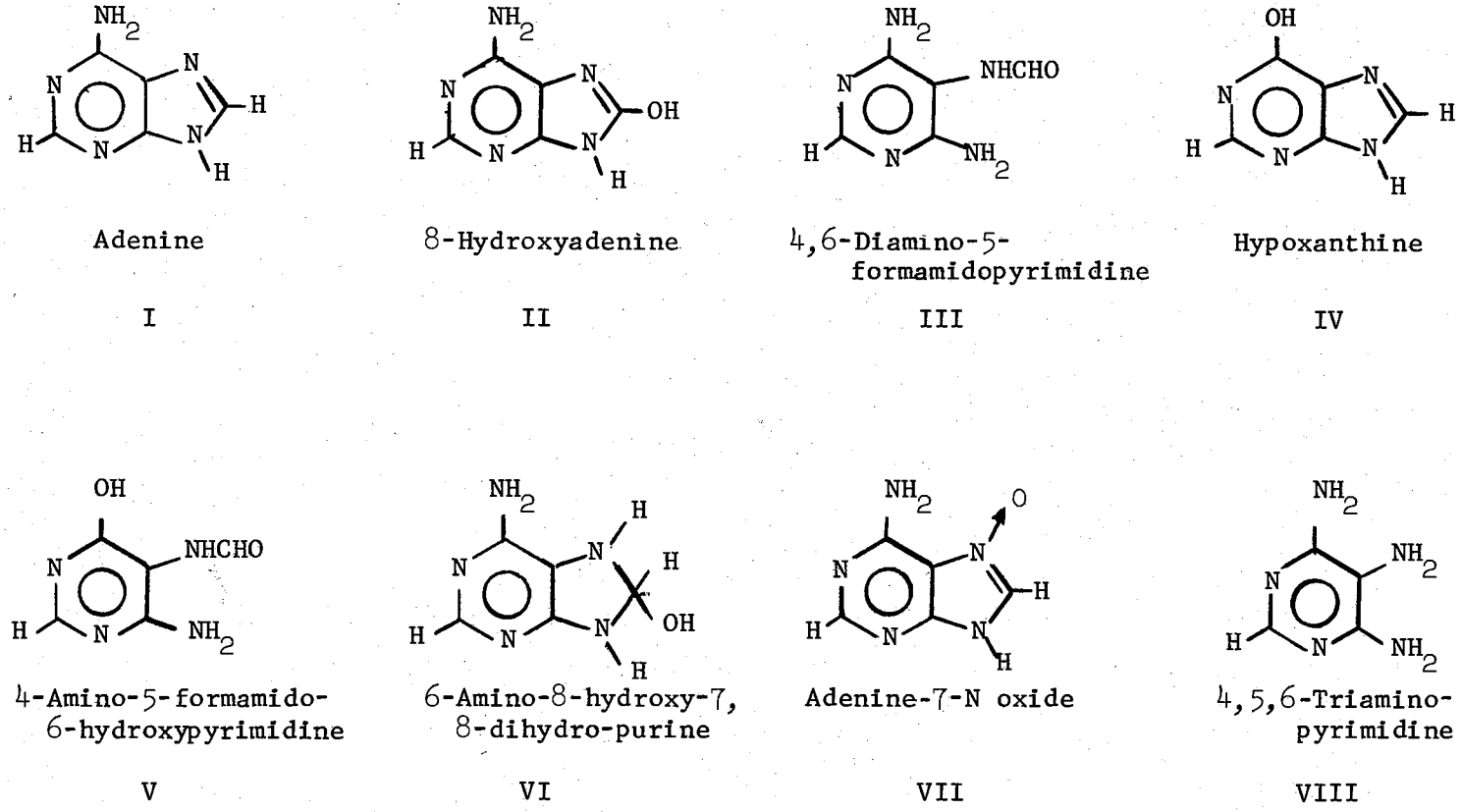


Figure 10. Some Major Products in the Radiolysis of Adenine

it may be expected that more and varied products are produced when oxygen is present. Since biological systems contain oxygen, it is important to study the radiolysis of DNA constituents in its presence.

8-Hydroxyadenine (II) and 4,6-diamino-5-formamidopyrimidine (III) (Figure 10) identified by Ponnamperuma, et al. (12) are produced with yield of 0.19 and 0.36 molecules/100 ev, respectively, at 10^6 rads in anoxic solutions of adenine. Minor amounts of hypoxanthine (IV) and 4-amino-5-formamido-6-hydroxypyrimidine (V) were observed.

Conlay (9) identified II and III in both aerated and deareated solutions of adenine. It has been suggested that III is the ultimate product derived from reaction of the hydrated electron (e^-_{aq}) with adenine (9,20). Since oxygen scavenges e^-_{aq}, Hems (20) postulated that III should not be formed in the presence of oxygen. Adenine reacts at almost the same rate with e^-_{aq} as oxygen does (6,15). Therefore, in solutions where adenine and oxygen are at least present in equal concentrations, the propensity for oxygen to scavenge e^-_{aq} should reduce the yield of III, provided the oxygen does not interfere in later steps of the mechanism. This undoubtedly happens to some extent, but evidently not enough to negate the production of III in the presence of oxygen. Other routes to III are apparently operative since Van Hemmen and Bleichrodt (10) showed that the production of III was not dependent on e^-_{aq}. Using low concentrations of adenine and high concentrations of N_2O, III was still produced even though most hydrated electrons are scavenged by the N_2O. III was produced only in minor yields in oxygen containing solutions suggesting (a) rapid reaction of III with radicals derived from oxygen, and/or (b) reaction of a precursor to III with oxygen or a reactive species derived from

oxygen.

These investigators (10) also reported the formation of II in both oxygen and non-oxygen containing solutions. II was postulated to arise via disproportionation of the hydroxy radical adducts of adenine. 6-Amino-8-hydroxy-7,8-dihydropurine (VI) was reported in deareated solutions but not in N_2O or oxygen containing solutions, which suggested that it was formed from electron adducts of adenine.

The only other ultraviolet absorbing product identified to date was adenine-7-N oxide (VII) (13). This product was described as "sensitive to ultraviolet light" and has not been reported in other investigations.

We have utilized thin-layer chromatography, autoradiography, and low-voltage mass spectrometry in an attempt to elucidate the products produced upon radiolysis of adenine. Several new products and their G-values are reported.

MATERIALS AND METHODS

Adenine obtained from Sigma Chemical Company was used in irradiations requiring only non-radioactive adenine. Adenine 8-^{14}C, 0.031 mc/mg, was obtained from New England Nuclear. Isoguanine, hypoxanthine, and 4,5,6-triaminopyrimidine were obtained from Sigma Chemical Company, Schwarz Bioresearch Inc., and Aldrich Chemical Company, respectively. 8-Hydroxyadenine and 4,6-diamino-5-formamido-pyrimidine were prepared by the method of Cavalieri and Bendich (27,28).

All irradiations were performed in a Gammacell 200 Cobalt 60 Unit (Atomic Energy of Canada Ltd.). Dose rates were determined by Fricke dosimetry (24) using $G(Fe^{3+}) = 15.5$. The dose rate at the time of

experimentation was between 2600 and 2900 rads/min^{-1}.

The water used was prepared by: 1) distillation, 2) passage through deionizing resins, 3) distillation from alkaline permanganate, 4) passage through activated charcoal (Darco, Grade G20), 5) distillation.

Solutions were saturated with oxygen by bubbling the gas directly into the solution in the Gammacell cavity. The flow rate was 1-2 ml/sec.

Oxygen-saturated (1.25×10^{-3} M) adenine solutions (2×10^{-3} M) containing 1 μc/ml of adenine 8-^{14}C were given total doses of 810 krads in 270 krad intervals. The irradiated solutions were analyzed by thin-layer chromatography using 10 x 10 cm glass plates precoated with "avicel" microcrystalline cellulose (Brinkmann Instruments, Inc.) without binder or fluorescent indicators. A plate was spotted with a total of 50 μl of radiolyzed solution in approximately 1 μl aliquots using warm air to dry the plate. Each plate was developed two-dimensionally using n-butanol, propionic acid, and water (14:9:10) in one direction and propanol, ammonium hydroxide, and water (6:3:1) in the other direction (33,34). Autoradiographs of the tlc plates were obtained by exposing the plates to Gaf non-screen x-ray film for varying periods of time, depending on the amount of radioactivity used. Radioactivity measurements were performed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer by mixing the spots scraped from the tlc plates in 10 ml of a mixture of 6 parts ethanol (abs.) and 4 parts toluene (reagent grade) containing 0.5% PPO and 0.025% POPOP.

Low resolution mass spectra were recorded on an LKB 9000 prototype

combination Mass Spectrometer-Gas Chromatograph at reduced electron voltages. Standard low voltage (10 ev) spectra of all irradiation products obtainable were recorded. The irradiated solutions were analyzed by evaporating one μl of the solution onto a stainless steel silanated wire gauze (5 x 3 mm). The gauze was then introduced into the direct probe of the mass spectrometer. As the temperature was increased from 25°-250°C, spectra of the compounds volatized were recorded. Spectra were recorded at source temperatures of 180°-230°C, and trap currents of 65 and 20 μa with the extraction plates near the block potential.

RESULTS AND DISCUSSION

Analysis of Radiolysis Products of Oxygenated Adenine Solutions

Table IV tabulates the R_f values obtained upon thin-layer chromatography of a number of purine and pyrimidine derivitives. For the purpose of comparison, literature R_f values (10) obtained from paper chromatography of these compounds are included.

The autoradiograph of the two-dimensional chromatogram obtained for a solution receiving 810 krads is shown in Figure 11. At this dose, 85% of the adenine is decomposed. The R_f values calculated from this chromatogram are included in Table IV.

Mass spectral (70 ev) identification of the individual components on the chromatograms proved to be impossible because of the impurities present in the solvents and/or on the plates in amounts about equal to the product. The presence of numerous fragments in the mass spectrum of purine and pyrimidine derivitives (35) suggested that accurate identification of molecular ions in the mass spectra of the radiolysis

TABLE IV

R_f VALUES OF STANDARD PURINES AND PYRIMIDINES AND THE RADIOLYSIS PRODUCTS OF A 2 X 10^{-3} M OXYGEN-SATURATED SOLUTION

Compound	Chromatography On Microcrystalline Cellulose		Paper Chromatography	
	System 1(a)	System 2(b)	System 1(a)	System 2(b)
Adenine	0.70	0.51	0.78	0.61
8-Hydroxyadenine	0.58	0.42	0.66	0.50
4,6-Diamino-5-formamidopyrimidine	0.43	0.50	0.58	0.50
4,5,6-Triamino-pyrimidine	0.62	0.59	---	0.40
Isoguanine	0.52	0.30	---	0.29
Hypoxanthine	0.70	0.52	0.59	0.34
Urea	0.61	0.63	---	---
Spot 1 (Figure 11)	0.70	0.51		
2	0.58	0.42		
3	0.61	0.63		
4	0.54	0.52		
5	0.61	0.51		
6	0.43	0.50		
7	0.38	0.55		
8	0.39	0.32		
9	0.35	0.27		

a) butanol, propionic acid, water (14:9:10)
b) propanol, ammonia, water (6:3:1)

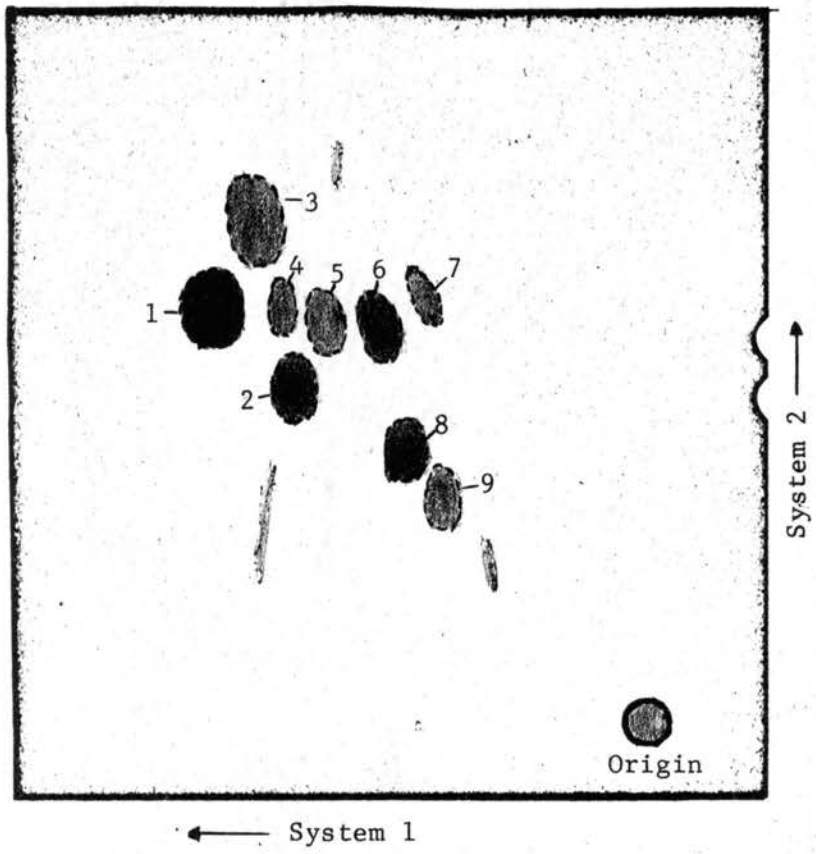


Figure 11. Autoradiograph of Chromatographed Adenine Given a Dose of 810 Krads Concentration = 2×10^{-3} M

mixture would not be possible. This difficulty was overcome by utilizing low voltage mass spectrometry. The data in Table V reveals that the 10 ev (nominal) spectra of the class of compounds of present interest is characterized by an absence of fragment ions. The requirement of intense molecular ions is met.

On the basis of the identical R_f values in both solvent systems (Table IV) and the presence of ions at m/e 135 and 108 (Table VI) at a probe temperature in excess of 130°C, Spot 1 is identified as unreacted adenine. Since the ratios of the ions at m/e 135 and 108 are the same in the standard spectrum of adenine (100:31, See Table V) and in the spectra of the radiolysis mixture (100:30), the ion at m/e 108 is reasonable assigned to the fragment resulting from loss of HCN from the adenine molecular ion (m/e 135) (35). Furthermore, no ion at m/e 108 is observed before m/e 135 appears.

The m/e 151 ion (Table VI) corresponds in mass to the molecular ion of 8-hydroxyadenine. This result, together with the good correspondence in R_f values between Spot 2 (Figure 11) and 8-hydroxyadenine (II, Figure 10), demonstrates that II is a product of the radiolysis. This result is in agreement with other investigations (9,10). The correspondence in R_f values of Spot 6 (Figure 11) and 4,6 diamino-5-formamido pyrimidine (III, Figure 10), the presence of an ion at m/e 153, and the ratio of the m/e 153 to 125 ion intensities in the spectrum of the mixture (100:31) and the authentic compound (100:31.5) at a probe temperature of 170°C identifies III as a product. III has been previously reported in deareated solution (9,10,12) and in areated solutions (9,10).

The ion at m/e 125 (Table VI) cannot be solely due to the m/e 125

TABLE V

SPECTRA OF SOME PURINE AND PYRIMIDINE DERIVITIVES

Compound	Probe Temp.#	Relative Abundance of the Ions*						
		153	151	135	125	108	60	17
Adenine	130°C	---	---	100	---	31	---	---
8-OH Adenine	220°C	---	100	---	---	---	---	---
4,6 Diamino-5-formamidopyrimidine	170°C	100	---	2.7	31.5	---	---	---
4,5,6 Triamino-pyrimidine	120°C	---	---	---	100	---	---	---
Urea	55°C	---	---	---	---	---	100	15
Isoguanine	230°C	---	100	4.5	---	---	---	---
Hypoxanthine	135°C	---	---	100	---	1.8	---	---

*The spectra do not include isotope peaks.
#Temperature at which the molecular ion is most intense.

TABLE VI

IONS IN 10 Ev SPECTRA OF THE PRODUCT MIXTURE FROM RADIOLYSIS OF ADENINE

m/e	Probe Temp. ($^{\circ}$C)*	Possible Structure
17	30° up	NH_3 fragment of urea-type compounds
45	30° up	$NH_2 - C(=O) - H$
46	30° up	$HO - C(=O) - H$
60	55° to 150°	$H_2N - C(=O) - NH_2$
86	130°	Unknown
88	130°	$H_2N - C(=O) - NH - C(=O) - H$
96	130° to 210°	A mono-hydroxypyrimidine
108	130° up	Adenine fragment
111	210° up	A mono-amino-mono-hydroxypyrimidine
125	120° and 170°	4,5,6-Triaminopyrimidine and 153 fragment
129	130° up	Unknown
135	130° up	Adenine
151	150° to 230°	8-Hydroxyadenine
153	140° to 210°	4,6-Diamino-5-formamidopyrimidine

*Temperature at which ion first appears.

fragment ion formed via loss mass 28 from the molecular ion of III since the ion at m/e 125 appears at a probe temperature of about 120°C, at which no 153 ion is present. If m/e 125 corresponds to a molecular ion, it must contain an odd number of nitrogens. A reasonable composition would be $C_4H_8N_5$. In composition, this corresponds to 4,5,6 triaminopyrimidine (VIII, Figure 10). If the ion at m/e 125 corresponds to the molecular ion of VIII, this would be the first time it has been identified in radiolyzed solutions of adenine. The possibility that m/e 125 corresponds either to a "false" molecular ion produced via loss of a neutral molecule from the true molecular ion at higher m/e values or to an even electron fragment ion containing an even number of nitrogens must be considered. Such a possibility is judged to be remote since a) no m/e 125 is observed in the spectra of I and II, b) the mass difference between m/e 129 (Table VI) and m/e 125 is illogical, and c) the absence of other peaks at m/e values in excess of m/e 129.

The spot at position 3 (Figure 11) and urea (Table IV) have identical R_f values in solvent systems 1 and 2. Furthermore, an ion at m/e 60 appears at a probe temperature of 55°C to 150°C in the radiolysis mixture. The m/e 60 ion corresponds in mass to the molecular ion of urea. It is therefore reasonable to assign Spot 3 to urea. This compound has not previously been identified in the radiolysis of neutral solutions of adenine. The formation of urea containing the 8-position carbon atom is a major process in the radiolysis, vide supra. Although all the urea produced does not necessarily have to contain C_8, reactions which ultimately lead to cleavage of the C_4-N_7 and the C_5-N_9 bonds in the imidazole ring must be important in the decomposition reactions of adenine. The present

data are insufficient to define the pathways leading to formation of urea-^{14}C; nevertheless, it appears reasonable to suggest that precursors should contain an intact imidazole ring. Thus II, IV, and VI in Figure 10 or precursors to these species are likely possibilities. Reactions which destroy the six-membered ring of I could also be a pathway(s) leading to urea-^{14}C. Further studies will be necessary to elucidate the mechanisms.

We now discuss the remaining ions observed in the spectra of the radiolysis solutions (Table VI). The absence of fragment ion at m/e 129 and 111 in the spectra of the reference compounds indicates that these may well be radiolysis products. At the present time, no information is available as to their elemental compositions. Therefore, the ions at m/e 45, 46, 86, 88, and 96 may correspond to radiolysis products or fragment ions of m/e 129 and/or 111. If the previously mentioned ions are molecular ions of radiolysis products, possible compositions are given in Table VI which are consistent with the elemental composition of adenine and the fact that oxygen is present in the radiolysis.

We believe that conclusive evidence for the formation of 8-hydroxyadenine, 4,6-diamino-5-formamidopyrimidine, and urea in the radiolysis of oxygen-saturated solutions of adenine has been presented. Furthermore, the probable production of 4,5,6-triaminopyrimidine and many other fragments of the purine ring has been shown.

Decomposition Yield of Adenine and Yields of the Products

The true initial yield of a 2×10^{-3} M oxygen-saturated solution of adenine was found to be c.a. 3.1 molecules/100 ev using a specific

colorimetric test to determine the amount of adenine decomposed in the radiolysis (32). This result was confirmed by counting the radioactivity in the spots containing the unreacted adenine in the chromatograms (liquid scintillation spectrometry) of the radiolyzed 2×10^{-3} M solution containing 1 μc/ml of adenine 8-^{14}C. The G-values calculated for adenine and the products at different doses are shown in Table VII. The G(-adenine) values are in agreement with those previously determined (32) in our laboratory but are substantially higher than other reports (9,10,13).

The percentage of radioactivity recovered for the solutions was 98.5% for the 135 krad solution, 96.0% for the 270 krad solution, 93.0% for the 540 krad solution, and 90.0% for the 810 krad solution. This is probably due to production of CO_2 from the eight-position carbon. The maximum G-value for CO_2 produced from the eight-position was .23 at 810 krads. The production increases linearly with dose.

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TABLE VII*

PRODUCT YIELDS FOR 2 x 10^{-3} M OXYGENATED ADENINE SOLUTIONS

Spot No. (Figure 11)	Yield (molecules/100 ev)			
	135 krads	270 krads	540 krads	810 krads
1	2.86	2.66	2.38	1.98
2	0.45	0.51	0.30	0.24
3	0.14	0.16	0.25	0.22
4	0.15	0.14	0.09	0.11
5	0.34	0.32	0.28	0.23
6	0.38	0.44	0.34	0.24
7	0.23	0.15	0.14	0.13
8	0.33	0.33	0.31	0.26
9	0.20	0.14	0.12	0.11

CHAPTER III

EXPERIMENTAL

Purification of Water

Water was purified by 1) distillation, 2) passage through deionizing resins (Ilko-way, Universal Model Ion X-Changer), 3) distillation from alkaline permanganate (10 grams NaOH plus 5 grams $KMnO_4$ per liter of solution), and 4) distillation from a high temperature total immersion heater. The water was stored in pyrex glassed-stoppered bottles after the final distillation. Glassware used in irradiations was rinsed thoroughly in deionized water and once again with triply-distilled water immediately before use.

Water used in irradiations involving mass spectrometric product analysis was purified by passing water from the permanganate distillation through activated charcoal (Darco, Grade G20) before the final distillation. This step was incorporated because the mass spectrometric analysis indicated small amounts of organic materials in the water after final distillation.

Preparation of pH 7 Buffer

Phosphate buffer solution, pH 7, was prepared from triply-distilled water by dissolving 2.7598 g of NaH_2PO_4 and 4.2588 g of Na_2HPO_4 in approximately 500 ml of water in a one-liter flask and bringing the final volume to the mark. The pH was checked on a

Beckmann Model Expandomatic pH Meter.

Preparation of Adenine Solutions

Adenine (Σ Grade obtained from Sigma Chemical Company) was used in all irradiations. Standard procedure was to make a 2×10^{-3} M stock solution of the adenine by adding 0.6755 g of adenine to approximately 100 ml of the pH 7 buffer solution in a 250 ml volumetric flask, dissolving the adenine, and filling to the mark with buffer. Solutions of lower concentration were then prepared by appropriate dilution with buffer solution. The stock solution was stored in a refrigerator for not more than three days.

Determination of Gammacell Dose Rate

The dose rate of the Gammacell (Gammacell 200, Atomic Energy of Canada Ltd. (23)) was determined by the method of Fricke (24) using $G(Fe^{+3}) = 15.5$. The ferrous ion dosimeter solution was prepared from 0.4 g Fe $(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.06 g NaCl, and 22.0 ml H_2SO_4 (conc.) per liter of solution. Triply-distilled water was used in preparing the solution. The solution was used immediately after preparation by placing 5 ml of dosimeter solution in each of seven 3-dram pyrex vials. The vials were placed in reproducible positions in the cell holder (23) and irradiated for 10 minutes. The absorbance at 304 nm was then determined on a Beckmann Model DU Spectrophotometer equipped with a Gilford Model 222 photometer and light source stabilizer. The dose rate was calculated using the following equation:

$$\text{Dose Rate (rads/min)} = \frac{2.84 \times 10^4 \ A^{\circ}304}{t}$$

where A^{o}_{304} = absorbance of the radiolyzed dosimeter solution at 304 nm, and

t = time in minutes the solutions were radiolyzed.

Experiments indicated that absorbance at 304 nm varied from 0.5 to 2% from vial to vial. This meant that the dose rate at various positions in the Gammacell irradiation chamber varied slightly (c.a. 0.5 to 1% or 25 to 50 rads/min at 3000 rads/min). Table VIII shows the absorbances at 304 nm for a typical determination. Since the variance was small from position to position, an average value of the absorbance of the seven vials was used to calculate the dose rate.

TABLE VIII

TYPICAL DETERMINATION OF THE GAMMACELL DOSE RATE

Vial Position	A^{o}_{304} (Run 1)
1	0.955
2	0.937
3	0.933
4	0.952
5	0.941
6	0.954
7	0.956
	Avg. = 0.947

Avg. of Run 2 - 0.943
Avg. of Run 3 - 0.945
Final Average - 0.945

Dose Rate - 2680 rads/min
Determined August 18, 1971

The value determined represents the dose at a position in the irradiation chamber at which a sample receives approximately 96% of the center dose rate as determined from an isodose plot provided with the Gammacell. Since all samples were irradiated in this position, a correction factor was not applied.

The dose rate calculated was slightly less than the true dose rate. It was not corrected for the dose received in the time interval when the sample chamber was being lowered or raised from the Gammacell radiation cavity. Experiments indicated that the amount of time the sample was in the field of the cobalt source before the timer began operation or after it had stopped was c.a. six seconds. The maximum dose received in this time would be 10% of the true dose per minute. However, the sample chamber was not in the maximum field of the source during much of this time. The isodose plot showed that a sample in the top part or bottom part of the chamber received approximately 70% of the center dose rate. The value of the dose received in this time interval was determined to be approximately 2% of the true dose rate. This value was determined by lowering and raising the Gammacell irradiation chamber containing dosimeter solution in and out of the radiation cavity twenty times and determining the average value of the dose received by the dosimeter. Since the dose calculated by this method was within experimental error of the Fricke determination itself, a correction was not applied.

If the dose rate at any given time is known, the dose rate before that time or the remaining dose rate may be calculated from the following expression:

$$N/N_o = e^{\frac{-0.693+}{T_h}}$$

where N = remaining radiation,

N_o = initial radiation,

t = time elapsed (years), and

T_h = half-life of Co (5.27 years).

Irradiations of Degassed Solutions

Degassing of solutions was accomplished on a high vacuum line using a freeze-thaw technique, i.e., freezing the solution and then thawing to drive out dissolved gases. Cells used for this purpose were 6 x 2 cm pyrex fitted with a 10/30 ground joint. The sealed tubes containing 5 ml solution were irradiated in the cell holder in the same position as the 3-dram vials.

Ultraviolet Spectra of the Adenine Solutions

Immediately after the irradiations, the radiolyzed solutions were diluted with pH 7 buffer and the spectra taken on the Beckmann DU, as previously described, on a Cary Model 14 recording spectrometer. One centimeter silica cells were used.

Specific Colorimetric Test for Adenine

A specific colorimetric test for adenine developed by Davis and Morris (26) was used to determine the amount of unreacted adenine. A 3.0 ml aliqout of the sample to be analyzed (diluted to at least 1×10^{-3} M) was transferred to a 10 ml volumetric flask, and 0.4 ml of 18 N sulfuric acid was added. The contents were mixed, and 0.2 ml

of 0.2 M potassium bromide solution was added. After shaking, 0.6 ml of 1 N potassium permanganate was added and the solution again shaken. After 5 minutes, 0.2 ml of 6% hydrogen peroxide solution was introduced to decolorize the excess permanganate. The final volume was then adjusted to 6.0 ml with distilled water and the absorbance measured at 328 nm within 15 minutes against a reagent blank. The concentration of the adenine was read directly from a calibrated graph of the absorbance versus concentration of adenine. This graph must be prepared in conjunction with the analysis of the radiolyzed solutions since factors such as room temperature, pressure, and small variances of time will affect the absorbance readings. Table IX shows a typical calibration graph for given concentrations of adenine.

TABLE IX

CALIBRATION GRAPH FOR COLORIMETRIC TEST

Adenine Conc. Moles/liter	A^{o}_{328}
1.000×10^{-3}	0.952
8.000×10^{-4}	0.750
6.000×10^{-4}	0.550
4.000×10^{-4}	0.340
2.000×10^{-4}	0.140
1.000×10^{-4}	0.038

Preparation of 4,6 Diamino-5-Formamidopyrimidine

4,6-Diamino-5-formamidopyrimidine was synthesized by the method

of Cavalieri, et al. (28). Five grams (.04 mole) of 4,5,6-triaminopyrimidine (Aldrich Chemical Company) was warmed gently in a boiling flask with 10 ml of 98% formic acid until all the solid was in solution. The excess formic acid was removed by evaporation at room temperature. Recrystallization of the crude product from absolute ethanol yielded 5.1 grams (90%). The ultraviolet spectrum at pH 7 yielded an extinction coefficient of 4,600 at 260 nm and 9,400 at pH 2 in agreement with Cavalieri, et al. (27).

Preparation of 8-Hydroxyadenine

8-Hydroxyadenine was synthesized by the method of Cavalieri, et al. (27). 4,5,6-Triaminopyrimidine (5 g, 0.04 mole) (Aldrich Chemical Company) was dissolved in 200 ml of 10% sodium hydroxide and phosgene bubbled in for two hours. After addition of 25 ml of 2 N sulfuric acid, the solution was heated and then decolorized with N orit A. Upon cooling, the crude 8-hydroxyadenine sulfate crystallized from the reaction mixture. Recrystallization twice from 2 N sulfuric acid yielded 1.65 grams (40%). To obtain the free base, the sulfate salt was reacted with $NaHCO_3$ (sat.) and washed thoroughly with distilled water. The ultraviolet spectrum at pH 7 had an extinction coefficient of 10,100 at 260 nm in agreement with Cavalieri, et al. (27).

Analysis of Radiolysis Products

After irradiation of adenine of appropriate concentration containing 1-2 μc adenine-8-14 C/ml, 50 μl of the radiolyzed solution was spotted on a 10 cm x 10 cm thin-layer plate. All plates were obtained from Brinkmann Instruments Inc. and were precoated with "avicel"

micro-crystalline cellulose without binder or fluorescent indicators, Brinkmann code name, Cell Plate 22. The solution was spotted with a 10 ml Hamilton microliter syringe, Model 701 SN, approximately 1 μl at a time, using a heat gun and warm air to dry the plate. The plates were developed two-dimensionally, using n-butanol, propionic acid, and water (14:9:10) in one direction, and propanol, ammonium hydroxide, and water (6:3:1) in the other direction.

Autoradiographs were obtained by exposing the plates to Gaf non-screen x-ray film for 24-48 hours. For an exposure period of 36 hours, 1 μc/μl of the radioactive solutions yielded very dense spots on the film of both adenine and the products. Radioactivity measurements were performed with a Packard Model 3320 Tricarb Liquid Scintillation Spectrometer. The spots on the plates were located by reference to the x-ray film of the tlc plate. The individual spots were then scraped from the tlc plates and mixed (sonicator) in 10 ml of a counting solution of 6 parts ethanol (abs.) and 4 parts toluene (reagent grade) containing 0.5% of PPO and 0.025% POPOP.

Calculation of G-Values

The decomposition yield of adenine or the production yield of the products was calculated from the following equation (36):

$$G = \frac{9.632 \times 10^{8}\ C}{D}$$

where G = G-value,

C = concentration of material in solution, (moles/liter), and

D = dose (rads/min).

Low Voltage Mass Spectra of the Radiolyzed Solutions

Low resolution mass spectra were recorded on an LKB 9000 prototype combination Mass Spectrometer-Gas Chromatograph (37) at reduced electron voltages. Standard low voltage (10 ev) spectra of all irradiation products obtainable were recorded. The irradiated solutions were analyzed by evaporating one ml of the solution onto a stainless steel silanated wire gauze (5 x 3 mm). The gauze was then introduced into the direct probe of the mass spectrometer. As the temperature was increased from 0-250^{o}C, spectra of the compounds volatized were recorded. Spectra were recorded at source temperatures of 180^{o} to 230^{o}C, and trap currents of 65 and 20 μa with the extraction plates near the block potential.

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