VRIJE UNIVERSITEIT TE AMSTERDAM

RADIATION DAMAGE IN ADENINE AND DNA EFFECTS OF SENSITIZING AND PROTECTING AGENTS

Academisch proefschrift

ter verkrijging van de graad van
doctor in de Wiskunde en Natuurwetenschappen aan de
Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus Mr. I. A. Diepenhorst,
hoogleraar in de faculteit der Rechtsgeleerdheid,
in het openbaar te verdedigen op vrijdag 25 januari 1974
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De Boelelaan 1105

door

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geboren te Epe

Promotor: Dr. H. Loman

Copromotor: Prof. Dr. Joh. Blok

S T E L L I N G E N

1. De bewering van Cole en Cole dat de vooruitgang van de wetenschap niet wordt beïnvloed door een selektieve vermindering van het aantal wetenschapsmensen is gebaseerd op ondeugdelijke argumenten.

J.R.Cole en S.Cole , Science 178 , 368 (1972)

2. Door de snelheidsverdeling in een mensenmenigte te beschrijven met de distributie theorie van Maxwell en Boltzmann voor ideale gassen , gaat Henderson volledig voorbij aan sociale relaties in die menigte.

L.F.Henderson , Nature 229 , 381 (1971)
L.F.Henderson en D.J.Lyons , Nature 240 , 353 (1972)

3. In het werk van Fielden en Lillicrap betreffende de radioluminescentie van DNA wordt ten onrechte geen rekening gehouden met de vorming van licht emitterende stralingsprodukten.

E.M.Fielden en S.C.Lillicrap , Current Topics in Radiation Research Quarterly 7 , 133 (1972)

4. Het verdient aanbeveling om te onderzoeken of de vergiftigingsverschijnselen van 'paraquat' bij de mens kunnen worden behandeld met 'superoxide dismutase'.

5. De toepassing op grote schaal van virussen bij de bestrijding van insekten is onverantwoord zolang de kennis over de soortspecificiteit van die virussen en de eigenschappen van hun DNA nog onvoldoende is.

6. De voordelen die de toepassing van pionen en zware ionen bij de radiotherapie van diepliggende tumoren kan bieden in vergelijking met de conventionele orthovolt- en megavolttherapie , voor wat betreft de relatieve dosis ter plaatse van de tumor , het zuurstof-effekt en de R.B.E. waarde , rechtvaardigen geenszins de ontwikkeling van deze techniek gezien de zeer hoge kosten die hieraan zijn verbonden.

7. Het gebruik van het geïsoleerde lichtgevende orgaan van de Photoblepharon Palpebratus bij de visvangst door de vissers van Banda is onvoldoende reden om aan hetzelfde orgaan bij het intakte dier een overeenkomstige funktie toe te schrijven bij het vinden van een prooi.

Aan de nagedachtenis van mijn vader

C O N T E N T S

C H A P T E R I Introduction

If one irradiates living cells with ionizing radiation , all functions of these cells may be affected , because ionizing radiation is non-specific in its action. Nevertheless it has been observed that some functions of the cell are more susceptible to radiation than others. In particular the loss of the capacity to reproduce is a very sensitive criterium in studies of the various parameters that modify the action of radiation on cells.
When cells are irradiated in the presence of small amounts of certain chemicals , the amount of radiation-induced cell-killing may be changed (protection or sensitization). Such chemicals may have important applications in chemoprophylaxis of radiation damage and radiation therapy of tumours.

The general problem of how ionizing radiation affects the survival of cells can be subdivided in several questions , which at the same time formulate a program to tackle the problem.

i) What are the important structures or molecules ?

ii) What is the nature of the damage that is produced in these structures ?

iii) What are the pathways that lead to this damage ?

iiii) How do sensitizers and protectors interfere with these pathways ?

It is now almost generally accepted that DNA , the molecule which contains the genetic information needed for virtually all functions of the cell , is the most critical target for the action of radiation on cells. The radiation damage which accumulates in DNA as a consequence of irradiation can be due to two processes. Radiation energy can be absorbed by the DNA itself (direct action) , or it can be absorbed by surrounding molecules like water or organic compounds ,

producing reactive intermediates which may react with DNA (indirect action). Although it is often very difficult to differentiate between the two types of radiation action , it has been shown that the water content in for instance bacterial spores determines the radiosensitivity to a large extent.

The present study is part of a research program concerning the reactions between DNA and radiation-induced free radicals (see e.g. Van der Schans 1969 and De Jong 1972). In the experiments that will be described here it was attempted to obtain more information on these reactions. This study was done with adenine , one of the four bases of naturally occurring DNA's. Furthermore the involvement of DNA in radiosensitization and radioprotection under conditions of direct and indirect action was examined. For these studies it proved useful to apply techniques like pulse radiolysis and rapid-mixing *). With these techniques it was possible to obtain some information about the pathways involved in the formation of radiation-induced damage in DNA.

The elementary processes involved in the absorption of energy by water are described in chapter II. Furthermore the literature on the radiation chemistry of DNA and its derivatives is briefly described in this chapter , whereas a short discussion on the effects of radiosensitizers and radioprotectors follows in chapter III. Technical details are given in chapter IV. The radiation chemical experiments with adenine are described in chapter V. In the last chapters the experiments are presented which were attempted to show , if DNA is involved in radiosensitization (chapter VI) and radioprotection (chapter VII).

*) These experiments were initiated during a stay in the "Gray Laboratory" in Northwood and were further evaluated in collaboration with the section "Stralingschemie" of the "Interuniversitair Reactor Instituut " in Delft.

C H A P T E R II Radiation chemistry of water and aqueous solutions of DNA and its constituents

The interaction of ionizing radiation with matter depends on energy and type of radiation. An elaborate treatment of this interaction and of the consequent processes is given by Draganić and Draganić (1971). In the present chapter a summary will be given of the reactions which occur in the radiolysis of water. In the presence of solutes the water radicals that are formed will react with these solutes. In general the resulting solute radicals will decay, forming stable products. The radiation chemistry of DNA and its purine and pyrimidine constituents has recently been reviewed by Blok and Loman (1973) and Scholes (1968) and will also be summarized shortly in this chapter.

II.1 Radiation chemistry of water

In our work two different types of ionizing radiation have been used , ^{60}Co-γ-rays (1.17 and 1.33 MeV) and 2-3 MeV electrons produced by a Van de Graaff accelerator or a linear accelerator.

γ-Rays interact with matter in three different ways , namely photoelectric effect , Compton effect and pair production. The extent to which these three processes contribute to the energy absorption depends on the incident energy. γ-Rays with an energy between 0.1 and 5 MeV are almost exclusively absorbed by Compton effect in water and biological materials.

The fast electrons that are produced by the interaction of γ-rays and water or by the above mentioned machines , lose their energy by collision and water molecules become ionized and excited. Due to the electrons which originate in the primary ionization , further ionizations and excitations may occur. It is obvious that the spatial distribution of ionizations and excitations depends on the inhomogeneous energy disposition. The regions with high concentrations of affected

water molecules have been called spur. In the spur the ionized and excited water molecules will decay. The H_2O^+ species formed in the reaction ,

$$H_2O \rightarrow H_2O^+ + e^-$$

will react according to the ion-molecule reaction ,

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH^\cdot .$$

Lampe , Field and Franklin (1957) have estimated that the rate constant of this reaction was $k = 7.5 \times 10^{11}\ M^{-1}sec^{-1}$. The above reaction shows the formation of the very important oxidizing hydroxyl radical ($OH^\cdot$). Some of the positive water ions can recapture an electron in the spur ,

$$H_2O^+ + e^- \rightarrow H_2O^* \quad \text{(geminate recombination)}$$

Electrons can also be captured by H_3O^+ to give H_3O. This unstable entity can undergo a dissociative reaction ,

$$H_3O \rightarrow H^\cdot + H_2O .$$

Most electrons will escape the forces of the positive ions in the spur and become hydrated. This hydration of the negatively charged electron is occurring due to the polar character of the water molecules and is still the subject of considerable discussion. Excited water will decay mainly according to

$$H_2O^* \rightarrow H^\cdot + OH^\cdot .$$

It seems however that the amount of excited water , which contributes to the formation of the radicals is relatively small.
The reducing radicals ($H^\cdot$ and e^-_{aq}) and the oxidizing radical ($OH^\cdot$) will react together or diffuse away from the spur. In the spur some molecular products are formed like hydrogen and hydrogen peroxide. Furthermore water will be reconstituted. In table I a summary is given of a number of important reactions which occur in the radiolysis of water.
In radiation chemical experiments it is sometimes useful to eliminate some of the radicals. For instance e^-_{aq} and $H^\cdot$ react with oxygen

Table I

Some important reactions in the radiation chemistry of water

	k x 10^{-9} ($M^{-1}sec^{-1}$)	pH	reference **)
$e^-_{aq} + e^-_{aq} \rightarrow H_2 + 2\,OH^-$	5	10-13	1
$e^-_{aq} + H^\cdot \rightarrow H_2 + OH^-$	25	10.5	2
$e^-_{aq} + OH^\cdot \rightarrow OH^-$	30	11	1,2
$e^-_{aq} + H_3O^+ \rightarrow H^\cdot + H_2O$	24	4-5	1
$e^-_{aq} + H_2O_2 \rightarrow OH^\cdot + OH^-$	12	7	1
$e^-_{aq} + H_2O \rightarrow H^\cdot + OH^-$	16 x 10^{-9}	8.5	3
$H^\cdot + OH^- \rightarrow e^-_{aq} + H_2O$	0.018	11.5	2
$H^\cdot + H^\cdot \rightarrow H_2$	13	0.4-3	4
$H^\cdot + OH^\cdot \rightarrow H_2O$	32	0.4-3	4
$H^\cdot + H_2O_2 \rightarrow H_2O + OH^\cdot$	0.16	0.4-3	4
$OH^\cdot + OH^\cdot \rightarrow H_2O_2$	4	7	5
$OH^\cdot + H_2O_2 \rightarrow H_2O + HO_2^\cdot$	0.045	7	5
$e^-_{aq} + O_2 \rightarrow O_2^-$	19	7	1
$H^\cdot + O_2 \rightarrow HO_2^\cdot$	19	2	6
$HO_2^\cdot \rightleftarrows O_2^- + H^+$	pK = 4.88		7
$HO_2^\cdot + HO_2^\cdot \rightarrow H_2O_2 + O_2$	7.6 x 10^{-4}	1	7
$HO_2^\cdot + O_2^- \rightarrow HO_2^- + O_2$	8.5 x 10^{-2}	5	7
$2\,O_2^- + H_2O \rightarrow HO_2^- + O_2 + OH^-$	*)	-	7

*) According to Czapski (1971) this reaction rate constant is very small and is difficult to measure due to interference with impurities.

**) References : 1, Gordon , Hart , Matheson , Rabani and Thomas (1963). 2, Matheson and Rabani (1965). 3, Hart , Gordon and Fielden (1966). 4, Fricke and Thomas (1964). 5, Schwarz (1962). 6, Sweet and Thomas (1964). 7, Czapski (1971).

as is shown in table I. The product of these reactions in neutral solutions is the peroxy radical (O_2^-). In most chemical systems the peroxy radicals combine to hydrogen peroxide and do not react with other solutes.

The hydrated electron can be converted into OH$^\cdot$ by nitrous oxide ,

$$e^-_{aq} + N_2O \rightarrow N_2 + OH^\cdot + OH^- \quad k = 8.7 \times 10^9\ M^{-1}sec^{-1}.$$

Very often t-butanol is used to scavenge OH$^\cdot$ radicals,

$$OH^\cdot + (CH_3)_3COH \rightarrow {}^\cdot CH_2(CH_3)_2COH + H_2O \quad k = 5 \times 10^8 M^{-1}sec^{-1}.$$

The t-butanol radical is unreactive. It is also possible to convert the hydrogen atom into the hydrated electron and vice versa , by changing the pH of the solution (see table I).

The number of free radicals (molecules , ions) that are formed or destroyed per 100 eV energy dissipated in the solution and have diffused out of the spur is called the G-value or yield. This value depends on the type of radiation , pH and other parameters. In table II the G-values for the important products of water formed by γ-radiation are given (Henglein , Schnabel and Wendenburg 1969).

Table II

G-values for the radiolysis products of water at pH 7

Radiolysis product	G-value
e^-_{aq}	2.65
H$^\cdot$	0.55
OH$^\cdot$	2.70
H_2O_2	0.70
H_2	0.45

It is easy to show that for the oxidizing products , the following relation exists ,

$$G(OH^{\cdot}) + 2\,G(H_2O_2) = G(-H_2O)$$

and for the reducing products ,

$$G(H^{\cdot}) + G(e^-_{aq}) + 2\,G(H_2) = G(-H_2O).$$

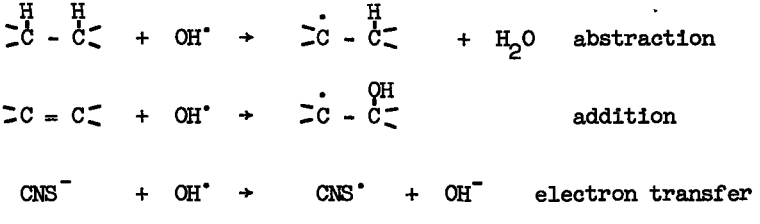
A combination of these two equations leads to the equation of material balance , which was first formulated by Allen (1954).

$$G(OH^{\cdot}) + 2\,G(H_2O_2) = G(H^{\cdot}) + G(e^-_{aq}) + 2\,G(H_2)$$

Obviously the values from table II have to satisfy the equation of material balance.

II.2 Radiation chemistry of DNA and its constituents

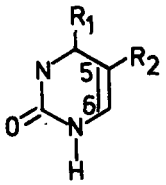
If solutes are present they will react with the free radicals formed in water. In general such a reaction involves abstraction , addition or electron transfer. For the reactions with $OH^{\cdot}$ radicals , examples are given below.



The solute radicals that are formed , undergo radical-radical reactions or decay unimolecularly to form another radical. It is also possible that the radicals react with solute molecules. Ultimately stable products are formed. In the radiolysis of DNA and its constituents similar reactions occur. For recent reviews see Blok et al.(1973) and Scholes (1968). In this paragraph some of the results are summarized.

II.2.1 Pyrimidines

The structure of the most important pyrimidines are given below. Thymine and cytosine occur in DNA. Uracil is one of the pyrimidine bases of ribonucleic acid (RNA).



thymine : R_1 = OH R_2 = CH_3

cytosine : R_1 = NH_2 R_2 = H

uracil : R_1 = OH R_2 = H

The rate constants for the reactions of water radicals with the pyrimidines are high (table III). Hydroxyl radicals react less rapidly with nucleic acid bases than hydrated electrons , but more rapidly than hydrogen atoms.

The radical anions that are formed by the reaction between pyrimidines and hydrated electrons are rapidly protonated (Hayon 1969 ; Theard , Peterson and Myers 1971). In the case of thymine it has been shown that the protonated radical is different from the hydrogen adduct of thymine (Loman and Blok 1968). Cadet and Teoule (1971[a]) have given the most complete account of the radiation products of thymine in deaerated solution. From the structures of the radiation products it is concluded that both hydroxyl radicals and hydrogen atoms attack thymine (Cadet and Teoule 1971[a]) , cytosine (Khattak and Green 1966[a]) and uracil (Khattak and Green 1966[b]) mainly at the C_5-C_6 double bond. According to Loman and Blok (1968) , the electron adduct of thymine does only contribute to the destruction of the chromophore when oxygen is present in the solution. Willson (1970) and Loman and Ebert (1970)

Table III

Rate constants for the reactions of water radicals with DNA derivatives at neutral pH

compound	k x 10^{-8} ($M^{-1}sec^{-1}$)			reference **
	$OH^{\cdot}$ *)	$H^{\cdot}$	e^{-}_{aq}	
adenine	54	0.83	90	1 , 2 , 3
thymine	54	8	170	1 , 4 , 5
guanine	105	-	-	5 , - , -
cytosine	53	1.2	130	1 , 6 , 3
d-adenosine	45	1.4	92	1 , 1 , 3
thymidine	49	2.5	-	1 , 1 , -
d-guanosine	-	-	-	- , - , -
d-cytidine	49	-	80	1 , - , 1
dAMP	35	-	-	7 , - , -
TMP	53	2.3	15	7 , 1 , 5
dGMP	68	-	-	7 , - , -
dCMP	50	-	-	7 , - , -
DNA	8	1	1.4	8 , 1 , 3

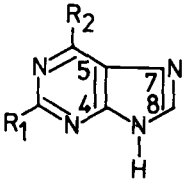
*) Reaction rate constants that have been determined with the thiocyanate method , were corrected according to Willson , Greenstock , Wageman and Dorfman (1971)

**) References : 1, Scholes (1968). 2, Neta and Schuler (1971). 3, Shragge , Michaels and Hunt (1971). 4, Loman and Blok (1968). 5, Anbar and Neta (1967). 6, Neta (1972). 7, Scholes , Shaw , Willson and Ebert (1965). 8 , Scholes , Willson and Ebert (1969).

have shown that thymine radicals react rapidly with oxygen and this feature explains the large differences in radiation products in aerated and deaerated solutions. In the presence of oxygen the main products of thymine are peroxy derivatives (Cadet and Teoule 1971[b]) which are not found in the absence of oxygen. In the case of cytosine and uracil the hydroperoxy derivatives are unstable (Daniels and Schweibert 1967 ; Schweibert and Daniels 1971).

II.2.2 Purines

The knowledge of the radiation chemistry of purines is much less complete than that of the pyrimidines. The structural formulas of some purines are given below



adenine	:	R_1	= H	R_2	=	NH_2
guanine	:	R_1	= NH_2	R_2	=	OH
xanthine	:	R_1	= OH	R_2	=	OH
hypoxanthine	:	R_1	= H	R_2	=	OH

Deoxyribonucleic acid contains adenine and guanine as the purine bases. As far as radiation chemistry is concerned , guanine has received little attention , because it is almost insoluble in a neutral aqueous solution.

As can be seen from table III , the reactions of water radicals with purine derivatives is fast. In view of this fact it is surprising that steady state radiolysis shows a very low yield of destruction of adenine (see V.2). Scholes , Ward and Weiss (1960) ascribed the low yields to the occurrence of reconstitution reactions. In chapter V , experi-

ments will be described which were done to gain more insight in this problem.

Scholes and Weiss (1952) have studied the radiolysis of adenine and have suggested on the basis of analogy with oxidation studies of uric acid that the C_4-C_5 double bond would be the major site of attack of OH· radicals. Holian and Garrison (1967[a,b]) have shown that the addition of OH· radicals to the C_4-C_5 double bond in purines occurs in oxygenated acid solutions of xanthine , hypoxanthine and adenine. In neutral oxygenated solutions they found the same result for xanthine and hypoxanthine , but could not obtain significant evidence in the case of adenine.

Le Roux , Boulanger and Arnaud (1971) have shown that in the case of xanthine in aerated and in deaerated solutions there is an attack at the C_4-C_5 double bond as well as on the N_7-C_8 double bond (formation of uric acid and 4-amino-5-formamido-2,6-dihydroxy-pyrimidine). In the work of Ponnamperuma , Lemmon and Calvin (1963) all the radiation products that have been found in oxygen free solutions of adenine have an intact C_4-C_5 double bond (8-hydroxy-adenine , 4,6-diamino-5-formamido-pyrimidine and hypoxanthine). In the presence of oxygen , Conlay (1963) has isolated only 8-hydroxy-adenine as a compound with an intact C_4-C_5 double bond. For adenine it can be concluded that in neutral solutions in the absence of oxygen hydroxyl radicals react mainly with the N_7-C_8 double bond. In chapter V , experiments will be described which give more quantitative data about the radiation products of adenine.

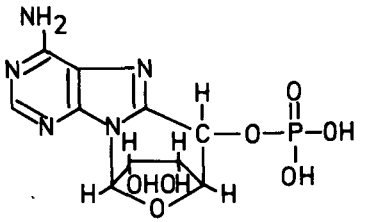
II.2.3 Nucleotides

Nucleotides can be regarded as the smallest structural units of DNA. They contain a purine or a pyrimidine base which is bound to a deoxyribose-5'-phosphate.

In the nucleotide , ionizing radiation will damage the base or the sugar and the damage in the sugar often leads to hydrolysis of the

phosphate ester bond. According to Scholes et al.(1960) , about 80 per cent of the water radicals react with the bases and about 20 per cent with the sugar moieties. It seems that almost every attack on the sugar moiety leads to liberation of a phosphate group (Scholes 1963 ; Ward 1971). According to Hems (1958 , 1960[a,b]) base damage is essentially the same for the nucleoside , nucleotide and free base at least in the absence of oxygen.

Keck (1968) has isolated 8,5'-cyclonucleotides as radiation products of nucleotides in the absence of oxygen. The structure of such a compound produced in the radiolysis of AMP is shown below.



II.2.4 Deoxyribonucleic acid

In 1952 Scholes and Weiss published a paper on the action of X-rays on nucleic acids in aqueous solution , in which they demonstrated that this action leads to base destruction , base liberation and release of inorganic phosphate.

In many experiments done in the last ten years , biologically active DNA has been used , isolated from bacteriophages, which means that it is able to propagate and form complete phage particles by infection of spheroplasts of the bacterial host. Such DNA was used in order to be able to study the biological significance of the different types of radiation-induced damage.

The inactivation by water radicals of such a DNA has been studied in

detail by Blok and coworkers (for a recent review see Blok et al. 1973). It has been shown that $OH^{\cdot}$ and $H^{\cdot}$ radicals inactivate DNA , whereas hydrated electrons although reacting rapidly with DNA do not destroy the biological activity. In oxygenated solutions the reducing radicals react at least partly with oxygen , forming O_2^- radicals which according to Blok , Luthjens and Roos (1967) do not contribute significantly to inactivation of DNA.

Furthermore it has been shown by De Jong , Loman and Blok (1972[a]) and De Jong (1972) that certain organic radicals (derived from amino acids) are able to inactivate biologically active DNA. The water radicals are known to introduce single-strand breaks , double-strand breaks and nucleotide damage in the DNA , where nucleotide damage is defined as damage which does not lead to breaks in the DNA. The contribution of these types of damage to the inactivation of PM2 DNA , as shown in table IV , has been determined by Van der Schans , Bleichrodt and Blok (1973).

Table IV

Contribution of the different kinds of radiation damage to the inactivation of PM2 DNA

	contribution (percent)	inactivation *) efficiency
nucleotide damage	87 ± 4	0.28 ± 0.02
double-strand breaks	4.5 ± 0.5	1
single-strand breaks	8.5 ± 4	0.02 ± 0.01

*) For experimental details and the basic assumptions underlying these figures , see Van der Schans et al. (1973).

As can be seen from the table , single-strand breaks and nucleotide damage are most frequently observed. Nevertheless only about 2 percent of the single-strand breaks and 28 percent of the nucleotide damage is inactivating the DNA , which means that the DNA is repaired to a considerable extent in the bacterial host. De Jong ,Loman and Blok (1972[b]) have shown that also damage sustained by organic radicals in DNA is repairable under certain conditions.

C H A P T E R III Radiosensitization and radioprotection

Various chemicals can increase or decrease the cellular radiosensitivity when present during irradiation (sensitizers and protectors). In this chapter some information about possible mechanisms of radiosensitization and radioprotection is given as an introduction to the experiments , presented in chapters VI and VII.

III.1 Radiosensitization

The study of radiosensitization was prompted by the notion that it might have implications for radiotherapy (Gray 1957) and by the discovery of other radiosensitizers than oxygen , e.g. N-ethylmaleimide (Bridges 1961) The sensitizing effect of oxygen on the survival of cells is a well documented phenomenon. Nevertheless the mechanisms underlying sensitization by oxygen or other sensitizers are still to a large extent unknown , although several hypotheses have been suggested (see the reviews by Bridges (1969) , Adams (1972[a]) and Emmerson (1972)).

1. Removal of intracellular protective substances
2. Reactions of sensitizers with transient radicals in target molecules
3. Interference with cellular repair processes
4. Fixation of radiation damage by transfer of an electron to sensitizers
5. Formation of toxic radiation products

These hypotheses will be briefly discussed below.

III.1.1 Removal of intracellular protective substances

It has been suggested that sulphydryl compounds are able to restore radiation-induced lesions (see III.2). Several SH compounds occur intracellularly. Therefore one would expect that pre-irradiation treatment of cells with compounds (e.g. N-ethylmaleimide) which react with intracellular SH groups would increase the radiosensitivity of cells. This was shown not to be true (Dewey 1965). Nevertheless , the reaction of N-ethylmaleimide with SH compounds contributes to some extent to

the sensitizing effect of this compound when present during irradiation (Müllenger and Ormerod 1969).

III.1.2 Reactions of sensitizers with transient radicals in target molecules

Howard-Flanders (1960) has offered an explanation for the cellular oxygen effect : free radicals formed by irradiation in target molecules react with oxygen to form peroxy radicals ,

$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot} .$$

This reaction is supposed to result in inactivation , which occurs in competition with chemical healing processes (see III.2) , which are therefore most effective in the absence of oxygen. This hypothesis can be extended to sensitizers from the N-oxyl radical type , like triacetoneamine-N-oxyl (TAN , 2,2,6,6-tetramethyl-4-piperidone-N-oxyl). TAN just like oxygen reacts fast with free radicals produced by the reaction between hydroxyl radicals and DNA derivatives (Roberts and Fielden 1971 ; Brustad , Bugge , Jones and Wold 1972). These reactions have rate constants of the order of 10^9 $M^{-1}sec^{-1}$ for oxygen and of the order of 10^8 $M^{-1}sec^{-1}$ for TAN. In this context it is worthwhile to mention that it has been shown by Brustad , Nakken and Jones (1971) ; Nakken , Sikkeland and Brustad (1970) and Brustad , Jones and Wold (1973) , that TAN is covalently bound to DNA irradiated in oxygen free solutions. N-ethylmaleimide too reacts with DNA radicals (Johansen , Ward , Siegel and Sletten 1968).

III.1.3 Interference with cellular repair processes

It has become clear that enzymatic repair processes play an important role in determining the sensitivity of cells to radiation. Therefore one would expect that sensitizers affect the repair reactions. Several compounds like caffeine (Sauerbier 1964) and chloram-

phenicol (Alexander , Dean , Lehman , Ormerod , Feldschreiber and Serianni 1970) are known to inhibit repair enzymes or to interfere with the synthesis of such enzymes. In these cases the sensitizer does not have to be present in the cell at the time of irradiation.
On the other hand , the observation of different degrees of sensitization of various bacterial mutants does not necessary mean that the sensitizer interferes with the repair system. It may as well be the result of the possibilities of the enzyme system to cope with the radiation-induced damage which may be different (III.1.2) in the presence or absence of the sensitizer (e.g. TAN (Emmerson 1968)).

III.1.4 Fixation of radiation damage by transfer of an electron to sensitizers

Adams and Dewey (1963) have noted that the radical anion formed by reaction of a hydrated electron and N-ethylmaleimide is a resonance-stabilized radical , implicating that it is a compound with a large affinity for electrons. The assumption that electron-affinity might be related in some way to sensitizing properties has led to the discovery of a large group of sensitizers which possess an electron acceptor group.
In a 'direct action model' (Adams 1968 ; Adams and Cooke 1969) it is assumed that the electron ejected by an ionization and the positive charge left on a target molecule may recombine after thermalization of the electron. However if the target molecule is surrounded by sensitizer molecules , removal of the electron from the target molecule will compete with charge recombination and thus with self-healing of the target molecule.
From electron spin resonance work it has been concluded that in freeze-dried mixtures of DNA and electron-affinic compounds , long-range electron transfer occurs towards compounds with high electron affinity (Nicolau , Körner and Cristea 1966 , 1967 ; Gregoli , Taverna and Bertinchamps 1970 ; Morley 1970[a,b]). Moreover the

radical yield was found to be higher in the presence than in the absence of these compounds.

III.1.5 Formation of toxic radiation products

It is already known for a long time that halogen containing sensitizers give rise to the formation of toxic products during irradiation. Dewey and Michael (1965) have shown that irradiated iodoacetamide is toxic for cells , in rapid-mixing experiments. The toxic products are of a transient nature because the toxicity dissappears in a few minutes.

Chemicals capable of increasing specifically the radiosensitivity of anoxic or hypoxic cells , as may be present in tumours , are of considerable interest in radiotherapy. In model systems , various compounds have been shown to act like oxygen at non-toxic concentrations. However , it is clear from the above discussion that there is not a unique mechanism for all the different compounds that have been investigated.
In chapter VI experiments will be described which were done in order to investigate the effects of sensitizers in the radiation chemistry of DNA.

III.2 Radioprotection

Protection against radiation damage has been the subject of many investigations. The reasons for the research in this area are obvious (see e.g. Bacq 1965). All known protective compounds that act in vivo are excellent radical scavengers , but not all radical scavengers are protecting in vivo. Although various organic compounds have been shown to act as protectors , the most efficient of these are the thiols such as cysteine and cysteamine.
The so-called hydrogen donation model (Howard-Flanders 1960) has

received much attention. The model postulates the elimination of a radical by hydrogen transfer from the sulphydryl group of the protector.

$$MH + OH^{\cdot} \rightarrow M^{\cdot} + H_2O$$

$$M^{\cdot} + RSH \rightarrow MH + RS^{\cdot}$$

This type of chemical repair has been observed in various model systems by pulse radiolysis (Adams , McNaughton and Michael 1968 ; Adams , Armstrong , Charlesby , Michael and Willson 1969). In these experiments the $RS^{\cdot}$ radical is observed as a complex ,

$$RSH \rightleftharpoons RS^{-} + H^{+}$$

$$RS^{\cdot} + RS^{-} \rightleftharpoons RSSR^{-} .$$

It still remains to be demonstrated that this mechanism acts intracellularly. In the same context , Ward (1971) has shown that several SH compounds are able to reduce the amount of phosphate that is released in the radiolysis of a deoxyribonucleotide (dCMP) in the absence of oxygen. This reduction was larger than would be expected from the scavenging of water radicals by sulphydryl compounds. A possible explanation is that radiation damage produced by hydrogen abstraction from the sugar moiety can be repaired by hydrogen transfer from a SH group. In the absence of SH groups the reaction would lead to release of inorganic phosphate.

De Jong , Loman and Blok (1972[a]) have shown that cysteamine is able to react with DNA radicals , thereby modifying the radiation damage. Cysteamine is also able to protect against the inactivation of biologically active DNA when the DNA is irradiated in the dry state (direct action , Hoff and Koningsberger 1970).

In a recent study with model compounds , Redpath and Willson (1973) showed that endogeneous redox compounds (e.g. vitamin C) may be involved in radioprotection and radiosensitization. They noted a general relationship between protection and reducing properties and between sensitization and oxidizing properties. Therefore knowledge of the

reactions between radicals and redox compounds could be valuable in the search for drugs effective in sensitization and protection.
In chapter VII experiments will be described which show that cysteamine is able to 'repair' radiation-induced radicals of biologically active DNA , even if it is added a few milliseconds after irradiation.

C H A P T E R IV Materials and methods

IV.1 DNA and its biological assay

Purified DNA of the bacteriophage PM2 was prepared and its biological activity assayed on spheroplasts as described by Van der Schans , Weyermans and Bleichrodt (1971) and Van der Schans , Bleichrodt and Blok (1973). For DNA of phage ΦX174 , methods were the same as those of Blok , Luthjens and Roos (1967). Double-stranded replicative form (RF) DNA of ΦX174 was isolated according to Jansz , Pouwels and Schiphorst (1966) *. DNA of phage λ was isolated as described by Kaiser and Hogness (1960) * and assayed according to Young and Sinsheimer (1967) using protamine sulphate to enhance the phage yield (Benzinger , Kleber and Huskey 1971).
The radiation damage in ΦX174 RF-DNA was investigated by assay on spheroplasts of Escherichia coli K12S uvr$^+$ and Escherichia coli K12S uvrA516.
Calf thymus DNA was obtained from Mann Research Laboratories. Before use this DNA was denatured and given the same treatment as used for the isolation of pure ΦX174 DNA.

IV.2 Chemicals

Paranitroacetophenone (PNAP) was purchased from Fluka A.G. , Triacetoneamine-N-oxyl (2,2,6,6-tetramethyl-4-piperidone-N-oxyl , TAN) was prepared according to Brière , Lemaire and Rassat (1966) *.
Its structure was checked by mass spectrometry and infrared analysis.
Cysteamine-HCl was obtained from EGA-Chemie , whereas benzophenone ,

*) I like to thank Dr G.Veldhuisen and Mr H.L.Heijneker (Medical Biological Laboratory TNO) for gifts of λ DNA and ΦX174 RF-DNA , and Mr G.R.van den Berg (Chemical Laboratory TNO) for the synthesis of TAN.

orotic acid , menadione and adenine were obtained from Sigma Chemical Company. Adenine was also obtained from Nutritional Biochemicals Corporation. 2-^{14}C-adenine was obtained from International Chemical and Nuclear Corporation ; 8-^{14}C-adenine , 2,8-^{3}H-adenine and $^{3}H-H_2O$ were bought from Radiochemical Centre (Amersham) , whereas 8-^{3}H-adenine was obtained from Schwarz BioResearch. All other chemicals were of analytical grade.

IV.3 Dosimetry

For steady state radiolysis , the so-called Fricke dosimeter has been used (Fregene 1967). The solution contains : 0.8 N H_2SO_4 , 10^{-3} M NaCl and 10^{-3} M $Fe(NH_4)_2(SO_4)_2$. During irradiation ferric ions are formed which can be measured spectrophotometrically. These measurements were done at 305 nm using $\varepsilon = 2200\ M^{-1}cm^{-1}$ as the value for the extinction coefficient at 25°. If necessary the optical densities were corrected for the decrease in light absorption of 0.7 % per degree under 25°. A yield of $G(Fe^{3+}) = 15.5$ has been used for ^{60}Co-γ-rays.

In pulse radiolysis experiments , thiocyanate was used as a chemical dosimeter. This method is based on the following reactions which occur in aerated solutions of 10^{-2} M KCNS ,

$$OH^{\cdot} + CNS^{-} \rightarrow CNS^{\cdot} + OH^{-}$$

$$CNS^{\cdot} + CNS^{-} \rightarrow (CNS)_2^{-} .$$

The $(CNS)_2^{-}$ radical anion absorbs light with a high extinction coefficient ($\varepsilon = 7100\ M^{-1}cm^{-1}$, Adams , Boag , Currant and Michael 1965), and is relatively long-lived. From the measurement one obtains the concentration of $OH^{\cdot}$ radicals and therefore the absorbed dose , using the well-know value of $G(OH^{\cdot})$, because in this system ,

$$G(OH^{\cdot}) = G((CNS)_2^{-}) .$$

Doses were determined by reference to the thiocyanate system , using a sandwich plate collector system , as described by Adams et al.(1965).

With conventional X-ray and γ-ray sources , the intensity of radiation is too small to obtain a measurable concentration of short-lived radicals. For a direct study of these radicals high dose rates are a prerequisite. Moreover the radiation dose must be delivered to the solution in a time that is short in comparison with the life time of the radical to be studied. Such high dose rates in a pulse can be obtained from e.g. a linear electron accelerator or a Van de Graaff accelerator. In the experiments described here , microsecond pulse radiolysis has been used and a schematic description of the experimental set-up is given in figure 1.

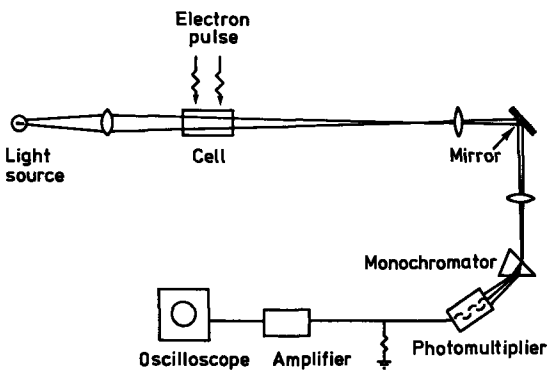


Figure 1. Basic arrangement for pulse radiolysis. For details see text.

By a pulse of electrons , radicals are formed in the solution in the cell. The occurrence of radicals can be studied by means of their absorption spectra obtained with a high intensity light source and a monochromator. The light beam emerges from the monochromator and falls on the cathode of a photomultiplier tube ; the output signal is amplified and displayed on an oscilloscope screen. The oscilloscope beam will move as a function of the changes in light absorption in the

cell and therefore the oscilloscope traces are kinetic records of the transient species (radicals or ions) formed or decaying during the chosen time interval.

It has been observed in most cases that the energy required to produce electronic excitation in a radical is less than that required to produce a similar excitation in the parent molecule. So the radicals usually absorb light at higher wavelengths than the parent molecules. Details about the optical systems and the sophisticated electronics in pulse radiolysis have been published by Keene (1965) , Michael (1969), Matheson and Dorfman (1969) , Luthjens and Schmidt (1973) and Luthjens (1973).

Oscilloscope traces which are obtained in pulse radiolytic experiments are photographed and optical densities can be calculated as a function of time. Attempts can be made to correlate the changes in optical density with kinetic equations of the type shown below , in order to obtain the relevant rate constants. In these equations ε represents the molecular extinction coefficient. OD_0 , OD_{oo} and OD_t are the measured optical densities at time 0 , infinite time and time t respectively. C represents the concentration, and l the length of the cell.

i) First order reaction ($A \rightarrow B$)

$$-dC_A/dt = kC_A$$

A absorbs only :

$$\ln OD_t = \ln OD_0 - kt$$

B absorbs only :

$$\ln (OD_{oo} - OD_t) = \ln OD_{oo} - kt$$

ii) Pseudo first order reaction ($A + B \rightarrow C$, with $C_A \ll C_B$)

$$-dC_A/dt = kC_AC_B$$

A absorbs only :

$$\ln OD_t = \ln OD_0 - kC_Bt$$

C absorbs only :

$$\ln (OD_{oo} - OD_t) = \ln OD_{oo} - kC_Bt$$

iii) Second order reaction between identical species

(A + A → B)

$$-dC_A/dt = kC_A^2$$

A absorbs only :

$$1/OD_t = 1/OD_0 + kt/\varepsilon_A l$$

B absorbs only :

$$1/(OD_{oo} - OD_t) = 1/OD_{oo} + 2kt/\varepsilon_B l$$

In our experiments a linear accelerator has been used delivering 0.2 μsecond pulses of 1.8 MeV electrons. Details have been published elsewhere (Adams , Boag and Michael 1965).

IV.5 Rapid-mixing apparatus

A rapid-mixing apparatus similar to the one described by Adams , Cooke and Michael (1968) was used and a schematic description is given in figure 2A and a photograph of the set-up in figure 2B.
Syringes A and B contain respectively a solution of DNA in buffer and a solution of the compound to be examined. Both syringes are connected to glass capillary tubes (C and D) of internal cross-section 0.3 mm^2. The tubes intersect at point E with a third capillary tube of cross-section 0.6 mm^2. A third syringe (F) containing the same DNA solution as A is connected to a capillary tube of cross-section 0.3 mm^2. The two DNA solutions are irradiated simultaneously over a 3 mm field (G) with a beam of 3 MeV electrons. For shielding against radiation a collimator consisting of 7 cm Pb covered with 1 cm Al was used. The syringes are driven by a constant speed stepping motor , displacing the contents of each syringe at a speed of 2 ml per second. The high flow rate ensures turbulant flow and efficient mixing within a millisecond.
At point H the glass tubes divide and are connected to silicon rubber tubing. With the aid of an electromagnetic shutter (I) , triggered two seconds after the combined start of irradiation and liquid flow , it is possible to collect that fraction of the DNA that passes the

electron beam at a constant speed.

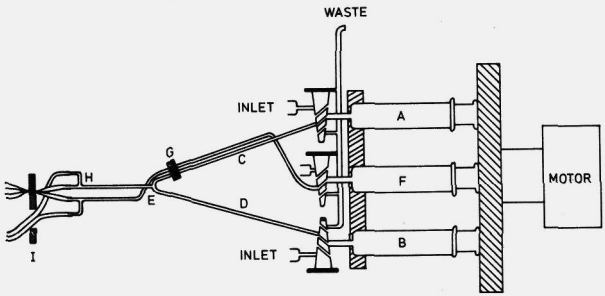


Figure 2A. Rapid-mixing apparatus (for details see text).

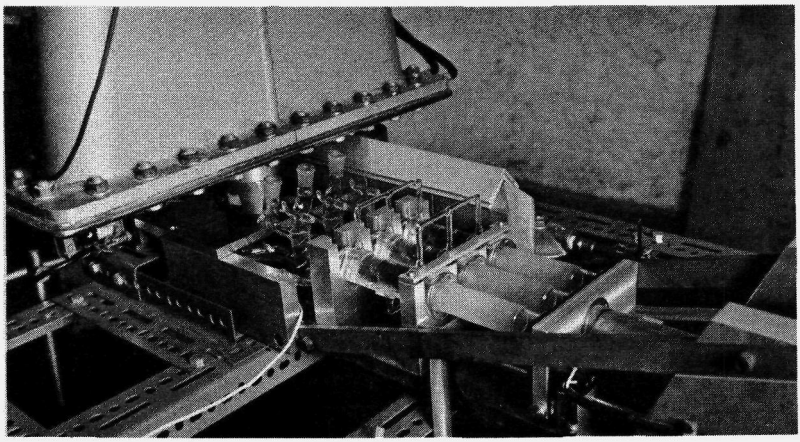


Figure 2B. A photograph of the rapid-mixing apparatus.

The 3 MeV electrons were obtained with a Van de Graaff accelerator. The electron beam current was measured in a deflected position with a Faraday target connected to an oscilloscope or current meter. During an experiment the electron beam was directed through a collimator. For alignment of the beam with regard to the collimator the current was collected on a target underneath the collimator and optimized with the beam positioner.
Relative doses in the experiments refer to currents measured with both targets. The ratio between these currents gives a continuous check on the alignment.

IV.6 Preparation of freeze-dried DNA samples

Aliquots of 0.5 ml of a mixture of biologically active DNA (10 µg/ml) and purified calf thymus DNA (100 µg/ml) and either PNAP , TAN (10 µg/ml) or no sensitizer in phosphate buffer (pH 7.1) , were rapidly frozen and freeze-dried , with the cooling trap at -60°. The ampullae containing the dry DNA were flushed several times with dry nitrogen , freeze-dried for another 4 hours and sealed under vacuum. After irradiation of the DNA the samples were dissolved in buffer and diluted sufficiently to ensure proportionality between yield of phage and concentration of DNA in the spheroplast assay (Blok 1967).

IV.7 Determination of the numbers of breaks in DNA samples.

1-2 ml of a solution containing at least 3 µg/ml DNA was layered on top of a sucrose gradient. The gradients were centrifuged overnight at about 24000 rpm in a 'swinging bucket' rotor (Spinco SW 27) according to Van der Schans et al. (1973). After centrifugation the tubes were pierced with a hypodermic needle through which carbon tetrachloride is led at a constant speed. The contents of the tube were led through a continuous-flow cell in a UV detection system.
An example of the analysis for circular double-stranded PM2 DNA is shown in figure 3. As can be seen , PM2 DNA exists in different forms.

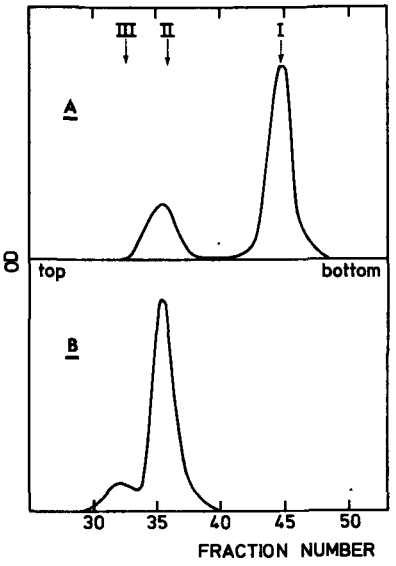


Figure 3. Sedimentation pattern of PM2 DNA in a sucrose gradient. Radiation doses : 0 krads (A) ; 1.5 krads (B).

Component I is a twisted cyclic double-stranded molecule. Introduction of one or more single-strand breaks converts component I into II which is no longer twisted. Component III is obtained by introduction of a double-strand break in I or II. It has been shown (Van der Schans et al 1973) that I and II are biologically active in the spheroplast assay , whereas III is inactive.

One may expect that the introduction of breaks in DNA by irradiation occurs at random and therefore the number of single-strand breaks per molecule follows the Poisson distribution. The same is true for double-strand breaks. If the average number of double-strand breaks per molecule is P_{ds} , then the fraction of DNA present as I and II is exp $(-P_{ds})$. In this way P_{ds} can be determined. The fraction of DNA present in the component I band is , exp $(-P_{ds}-P_{ss})$ where P_{ss} is the average number of single-strand breaks per molecule.

With single-stranded ΦX174 DNA the number of breaks has been determined in a similar way. In this case unbroken molecules and molecules with one break sediment in one band. From the fraction of the molecules which sediment in this band , $(P_{ss}+1)\exp(-P_{ss})$, the average number of single-strand breaks per molecule can be calculated.

IV.8 Chromatography

Irradiated solutions of adenine were analysed by eluting the solutions on a column (length : 30-100 cm ; diameter 1.5 cm) of Sephadex G-10 (Pharmacia Fine Chemicals) , which was equilibrated with 3 mM sodium chloride or 0.1 M ammonium formate (pH 3.5).
Paper chromatography was done with Whatman-1 paper using various solvents.

1. n-butanol saturated with 1.5 N NH_4OH
2. n-butanol - H_2O (43 : 7) V/V
3. isopropanol - saturated $(NH_4)_2SO_4$ - 1 M CH_3COONa (1 : 40 : 9) V/V
4. propanol - NH_3 - H_2O (6 : 3 : 1) V/V
5. n-butanol - diethylene glycol - H_2O (4 : 1 : 1) V/V , NH_3 in the vapour phase
6. n-butanol - propionic acid - H_2O (14 : 9 : 10) V/V
7. H_2O , containing NH_3 pH 10

IV.9 Other experimental details

A ^{60}Co-γ-ray source (Gamma cell 100 , Atomic Energy of Canada Ltd.) has been used for steady state radiolysis , with dose rates varying between 5 and 100 rads per second.
During irradiation , solutions were flushed with oxygen , nitrogen or nitrous oxide , saturated with water vapour. If necessary , the

absence of oxygen ($\leq 10^{-8}$ M) was checked by means of a Hersch cell (Hersch 1960).

The water used was deionized , distilled from alkaline permanganate and distilled once more without additives. Glassware was cleaned with tap-water , rinsed thoroughly with deionized water and finally with redistilled water.

Hydrogen peroxide was determined according to Allen , Hochnadel , Ghormley and Davies (1952). Radioactivity measurements were performed with a liquid scintillation counter (Nuclear Chicago). 0.5 ml of an aqueous solution or a dry piece of paper cut from a chromatogram was added to 12 ml of a mixture of ten parts toluene containing 0.01 % POPOP (W/V) and 0.6 % PPO (W/V) and three parts Triton X-100.

Optical densities were determined with a Zeiss PMQ II spectrophotometer. Absorption spectra were taken with a Beckman Dk-2A spectrophotometer. The mass spectra *) were obtained with a Jeol JMS-01SG-2 or a MS 902 (AEI). Samples were introduced on the direct insertion probe (sample temperature between 120 and 180° ; chamber temperature between 150 and 200°).

*) I am indebted to Dr H.A.H.Craenen (Chemical Laboratory TNO) and to Dr W.Heerma (Analytical Chemistry Laboratory , The University , Utrecht) for the mass spectrometric analyses.

C H A P T E R V Radiation chemistry of adenine *)

In this chapter experiments are described that were carried out to obtain more information on the radiation products of adenine and on the chemical pathways leading to these products. Furthermore some reactions of the adenine radicals with radiosensitizing and radioprotecting agents will be described.

V.1 Pulse Radiolysis

In aqueous solution nucleic acid derivatives react rapidly with the radicals from water radiolysis (e^-_{aq} , $OH^\cdot$ and $H^\cdot$, see table III). These reactions lead to the formation of short-lived radicals , which absorb light in the visible and near-UV region of the spectrum. These absorbing radicals can be observed and their reactions followed by the kinetic spectrophotometric method , briefly discussed in IV.4.

V.1.1 Spectra and reactions of the hydroxyl adduct of adenine

In figure 4 the transient absorption spectra are shown , obtained by pulsing an adenine solution saturated with N_2O. These spectra are believed to pertain to the hydroxyl adduct of adenine for the following reasons.
In the presence of N_2O the hydrated electrons are scavenged almost completely giving additional $OH^\cdot$ radicals. $H^\cdot$ radicals which are also present , may contribute to the absorption spectrum by the formation of hydrogen adducts , but this contribution must be small because it can be calculated from the reaction rate constant (table III) that the

*) Some results that are described in this chapter , have been published (Van Hemmen and Bleichrodt 1971 ; Van Hemmen 1971 ; Adams , Greenstock, Van Hemmen and Willson 1972).

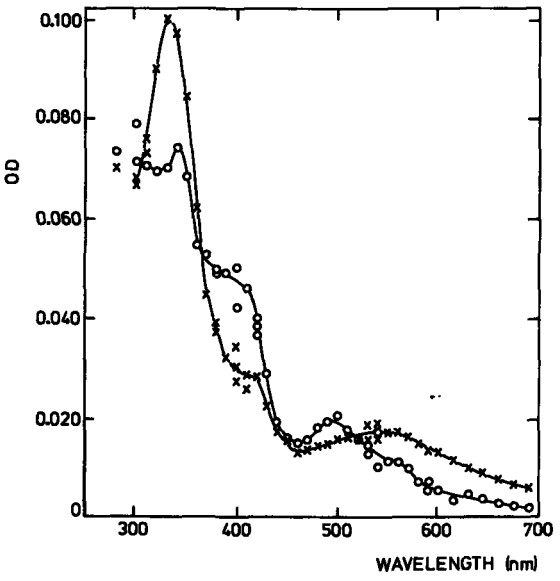


Figure 4. Absorption spectra of 3.8×10^{-4} M adenine , saturated with N_2O (2.8×10^{-2} M) , 2 μsec (o) and 10 μsec (x) after a pulse of about 2.2 krads.

half-life of formation of the hydrogen adduct is about 22 μsec in this solution , whereas the spectra have been taken at 2 and 10 μsec after the pulse. Furthermore the number of $OH^{\cdot}$ radicals formed is about ten times as large as the number of $H^{\cdot}$ radicals , because (see table II)

$$\frac{G(e^-_{aq}) + G(OH^{\cdot})}{G(H^{\cdot})} \approx 10$$

It can be calculated that in a neutral solution of 2.5×10^{-3} M adenine containing 2.5×10^{-1} M t-butanol and 2.8×10^{-2} M N_2O as scavengers for hydroxyl radicals and hydrated electrons respectively , almost equal numbers of $OH^{\cdot}$ and $H^{\cdot}$ radicals react with adenine. In this case spectra were observed having the same shape as those in figure 4 , indicating that the hydrogen adduct of adenine is not

contributing significantly to the spectra observed in figure 4 or that it has the same absorption spectrum. The t-butanol radicals that are formed in the solution do not absorb light above 300 nm (Simic , Neta and Hayon 1969).

As can be seen in figure 4 , the spectrum changes with time. At 330 and 600 nm the absorption still increases after 2 μsec , whereas at 400 nm the absorption decreases. In figure 5 oscilloscope traces are shown to illustrate the time dependence at 400 nm.

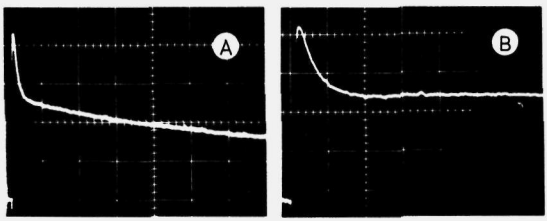


Figure 5. Oscilloscope traces at 400 nm ; adenine (3.8×10^{-4} M) saturated with N_2O. Ordinate : 2.5 % absorption per large division. Abscissae : 100 μsec per large division (A) and 10 μsec per large division (B).

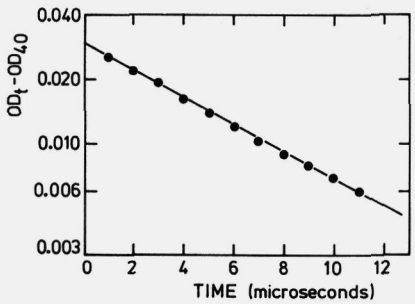


Figure 6. First order plot of the fast decay at 400 nm. Adenine (2.7×10^{-3} M) saturated with N_2O. Dose about 2 krads. OD_{40} is the optical density at t = 40 μsec .

The oscilloscope trace in figure 5A shows the decay at a long time scale. The slow decay was shown to follow second order kinetics. The fast decay in the spectrum at 400 nm (figure 5B) was shown to be of first order (figure 6). In table V it is shown that the decay is not dependent upon the concentration of adenine in the solution. Furthermore the half-life of formation or decay at various wavelengths is the same throughout the spectrum. Therefore the first order reaction may represent an intramolecular rearrangement.

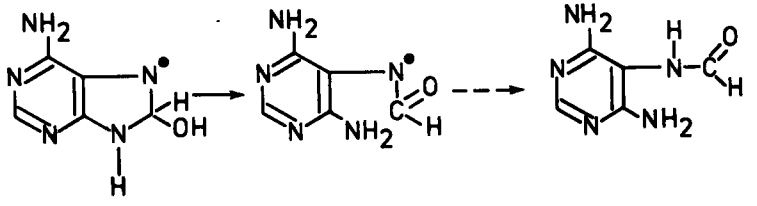
Table V

Half-life of the first order decay of the hydroxyl adduct of adenine as a function of the adenine concentration , measured at 400 nm.

adenine (M x 10^4)	$t_{1/2}$ (µsec)
1	4.4
2.7	4.3
8.1	4.5
10	4.4
27	4.8
81	4.1

Willson (1966) has found similar first order kinetics for the decay of hydroxyl adducts in some other purine derivatives like adenosine and dAMP. Roberts and Fielden (1971) noted a fast first order decay in irradiated DNA solutions. Furthermore it is known that in the radiolysis of adenosine , dAMP and DNA , ring opening occurs , which results in the formation of formamido-pyrimidine derivatives (Hems 1960).
In the γ-radiolysis of purine , no formation of a formamido-pyrimidine derivative has been detected (own observations) ; also the hydroxyl adduct of purine has been studied and no first order kinetics could be observed in pulse radiolytic experiments. These observations suggest that ring opening leading to the formation of formamido-pyrimidines is

correlated to the rapid changes in the absorption spectra of the hydroxyl adducts of purine derivatives.



It is tentatively suggested that the initial hydroxyl adduct of adenine is formed by addition of an OH$^{\cdot}$ radical to the 8-position of adenine. The adenine radical may then rearrange to form another radical in which the ring is opened. In the above scheme this is shown. It should be mentioned that the structure of the intermediate radical is purely hypothetical.

A reaction that may be important in radiosensitization is the reaction with oxygen. Willson (1970) has shown that various radicals of pyrimidines and purines react rapidly with oxygen ($k \approx 10^9\ M^{-1} sec^{-1}$). He did not investigate the reaction between the hydroxyl adduct of adenine and oxygen.

In figure 7 the absorption spectra obtained 100 µsec after pulse irradiating an adenine solution in the presence and absence of oxygen is shown. It is evident that oxygen reacts with an adenine radical , because the absorption spectra in figure 7 depend upon the concentration of oxygen in the solution. This is shown even more clearly in figure 8 , where oscilloscope traces are presented which show the time dependence of the hydroxyl adduct of adenine in the absence and presence of oxygen. In figure 8A the decay of the hydroxyl adduct of adenine is shown in the absence of oxygen. In the presence of a small amount of oxygen (figure 8B) , the initial radical concentration is lowered , indicating that the radical is reacting fast with oxygen. This is even

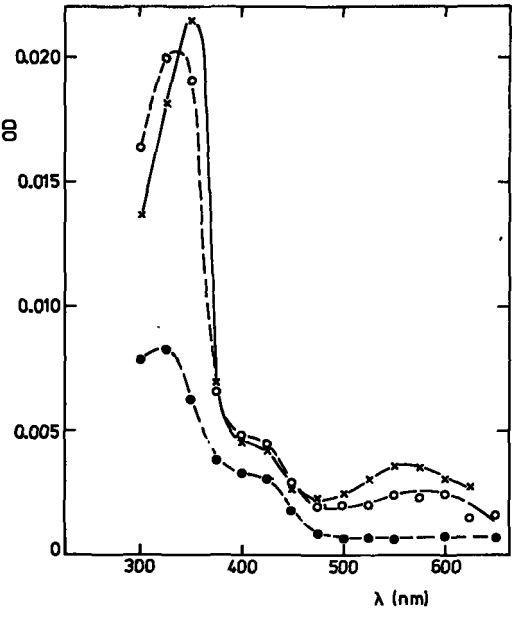


Figure 7. Absorption spectra of adenine solutions (3×10^{-4} M), 100 μsec after a pulse of about 2 krads.
x-x : 2.8×10^{-2} M N_2O.
o-o : 2.2×10^{-2} M N_2O and 2.5×10^{-4} M O_2.
●-● : 1.25×10^{-3} M O_2.

more clear from figure 8C, where adenine is irradiated in the presence of pure oxygen. Furthermore from the last trace it is evident that a new absorption is growing in.

Although it is pretty sure that the hydroxyl adduct of adenine reacts with oxygen, it is obvious that more experiments are needed to solve the reaction mechanism. In fact these experiments were done during a stay in the 'Gray Laboratory' and at this point the experiments were stopped in view of the complex character of the chemistry involved.

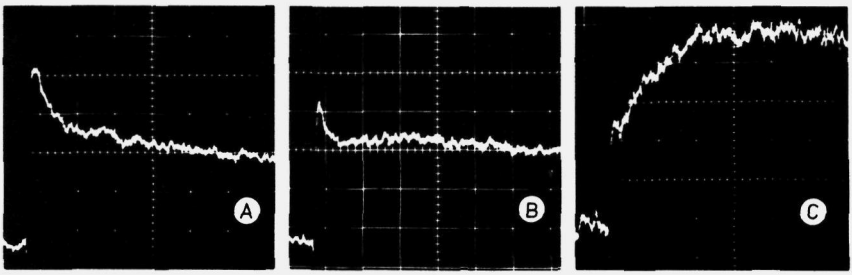


Figure 8. Oscilloscope traces of adenine solutions (3×10^{-4} M) at 425 nm , after a pulse of about 2 krads under different gas conditions. Abscissae : 20 µsec per large division. Ordinate :
A : 0.4 % absorption per large division (2.8×10^{-2} M N_2O)
B : 0.4 % absorption per large division (2.2×10^{-2} M N_2O and 2.5×10^{-4} M O_2)
C : 0.2 % absorption per large division (1.25×10^{-3} M O_2).

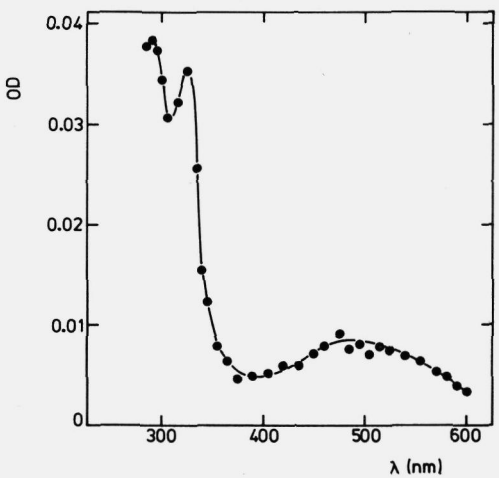


Figure 9. Absorption spectrum of the electron adduct of adenine (3.2×10^{-4} M) in the presence of t-butanol (10^{-1} M) , saturated with N_2 , 3 µsec after a pulse of about 2.3 krads.

Oxidation of the hydroxyl adduct of adenine by another radiosensitizer , PNAP could not be observed, by looking at the formation of the electron adduct of PNAP (see V.1.2) , giving an upper limit of about 3×10^7 $M^{-1}sec^{-1}$ for the corresponding bimolecular rate constant.
The reaction of the hydroxyl adduct of adenine with cysteamine (a radioprotecting agent) could be observed by the formation of the $RSSR^-$ radical anion (see III.2). The rate constant for this reductive process was determined as $k = 7.3 (\pm 1.2) \times 10^7$ $M^{-1}sec^{-1}$.

V.1.2 Spectrum and reactions of the electron adduct of adenine

If a solution of adenine saturated with nitrogen is irradiated in the presence of t-butanol , the hydroxyl radicals react with the alcohol and the hydrated electrons react with adenine. In figure 9 the absorption spectrum of the electron adduct of adenine , 3 μsec after the pulse is shown. The hydrogen adduct of adenine does not contribute significantly to the absorption spectrum in figure 9 , for similar reasons as discussed in V.1.1 .
The reactions of the electron adduct have been investigated and special attention has been given to the reactions with sensitizers. It can be concluded from figure 10 that the electron adduct of adenine can undergo electron transfer reactions to PNAP.
This follows from the fact that in the solution , containing adenine , PNAP and t-butanol , the hydrated electrons will react initially with adenine. However , an increase in the concentration of $PNAP^-$ is observed in the next microseconds , due to the following reaction ,

$$A^{(-)} + PNAP \rightarrow A + PNAP^-.$$

The spectra in figure 10 have been taken 10 μsec after the pulse ; at that time the reaction is over and all hydrated electrons are located at PNAP. The reaction follows pseudo first order kinetics and it is shown in figure 11 that the first order rate constant increases linearly with the concentration of PNAP , giving a bimolecular rate constant , $k = 5.4 \times 10^9$ $M^{-1}sec^{-1}$.

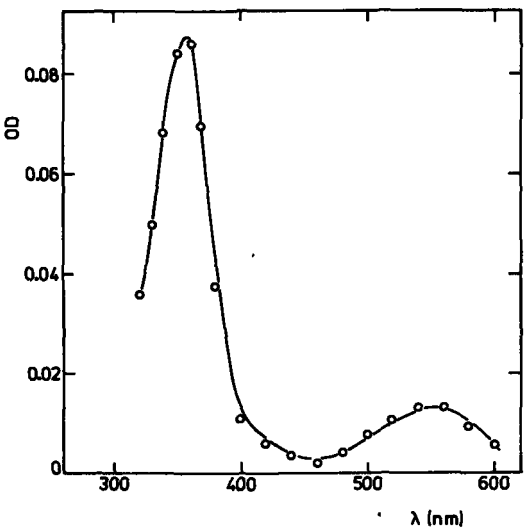


Figure 10. Electron transfer to PNAP. Absorption spectra of oxygen-free solutions , containing 10^{-4} M PNAP and 1 M t-butanol. Solid line , no adenine. o-o : 5×10^{-3} M adenine present. Dose : 1 krad. Spectra taken 10 µsec after the pulse.

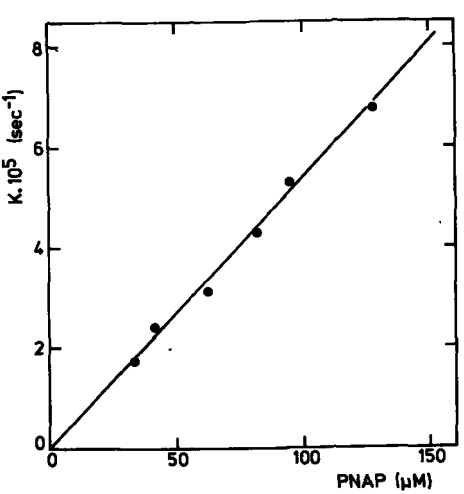


Figure 11. Pseudo first order dependence of the rate constant of electron transfer on the concentration of PNAP in oxygen-free solutions. Adenine : 5×10^{-3} M ; t-butanol : 1 M.

Electron transfer reactions are not unique for the electron adduct of adenine. In table VI it is shown that apart from adenine also some adenine derivatives show these reactions and to various acceptor molecules.

Table VI

Rate constants for electron transfer *)

Donor radical	PNAP pH 7	menadione pH 7	benzophenone pH 12	orotic acid , pH 7
adenine	5.4	4.2	2.7	3.5
adenosine	5.4	3.4	-	2.8
5'-AMP	4.6	2.9	-	1.8
dAMP	4.3	2.9	-	1.9

*) Units : 10^9 M^{-1} sec^{-1}

The electron adduct does not only react with PNAP , but also with the best known sensitizer , oxygen , although it is unknown whether this reaction involves electron transfer. The rate constant for this reaction has been determined by competition with electron transfer to PNAP

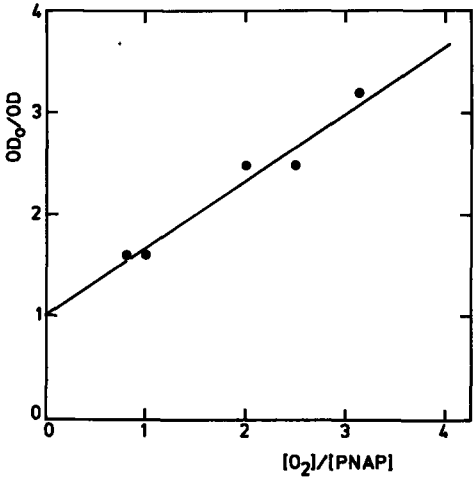


Figure 12. Competition plot for the reaction of the electron adduct of adenine with O_2 and PNAP. For details see text.

$$A^{(-)} + PNAP \xrightarrow{k_2} A + PNAP^-$$

$$A^{(-)} + O_2 \xrightarrow{k_1} \text{products}$$

If OD_o is the magnitude of absorption of $PNAP^-$ in the absence of O_2 and OD the magnitude of absorption in the presence of oxygen with various concentrations of PNAP , then the following relation holds , if there is simple competition between O_2 and PNAP for $A^{(-)}$.

$$\frac{OD_o}{OD} = 1 + \frac{k_1 \times [O_2]}{k_2 \times [PNAP]}$$

From the plot in figure 12 , a value for $\frac{k_1}{k_2}$ is obtained. From the value for k_2 (table VI) the reaction rate was determined to be $k_1 = 3.7\ (\pm 0.6) \times 10^9\ M^{-1}sec^{-1}$.

V.2 Steady state radiolysis

V.2.1 Identification of the radiation products

If a solution of adenine , irradiated with γ-rays under nitrogen with a dose of 2 Mrads,is subjected to chromatography on G-10 using a 3×10^{-3} M solution of sodium chloride as effluent , six ultraviolet light absorbing peaks are observed , as shown in figure 13.
Components I and II in figure 13 do not show the characteristic absorption bands of purines and pyrimidines in the wavelength region between 240 and 360 nm. This indicates that these compounds have lost the aromatic structure as present in a purine. Both compounds are found when 2-^{14}C-adenine (label in the pyrimidine ring) or 8-^{14}C-adenine (label in the imidazole ring) are irradiated. Therefore I and II possibly contain both C atoms.
In figure 14 , dose effect curves for the formation of I and II in the radiolysis of adenine are shown. Using labeled adenine the yields of components I and II were found to be almost independent of the adenine concentration. Because of their low yields ($G < 0.05$) these products

will not be discussed further.

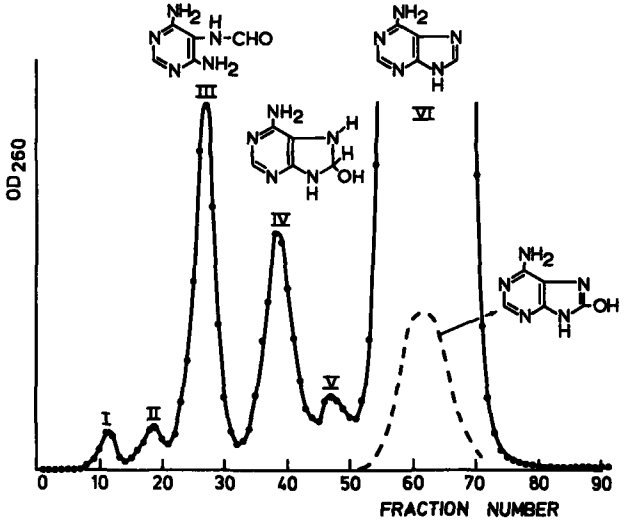


Figure 13. Typical elution diagram of adenine irradiated under N_2 , on Sephadex G-10 ; adenine concentration : 3×10^{-3} M ; Dose : 2 Mrads ; elution with 3×10^{-3} M NaCl.

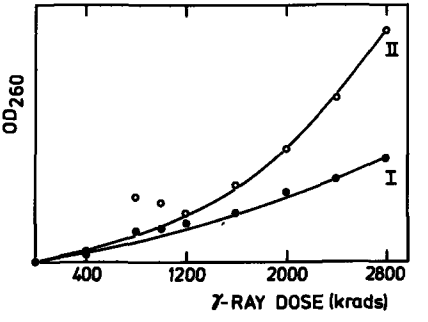


Figure 14. Dose effect curves of components I and II. Irradiation of adenine (3×10^{-3} M). Chromatography as in figure 13.

Fractions in the other peaks in figure 13 have been collected , freeze-dried and subjected to ascending paper chromatography. Except for peak VI all peaks were chromatographically pure. The R_F values have been summarized in table VII together with the R_F values of some selected compounds.

Table VII

R_F-values of some purine and pyrimidine derivatives

compound	solvent number (see IV.8)						
	1	2	3	4	5	6	7
III	0.10	0.12	0.40	0.50	0.34	0.53 0.25	0.63
IV	0.30	0.45	0.13	0.61	0.50	0.76	0.38
V	-	-	-	-	0.50	0.59	-
	0.31	0.46	0.14	0.61	0.50	0.78	0.40
VI	0.12	0.36	0.34	0.50	0.32	0.66 0.55	0.66
adenine	0.31	0.46	0.14	0.61	0.50	0.78	0.40
8-hydroxy-adenine	0.11	0.36	0.34	0.50	0.32	0.66	-
4,6-diamino-5-formamido-pyrimidine	0.09	0.10	0.40	0.50	0.20-0.35	0.58	0.59
hypoxanthine	0.10	0.23	0.34	0.55	0.36	0.59	0.60
4,5,6-triamino-pyrimidine	0.20	-	0.40	0.58	0.45	un-stable	0.50
isoguanine	0.03	0.11	0.29	0.42	0.27	-	0.41

It should be noted here that neither hypoxanthine nor adenine-7-N-oxide , which have been identified by Ponnamperuma , Lemmon and Calvin (1963) and Rhaese (1968) respectively as radiation products of adenine , could be observed in the present study.
The chromatographic behaviour of component III resembles that of 4,6-diamino-5-formamido-pyrimidine , which was synthesized according to the method of Cavalieri , Tinker and Brown (1949). Furthermore the absorption spectra at different pH were identical with those of

the synthesized product. Also the mass spectra of III and 4,6-diamino-5-formamido-pyrimidine were virtually the same (figure 15).

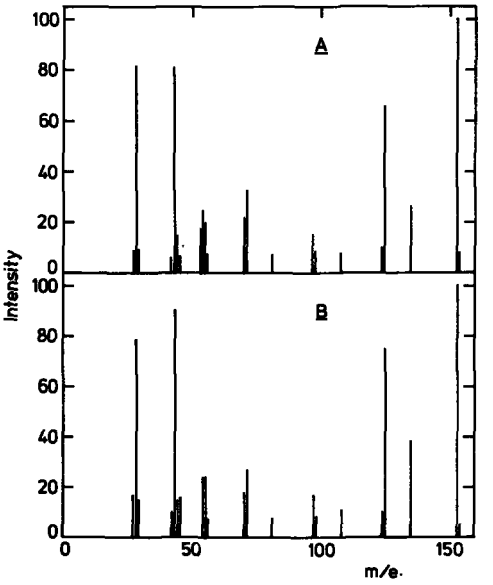


Figure 15. **Mass spectra of component III (A) and 4,6-diamino-5-formamido-pyrimidine (B). The spectrum (75 eV) is plotted in terms of relative abundance with the highest peak taken as 100. Intensities smaller than 5 % are omitted.**

An anomaly was noted with component III in solvent 6. With this solvent , two ultraviolet light absorbing spots appeared (table VII). Apparently this was due to the presence of sodium chloride in the freeze-dried column eluate , as also two spots appeared when the synthesized product was cochromatographed with sodium chloride in solvent 6. From the above results it is concluded that component III is 4,6-diamino-5-formamido-pyrimidine.

The absorption spectra of component IV are shown in figure 16. The absorption spectrum changes with time in dilute acid solution. Gel filtration and paper chromatography show that the reaction product under these conditions is adenine. Adenine is also obtained when a neutral

solution of IV is heated to 80°.

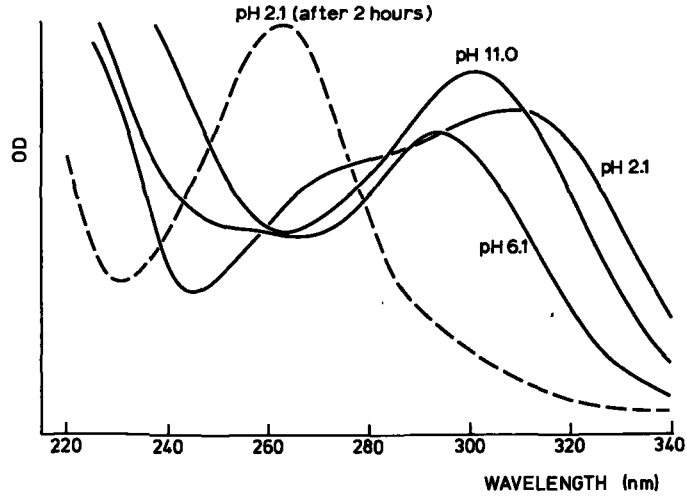


Figure 16. (——) Absorption spectra of component IV at various pH. (---) Absorption spectrum at pH 2.1 after 2 hours at room temperature.

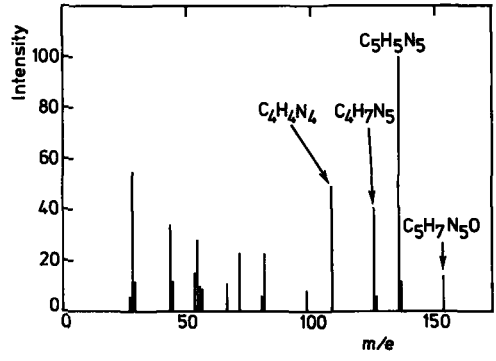


Figure 17. Mass spectrum of component IV. The spectrum (75 eV) is plotted in terms of relative abundance with the highest peak taken as 100. Intensities smaller than 5 % are omitted.

Exact masses , determined with a high resolution mass spectrometer, showed that the product in peak IV is hydrated adenine ($C_5H_7N_5O$ = $C_5H_5N_5 + H_2O$, figure 17). In the mass spectrometer , component IV easily loses water or it loses CO which results in the formation of $C_4H_7N_5$. This means that the OH group is bound to one of the carbon atoms in the original molecule. As discussed in II.2.2 , the 8-C atom is the carbon atom most susceptible to radical attack. To determine whether this atom is involved , the hydrated adenine was isolated from an irradiated mixture containing about equal amounts of 8-^{12}C- and 8-^{14}C-adenine. Mass spectrometric analysis of the hydrated adenine showed that the fragment which had lost the CO , contained no longer the 8-C atom. From these data it is concluded that component IV is 6-amino-8-hydroxy-7,8-dihydropurine.

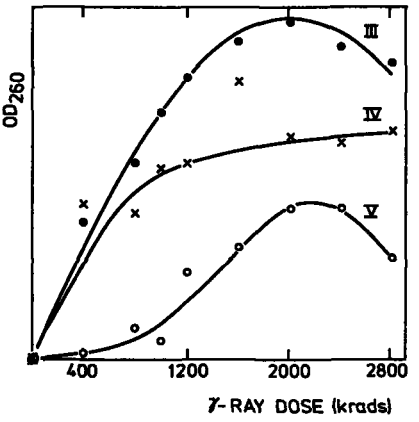


Figure 18. Dose effect curves of the components III , IV and V , determined after irradiation of adenine and separation of the products according to figure 13. Ordinate relative units.

Component V was found to be the main product in the radiolysis of 4,6-diamino-5-formamido-pyrimidine (III) , which suggests that V is a secondary radiation product of adenine formed via the formamido-pyrimidine. Comparison of the dose effect curves given in figure 18 gives other evidence that V is a secondary radiation product of adenine ,

because its dose effect curve has an initial slope of about zero in contrast with those of the other compounds.

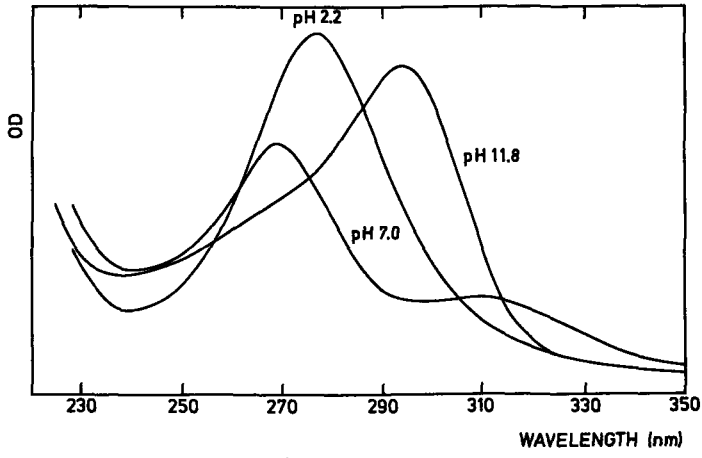


Figure 19. Absorption spectra of component V at various pH.

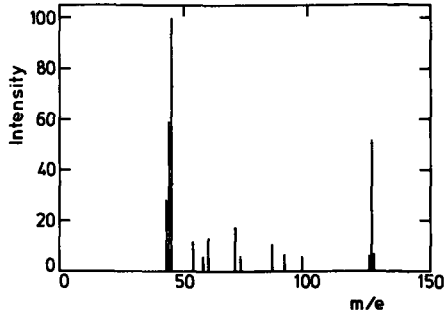


Figure 20. Mass spectrum of component V. The spectrum (70 eV) is plotted in terms of relative abundance with the highest peak taken as 100. Intensities smaller than 5 % are omitted.

By using 8-^{14}C-adenine it was found that component V has lost the 8-C atom. Nevertheless it still contains an ultraviolet light absorbing chromophore , as shown in figure 19 , indicating that the pyrimidine structure may be intact.

The elementary composition , determined with a high resolution mass spectrometer was shown to be $C_4H_6N_4O$ (figure 20) , suggesting that V is a diamino-monohydroxy-pyrimidine. If this is correct component V can be identified as 4,6-diamino-5-hydroxy-pyrimidine on the basis of the absorption spectra , because the other possible isomers have published spectra which are different from those in figure 19.

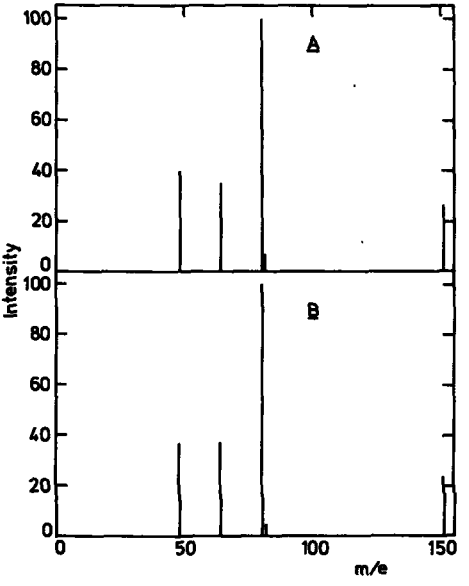


Figure 21. Mass spectra of one of the compounds from peak VI (see figure 13)(A) and 8-hydroxy-adenine (B). The spectra (75 eV) are plotted in terms of relative abundance with the highest peak taken as 100. Intensities smaller than 5% are omitted.

The fractions of peak VI contain adenine and 8-hydroxy-adenine. These compounds can be separated by gel filtration using 0.1 M ammonium formate (pH 3.5) or by paper chromatography. At pH 3.5 adenine is protonated and is eluted with a much smaller elution volume than at neutral pH. The compounds from peak VI were identified by comparison of their chromatographic behaviour (table VII) with that of adenine and 8-hydroxy-adenine , synthesized according to the method of Cavalieri and Bendich (1950). The identification was further confirmed by comparison of absorption spectra at various pH and by mass spectrometric analysis as is shown for 8-hydroxy-adenine in figure 21.

So far the discussion of the radiation products of adenine was confined to compounds which could be found by ultraviolet light absorption measurements , but it has also been observed (Goedbloed and Van Hemmen 1968) that several products found in the radiolysis of nucleic acid derivatives show a fluorescence. This is also true for adenine.
In figure 22 an elution diagram is shown which indicates that various fluorescent compounds are formed. One of the peaks (designated with X) was shown to occur with a yield $G < 0.005$. The same results have been found for another fluorescent compound that is observed after irradiation of adenine under N_2O and which could be isolated. No further attention was paid to these or other fluorescent compounds because of their low yields.

V.2.2 Yields of the radiation products

Using ^{3}H- or ^{14}C-adenine as a tracer the G-values of the radiation products of adenine were determined from the peak areas after separation on G-10. The amount of 8-hydroxy-adenine was determined after paper chromatography of peak VI (figure 13) in solvent 6 (see IV.8).
The spot of 8-hydroxy-adenine on the paper was located by means of autoradiography. The ^{14}C-activity was determined by immersing the paper into the scintillation liquid. The ^{3}H-activity was eluted quantitatively from the paper with 0.1 N HCl and the radioactivity was determined

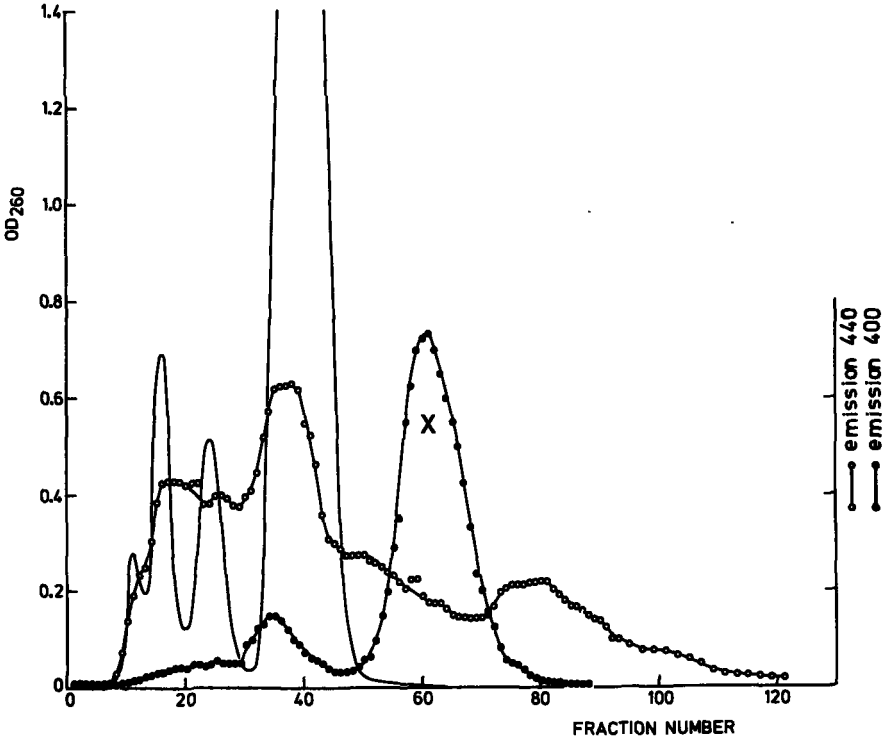


Figure 22. Adenine (10^{-3} M) irradiated with 1 Mrad (N_2). Chromatography on G-10 with 0.1 M ammonium formate (pH 7.0) as effluent. The light emission at 400 nm was excited at 300 nm , whereas the emission at 440 nm was excited by light of 360 nm.(For further experimental details see Goedbloed and Van Hemmen 1968).

in the eluate.

During our studies it was observed that the number of adenine molecules destroyed per 100 eV energy dissipated in the solution was dependent upon the adenine concentration. Therefore the experiments were to include the effect of concentration on the yield of the products. In figure 23 , the amount of adenine destroyed and the yields of

4,6-diamino-5-formamido-pyrimidine , 8-hydroxy-adenine and 6-amino-8-hydroxy-7,8-dihydropurine both for irradiation under N_2 and N_2O with a dose of 50 krads , are plotted versus the concentration of adenine. At this small dose the yield of component V is negligible.

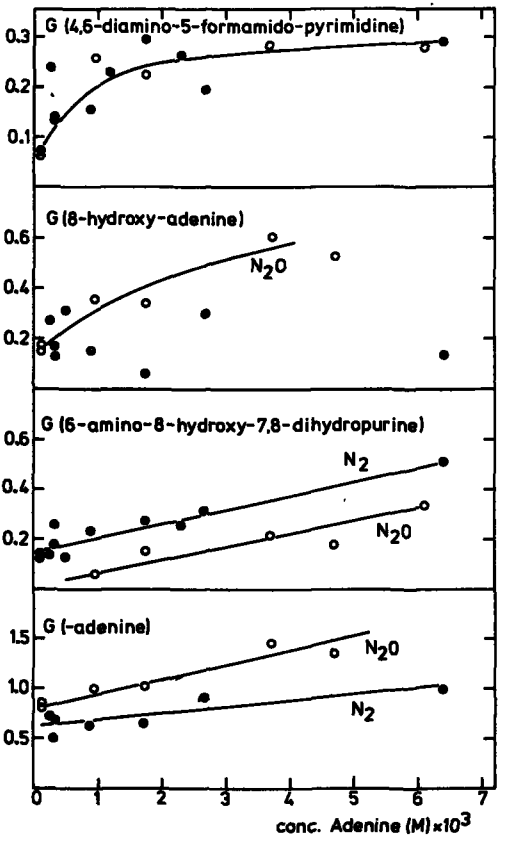


Figure 23. Yields (G-values) of some radiation products of adenine and yield of destruction of adenine against the concentration of adenine. N_2 (●) and N_2O (○).

In the absence of secondary solutes , all primary water radicals react with adenine. The adenine radicals react with each other , which ultimately results in the formation of stable products. Since radical-radical reactions do not depend upon the concentration of the parent molecule , whereas the formation of 4,6-diamino-5-formamido-pyrimidine , 6-amino-8-hydroxy-7,8-dihydropurine and 8-hydroxy-adenine and the destruction of adenine do show a dependence upon the adenine concentration, reactions of adenine radicals with adenine have to be assumed. If both types of reactions occur one expects an effect of dose rate on the yield of the radiation products and of the destruction of adenine. No significant differences have been observed however with dose rates varying between 0.3 and 3.4 krads per minute. This indicates , if the above assumption is true , that the radical-radical reaction is at least 100 times faster than the reaction with adenine.

It is seen in figure 23 that the points representing the yields of 4,6-diamino-5-formamido-pyrimidine under N_2 and N_2O scatter around the same line. At the adenine concentrations used , most hydrated electrons are converted into $OH^{\cdot}$ radicals in solutions saturated with N_2O. This means that hydrated electrons are not essential for the formation of 4,6-diamino-5-formamido-pyrimidine. In agreement with Hems (1960[b]) no 4,6-diamino-5-formamido-pyrimidine has been found among the radiation products after irradiation under oxygen. This may be due to the reaction of O_2 with hydrogen atoms (if these are required for the formation of this product) or with adenine radicals preventing one or more steps in the chain of reactions. At high concentrations of adenine and low concentrations of O_2 , some production of 4,6-diamino-5-formamido-pyrimidine was observed , perhaps as a consequence of competition between adenine and oxygen for reaction with adenine radicals.

8-Hydroxy-adenine was observed to be the main product when adenine was treated with Fenton's reagent (Nofre , Lefier and Cier 1961 ; own observations). In this system the $OH^{\cdot}$ radical is supposed to be the active agent,

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} .$$

In figure 23 it can be seen that under N_2O the amount of 8-hydroxy-adenine is higher than under N_2 , indicating that hydrated electrons are not necessary for the formation of this compound. In contrast with 4,6-diamino-5-formamido-pyrimidine , it is also formed in the presence of oxygen. Apparently H$^{\cdot}$ radicals are also not required for the production of 8-hydroxy-adenine. It seems reasonable to conclude from these data that part of the amount of 8-hydroxy-adenine is formed via a disproportionation reaction between hydroxyl adducts (Keck 1968) as shown in figure 24.

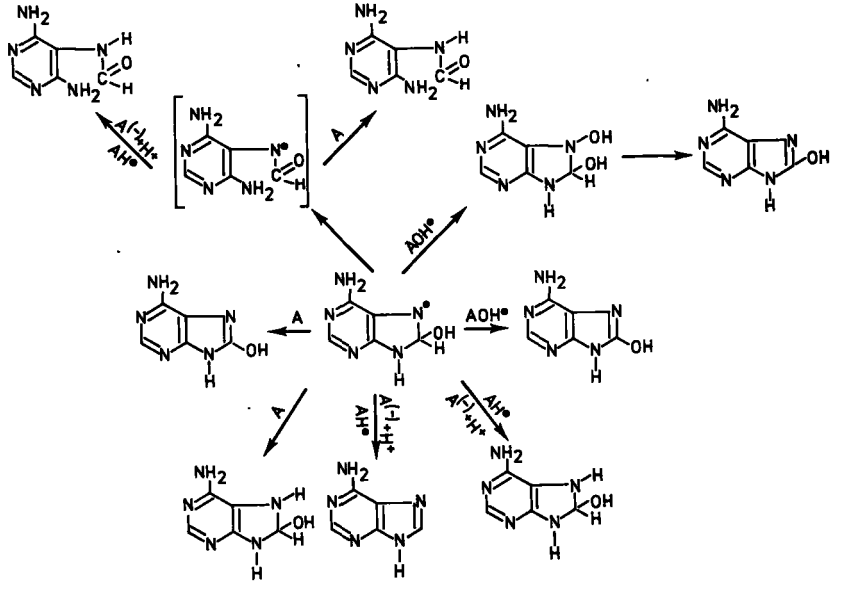


Figure 24. A proposal for the pathways in the formation of products. In this scheme the hydroxyl adduct of adenine takes a central position. The 8-position is of the highest electron density in the molecule (Pullman and Pullman 1961). The oxidizing OH$^{\cdot}$ radical will therefore probably add to this position. In the scheme the reactions occur by hydrogen or hydroxyl transfer. The electron adduct is assumed to be rapidly protonated and to react like the hydrogen adduct.

Because under N_2O , the yield of 8-hydroxy-adenine is strongly dependent upon the concentration of adenine another possibility is that adenine reacts with the hydroxyl adduct of adenine by hydrogen atom

transfer.

The yield of 6-amino-8-hydroxy-7,8-dihydropurine increases with adenine concentration (figure 23). Irradiation in the presence of N_2O gives a much lower yield than in the presence of N_2. This suggests that hydrated electrons are involved in its formation. Under conditions where no electron adduct of adenine can be formed (e.g. high concentrations of N_2O , H_2O_2 or O_2 and low concentrations of adenine) no 6-amino-8-hydroxy-7,8-dihydropurine has been found. These experiments and the structure of this compound suggest that it is formed via reactions shown in figure 24.

V.2.3 The yield of destruction of adenine

G-values for the destruction of adenine as reported in the literature are given in table VIII. These values are remarkably low in comparison with the yields of the primary radicals formed in water (table II). As already shown in figure 23 , the destruction of adenine is increased when irradiation takes place under N_2O (which converts e^-_{aq} into $OH^\cdot$) compared with irradiation under N_2, and is even more increased when high concentrations of H_2O_2 are present in the solution (table IX). H_2O_2 converts both e^-_{aq} and $H^\cdot$ into $OH^\cdot$ radicals :

$$H^\cdot + H_2O_2 \rightarrow OH^\cdot + H_2O$$

$$e^-_{aq} + H_2O_2 \rightarrow OH^\cdot + OH^- \ .$$

But also under these conditions the destruction of adenine is still lower than would be expected from the yields of the water radicals. These low yields suggest that combination reactions between the radical adducts of adenine leads to a certain amount of reconstituted adenine (Scholes , Ward and Weiss 1960).

The presence of oxygen clearly enhances the destruction of adenine (table IX). As oxygen reacts with the reducing radicals (e^-_{aq} and $H^\cdot$) producing O_2^- (see table I) , reaction of O_2^- radicals with adenine might perhaps explain this larger destruction of adenine.

Table VIII

G-values for the destruction of adenine in neutral solutions

adenine ($M \times 10^4$)	Dose	gas condition	G(-A)	references
20		O_2	1.1 initial	1
2		O_2	1.09 initial	1
0.2		O_2	0.65 initial	1
10	0.44 Mrads	N_2	0.35	2
10	0.3 Mrads	O_2	0.85	2
70	1 Mrad	N_2	0.97	3
70	2 Mrads	N_2	0.89	3
70	5 Mrads	N_2	0.60	3
80	5 Mrads	?	0.42	4
100	0.5 Mrads	?	1.5	5
100	5 Mrads	?	0.2	5
0.5		air	0.16 initial	6

References : 1, Scholes , Ward and Weiss(1960). 2 , Conlay (1963). 3 , Ponnamperuma , Lemmon and Calvin (1963). 4 , Rhaese (1968). 5 , Fahr (1968). 6 , Barzscz and Shugar (1972).

This possibility could be excluded by the irradiation of adenine in the presence of O_2 or of mixtures of O_2 and N_2O . Each O_2^- radical formed , yielded the equivalent number of H_2O_2 molecules. This follows from the data of table X which show that the experimental yields are close to the values that have been calculated from known yields and rate constants of e^-_{aq} and $H^\cdot$ with oxygen and adenine with the assumption that no reactions occur between adenine and O_2^- .
The G-values for the destruction of adenine shown in table IX have been determined by summation of the yields of the radiation products , because a direct determination from the decrease in radioactivity of the adenine peak at the low dose (50 krads) used , would have been very inaccurate.

Table IX

Destruction of adenine under various conditions *). Dose 50 krads

adenine ($M \times 10^4$)	O_2 ($M \times 10^4$)	N_2O ($M \times 10^3$)	H_2O_2 ($M \times 10^4$)	$G(-^{14}C\text{-}A)$	$G(-^{3}H\text{-}A)$
1.0	-	-	-	0.6 **	1.1 **
2.1	1.1	-	-	0.7	
9.3	1.2	-	-	1.0	
3.4	2.4	-	-	0.8	
2.1	3.2	-	-	0.7 **	1.1 **
2.1	9.9	-	-	0.8 **	1.1 **
1.2	12.5	-	-	1.2	
1.3	-	28	-	0.8	
1.0	1.3	21	-	1.1	
4.9	5.5	17	-	1.6 **	2.3 **
1.0	7.0	12	-	1.5	
4.9	9.5	9.2	-	2.0 **	3.1 **
11	-	-	3.5	0.8	
11	-	-	12	0.9	
3.5	-	-	53	0.9	
11	-	-	90	1.2	
27	-	-	390	1.8 **	2.1 **
10	12.5	-	450	2.4 **	2.4 **
12	12.5	-	60	2.1 **	2.2 **

*) The G-values have been determined by summation of the yields of the radiation products.

**) Mixtures of 8-^{14}C- and 2,8-^{3}H-adenine were irradiated.

Table X

Production of H_2O_2 in irradiated adenine solutions

adenine (M x 10^4)	N_2O (M x 10^2)	O_2 (M x 10^4)	G(H_2O_2)	
			experiment	calculated
1.3	-	12.5	2.31	2.30
1.3	1.1	8.3	1.20	1.17
1.3	2.1	1.5	1.03	1.00

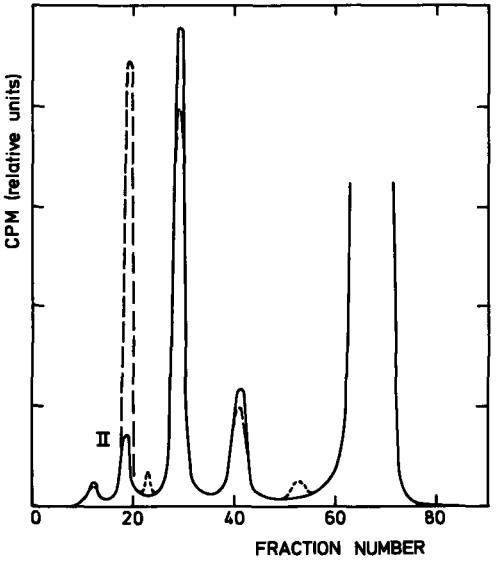


Figure 25. Elution diagram of an irradiated mixture of 8-^{3}H- and 8-^{14}C-adenine (3 x 10^{-4} M). Dose : 83 krads under N_2. ^{3}H : --- ; ^{14}C : ——. Elution technique as in figure 13.

The difference between the G-values for destruction of 2,8-^{3}H- and 8-^{14}C-adenine is largely due to the presence of one product which contains no carbon atom. Because the tritium used in these experiments

contained about 2 % radioactive impurities (for which corrections have been applied) , the results were checked by repeating some experiments with a mixture of 8-^{3}H- and 8-^{14}C-adenine , which contained less than 1 % radioactive impurities. A representative experiment is shown in figure 25. A large ^{3}H peak is observed and the same fractions also contain a small amount of ^{14}C activity , due to the presence of component II (see figure 13). In control experiments the ^{3}H peak was shown to be eluted with the same volume as ^{3}H-H_2O. Except the small amount of tritium label belonging to component II , the tritium is volatile and is easily removed by freeze-drying. From this evidence it is concluded that H_2O is a radiation product of adenine.

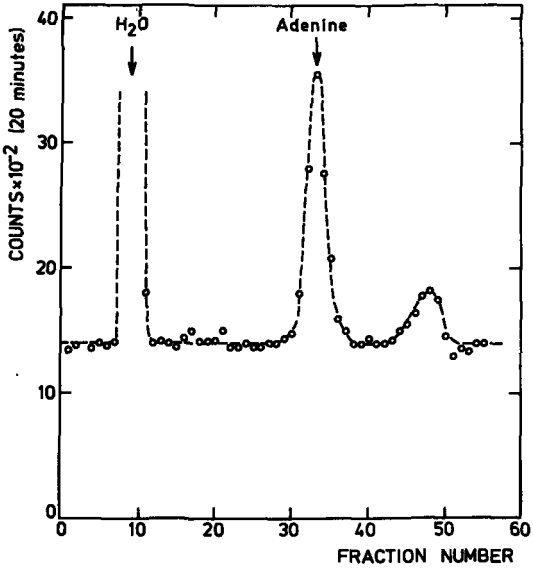


Figure 26. Elution diagram of adenine (6 x 10^{-4} M) irradiated in ^{3}H-H_2O (5 mCi/ml). Dose : 158 krads (N_2). Elution technique as in figure 13.

During irradiation of mixtures of ^{3}H- and ^{14}C-adenine it was observed that the specific activity of ^{14}C-adenine remained constant , whereas

the ^{3}H specific activity of adenine decreased at high doses. This again is an argument for the occurrence of reconstitution reactions in the radiolysis of adenine.

Reconstitution of adenine can also be shown by the production of tritiated adenine when adenine is irradiated in the presence of $^{3}H\text{-}H_2O$ of sufficiently high specific activity. Such an experiment is shown in figure 26. After irradiation the solution was freeze-dried to remove the greater part of the tritiated water. The peak in fraction 33 , which was not found in the unirradiated control , was shown to be adenine by paper chromatography. The peak around fraction 48 has not been identified.

V.3 Summary

Gel chromatography of adenine solutions , irradiated with γ-rays in the absence of oxygen shows the presence of six radiation products. Two of these do not show the characteristic ultraviolet absorption spectrum of pyrimidines and purines and are formed with a yield $G < 0.05$. Evidence obtained from UV absorption spectra , chromatographic data and mass spectra is presented which shows that the other products are 8-hydroxy-adenine , 4,6-diamino-5-formamido-pyrimidine , 6-amino-8-hydroxy-7,8-dihydropurine and possibly 4,6-diamino-5-hydroxy-pyrimidine. From experiments in which adenine was irradiated together with several radical scavengers it was concluded that 8-hydroxy-adenine is formed via reactions of $OH^{\cdot}$ radicals , while 6-amino-8-hydroxy-7,8-dihydropurine is only formed when also electron adducts of adenine are present. For the formation of 4,6-diamino-5-formamido-pyrimidine no hydrated electrons are required. Possible pathways for the formation of these compounds are presented.

Experiments with ^{3}H- and ^{14}C-adenine as tracers have clearly shown that in the anoxic radiolysis of adenine reconstitution reactions occur which give rise to the formation of water and repair of adenine radicals. It is also shown that reconstitution reactions occur when

oxygen is present.

The adenine radicals which are intermediates in the formation of the ultimate products were studied with the aid of pulse radiolysis. It was found that initially the hydroxyl adduct decayed unimolecularly and some arguments are given which suggest that this reaction is an early step in the formation of 4,6-diamino-5-formamido-pyrimidine. Further it was shown that the hydroxyl adduct reacts with the protecting agent cysteamine by hydrogen transfer from cysteamine to the adenine radical. The electron adduct of adenine reacts rapidly with electron-affinic compounds like paranitroacetophenone. In the latter case this reaction was shown to occur by electron transfer.

C H A P T E R VI Influence of the radiosensitizers paranitroacetophenone , triacetoneamine-N-oxyl and oxygen on the effects of ionizing radiation on DNA *)

In the present chapter some experiments are reported that were carried out to study the sensitization of DNA to the action of γ-rays by means of the sensitizers oxygen , TAN and PNAP. TAN is an example of a sensitizer of the class of stable nitroxide radicals and PNAP is an electron-affinic sensitizer.
The conditions of the experiments were chosen in such a way that the influence of these sensitizers under conditions of direct and indirect action could be studied separately.

VI.1 Influence of PNAP and TAN on the inactivation of DNA by the direct action of radiation

In a 'direct action model' (Adams 1968) an ionization in a target molecule produces a positive ion and an electron which after thermalization will migrate to a site with high electron-affinity on the molecule. In the presence of substances of high electron-affinity in the vicinity of the target molecule , electron transfer to these molecules could occur in competition with charge recombination , a process which contributes to self-healing. It has been shown that in solution electron transfer from electron adducts of nucleotides to sensitizers occurs readily (Adams , Greenstock , Van Hemmen and Willson 1972). Adams , Asquith , Dewey , Foster , Michael and Willson (1971) have shown that the electron-affinity of PNAP approaches that of oxygen , the best radiosensitizer in vivo , known so far. Therefore the inves-

*) The work described in this chapter has been published (Van Hemmen , Meuling and Bleichrodt 1973).

tigation of the sensitizing action of PNAP on a target molecule like DNA is of interest.

If the biological activity of freeze-dried single-stranded DNA of the bacteriophage ΦX174 is determined as a function of dose , one obtains curves which in stead of sensitization show a small protection by the presence of PNAP (figure 27) or TAN.

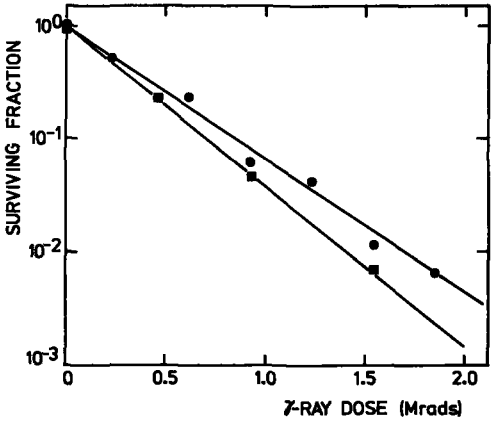


Figure 27. Inactivation of ΦX174 DNA , freeze-dried in the absence (■) and in the presence (●) of PNAP (9 % w/w). Samples containing calf thymus DNA as a carrier were irradiated with γ-rays in vacuo. The biological activity of the DNA was assayed on spheroplasts of E.coli K12S uvr$^+$.

The amount of PNAP present in the DNA sample (9 % w/w) is expected to be high enough to give the maximum increase in radical yield (quoted by Adams 1972[a]). In single-strand DNA of ΦX174 , every break is lethal and possibly every hit. Therefore one might think that no sensitization can occur in such a DNA. For this reason double-stranded DNA of the bacteriophage PM2 was used. Van der Schans et al. (1973) have shown that in this DNA not every radiation-induced hit is lethal. As is shown in figure 28 again no sensitization was detected.

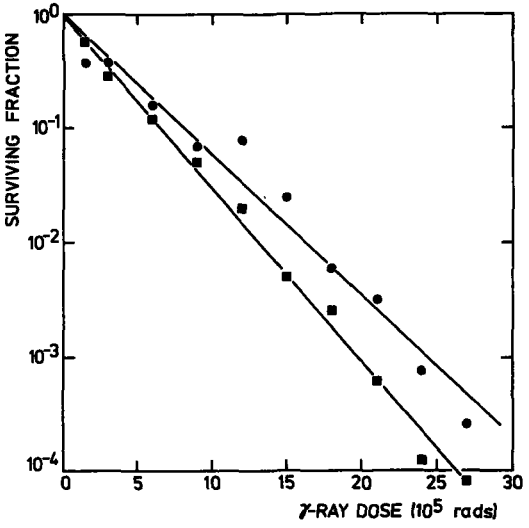


Figure 28. Inactivation of PM2 DNA , freeze-dried in the absence (■) and in the presence (●) of PNAP (9 % w/w). Samples containing calf thymus DNA as a carrier , were irradiated with γ-rays in vacuo. The biological activity of the DNA was assayed on spheroplasts of Pseudomonas BAL31/PM2.

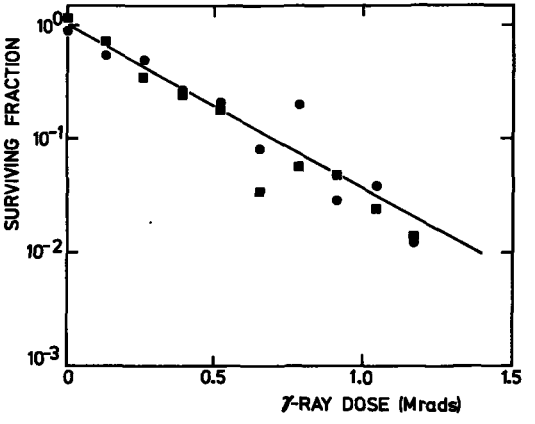


Figure 29. Inactivation of λ DNA , freeze-dried in the absence (■) and in the presence (●) of PNAP (9 % w/w). Samples containing calf thymus DNA as a carrier , were irradiated with γ-rays in vacuo. The biological activity of the DNA was assayed on spheroplasts of E.coli B206/λ .

Also in the case of the relatively large DNA of the bacteriophage λ no sensitizing effect of PNAP on the survival of the DNA has been detected (figure 29). Again in this DNA not every hit is lethal (Ginoza 1967 ; Bleichrodt , Verheij and De Jong 1972).

Since in general almost every additive is protecting in the γ-radiolysis of DNA in solution , small amounts of water still present in the freeze-dried samples of DNA can mask the sensitizing effect of PNAP and TAN. Hotz and Müller (1968) have shown that the radiosensitivity of ΦX174 DNA is not dependent upon small amounts of water in the sample. According to Ginoza (1963) the sensitivity of this DNA is the same in the solid state as in frozen solution , if nutrient broth is present. Although the reason for the small protection (figure 27 and 28) is unknown, it is highly improbable therefore that the observed protection is due to radical scavenging by the sensitizers. From the absence of any sensitization by PNAP and TAN of the various biologically active DNA's under conditions of almost complete direct action and under conditions where an increase in radical yield due the presence of sensitizers is easily detected by electron spin resonance techniques (see III.1.4) , it is concluded that the model of Adams does not hold in the case of dried DNA.

VI.2 Influence of PNAP , TAN and O_2 on the inactivation of DNA by the indirect action of radiation

Figure 30 shows that the presence of PNAP protects against the inactivation of a solution of ΦX174 DNA. This is not surprising because a large part of the water radicals is scavenged by PNAP and therefore possible sensitizing properties will be overshadowed by the protection.
This protective effect can be eliminated in rapid-mixing experiments , in which buffered DNA solutions are irradiated and then mixed thoroughly in about 1.5 msec with a sensitizer dissolved in the same buffer. At this stage the sensitizer can only react with DNA radicals.

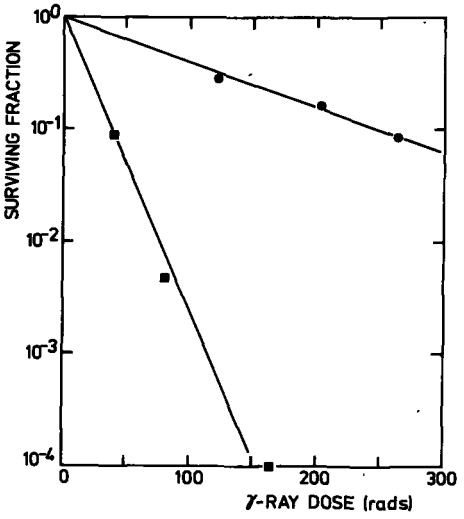


Figure 30. Inactivation of ΦX174 DNA (3.5 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 , under N_2 in the absence (■) and in the presence (●) of 10^{-4} M PNAP , with γ-rays. The DNA was assayed on spheroplasts of E.coli K12S uvr$^+$.

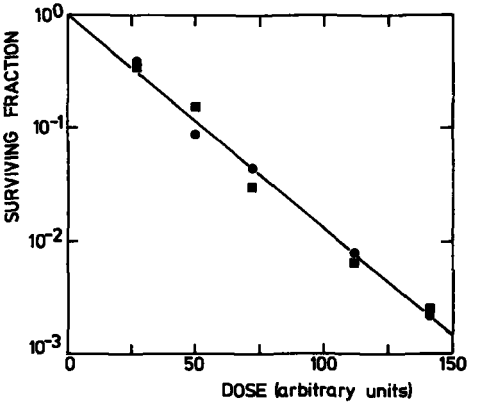


Figure 31. Inactivation of ΦX174 DNA (0.1 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 , containing denatured calf thymus DNA (1 μg/ml) as a carrier in the rapid-mixing device. Two solutions saturated with N_2O were irradiated simultaneously. One of these was mixed with 3×10^{-3} M PNAP in phosphate buffer , approximately 1.5 msec after irradiation (●). The mixed and the unmixed solutions were assayed on spheroplasts of E.coli K12S uvr$^+$.

Figure 31 shows such an experiment , in which an oxygen-free solution of PNAP was added to an irradiated solution of ΦX174 DNA saturated with N_2O. No effect of PNAP was seen. In control experiments in which the DNA was mixed with a pure buffer solution , in stead of a solution of PNAP , the survival of the DNA was exactly the same. Similar results were obtained for the sensitizers oxygen and TAN. Since in single-stranded DNA every hit may be lethal (see VI.1) , so that sensitizers cannot increase lethality , the experiments have been extended to double-stranded ΦX174 RF-DNA and PM2 DNA. Again no effect of the sensitizers was observed.

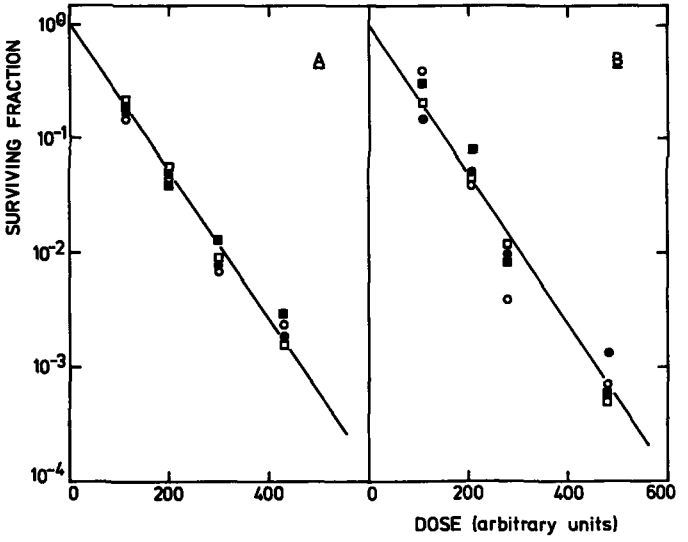


Figure 32. Inactivation of ΦX174 RF-DNA (0.3 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 , containing calf thymus DNA (1 μg/ml) as a carrier in the rapid-mixing device. Two solutions saturated with N_2O were irradiated simultaneously in A and in B.
One of the solutions (circles) was mixed with phosphate buffer (A) or with 10^{-2} M TAN in buffer (B). The mixed and the unmixed (squares) solutions were assayed on spheroplasts of E.coli K12S uvr$^+$(closed symbols) or of E.coli K12S uvrA516 (open symbols).

In pulse radiolysis experiments with DNA solutions , relatively long-lived DNA radicals have been observed (Myers , Hollis and Theard 1968 ; Scholes , Willson and Ebert 1969 ; Roberts and Fielden 1971).
Brustad et al. (1971 , 1973) have shown in rapid-mixing experiments that TAN is covalently bound to irradiated calf thymus DNA even minutes after irradiation. If binding of TAN to DNA has occurred in our experiments , this reaction apparently has no influence on the survival of the biologically active DNA.
In the assay that has been used to determine the biological activity of the DNA it is possible that some radiation damage is repaired by the bacterial spheroplast. To investigate whether the negative results in the experiments with the sensitizers could be due to repair of the lesions resulting from binding of the sensitizer to DNA , ΦX174 RF-DNA was irradiated in the rapid-mixing apparatus , mixed with TAN (figure32) or another sensitizer and tested on spheroplasts of bacteria deficient and proficient in excision repair. Again there was no effect , neither in the control experiments with buffer nor with the sensitizers.

When a solution of RF-DNA and TAN is irradiated with γ-rays under steady state conditions , radiation-induced reactions between TAN and DNA do occur since under these conditions some of the lesions are repaired by the excision repair system (figure 33). In the absence of TAN no difference is observed between the survival of the irradiated DNA on the wild type strain and the repair deficient strain.
Similar results have been obtained by De Jong , Loman and Blok (1972[b]) for irradiation of RF-DNA in the presence of phenylalanine.
In contrast to TAN , PNAP has only a very slight effect (figure 34) , whereas oxygen has no effect at all.
From the above experiments it is concluded that in solution radiation-induced reactions between sensitizers and DNA can occur. At least some of the lesions can be repaired by the excision repair system. Furthermore reactions of long-lived DNA radicals with PNAP , TAN or oxygen do not seem to be biologically significant.

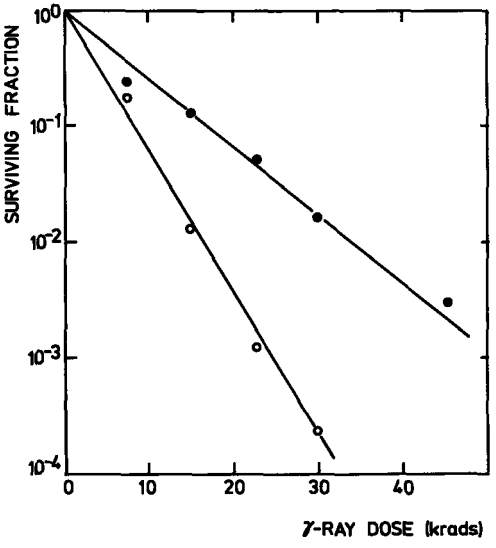


Figure 33. Inactivation of ΦX174 RF-DNA (4 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 , in the presence of 3.5×10^{-3} M TAN with γ-rays under N_2O. The DNA samples were assayed on spheroplasts of E.coli K12S uvr$^+$ (●) and K12S uvrA516 (○).

VI.3 Discussion

Oxygen , PNAP and TAN sensitize cells to ionizing radiation. With regard to some subcellular systems however they behave differently (Van Hemmen , Meuling and Bleichrodt 1973).

TAN reacts with DNA if it is present during irradiation. When it is mixed with DNA 1.5 msec after irradiation of the DNA , however , no sensitization occurs , suggesting that the binding of TAN with long-lived DNA radicals that has been observed by Brustad et al. (1971 , 1973) has little biological significance. Also rapid-mixing of irradiated DNA with a solution containing PNAP or oxygen does not affect the survival of the DNA.

Neither TAN nor PNAP exert a sensitizing effect when present during irradiation of biologically active DNA in the dry state showing that the 'direct action model' of Adams does not hold for DNA under our experimental conditions. This may indicate that this model also does not apply to DNA in a living cell and that cell components different from DNA are involved in the expression of sensitizers *in vivo* (cf Alper 1971).

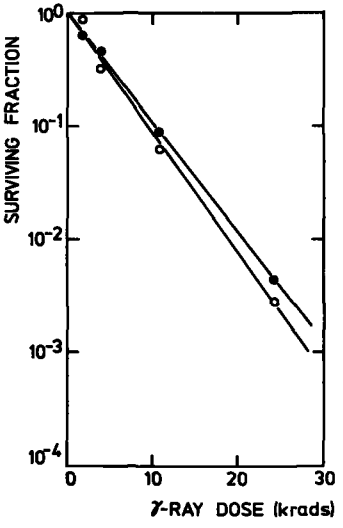


Figure 34. Inactivation of ΦX174 RF-DNA (3.5 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 , in the presence of 2.5 x 10^{-3} M PNAP with γ-rays under N_2O. DNA samples were assayed on spheroplasts of *E.coli* K12S *uvr*$^+$ (●) and K12S *uvrA516* (○).

From the present data it cannot be excluded however that damage in the DNA in a cell is modified by subsequent reactions in which the sensitizers play a role. In fact there is some evidence that DNA is influenced by PNAP (Dugle and Chapman 1971) in mammalian cells in tissue culture , when these cells are irradiated. Furthermore it has recently been shown (Van Hemmen , Meuling , Van der Schans and Bleichrodt 1973) that PNAP , TAN and oxygen sensitize biologically

active DNA dissolved in an extract of bacterial or mammalian cells. Under these conditions it could be shown that radicals from cellular components are involved in the sensitization of the DNA.

C H A P T E R VII The protective action of cysteamine in aqueous solutions of DNA *)

The presence of sulphydryl compounds in mammalian cells and bacteria results in a decrease of the radiation-induced cell killing. Many experiments have been carried out to measure the protective action on various cell components (e.g. DNA) in vitro. Unfortunately many of these experiments do not give information on the specific action of sulphydryl compounds in vivo. A large protection in DNA solutions is not surprising because SH compounds react rapidly with oxidizing and reducing radicals. The same is true for a number of other organic compounds that are present in the cell. Nevertheless a comparatively small concentration of SH compounds produces an appreciable reduction of the radiosensitivity of the cells.
The hydrogen donation model (see III.2) has received most attention in studies regarding radioprotection. Blok (1967) was the first to study the radioprotection of biologically active DNA by cysteamine. His experiments showed that the protection is dependent upon pH which is in fact required by the hydrogen donation model.
In this chapter , rapid-mixing experiments will be described which show that biologically active DNA , irradiated in vitro , is liable to radical repair by cysteamine.

VII.1 Experiments with PM2 DNA

When biologically active DNA is irradiated in solution , the presence of cysteamine exerts considerable protection (Blok 1967 ; De Jong 1972). This protection is largely due to the scavenging of water ra-

*) Some of the results described in this chapter have been submitted for publication (Van Hemmen and Meuling 1973 ; Van Hemmen , Meuling , De Jong and Luthjens).

dicals by cysteamine. The protection by radical scavenging can be eliminated in rapid-mixing experiments , where buffered DNA solutions are irradiated and immediately thereafter mixed with a solution of cysteamine.

The effects of γ-radiation on PM2 DNA in solution have been studied by Van der Schans et al. (1973) and they noted that the inactivation of the DNA was due to single-strand breaks , double-strand breaks and nucleotide damage (see II.2.4). When PM2 DNA is irradiated in the absence of oxygen in the rapid-mixing device and if within 1.5 msec after irradiation the DNA is mixed with buffer or a buffered solution of cysteamine , results are obtained as shown in figure 35. Apparently cysteamine is capable to react with DNA radicals , as follows from the increase in biological activity (figure

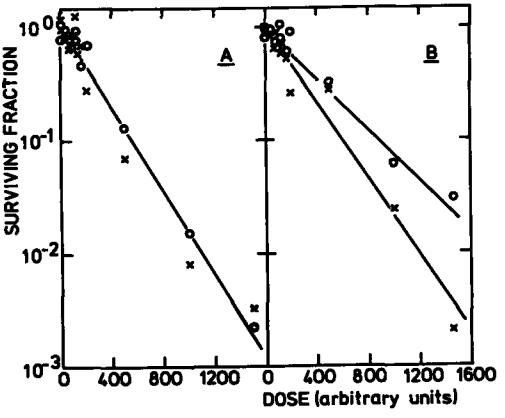


Figure 35. Inactivation of PM2 DNA (4 µg/ml) in 10^{-2} M phosphate buffer pH 7.1 in the rapid-mixing device. Two solutions saturated with N_2O were irradiated simultaneously in A and B. One of the solutions (o) was mixed with buffer (A) or with 10^{-2} M cysteamine in buffer (B) , 1.5 msec after irradiation. The other solutions were left unmixed (x). The DNA was assayed on speroplasts of Pseudomonas BAL31/PM2.

35B). The question arises which type of damage is affected by cysteamine. In figure 36 it is shown that the average number of single-strand breaks per DNA molecule decreases in comparison with control experiments in which the DNA was left unmixed or had been mixed with plain buffer.

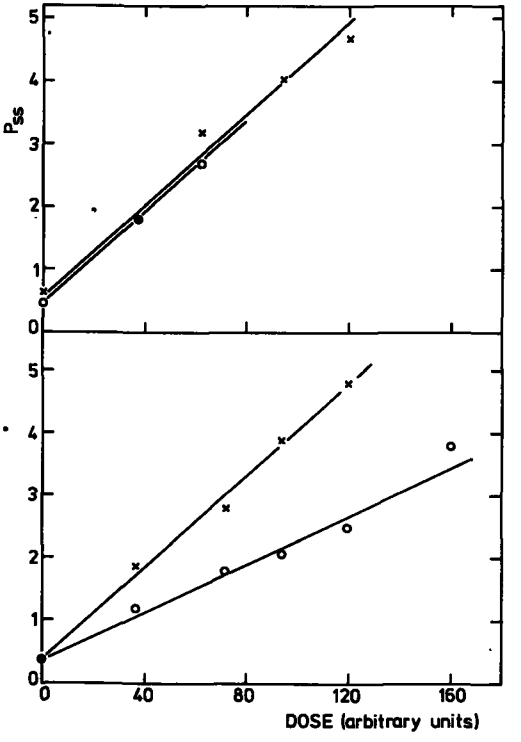


Figure 36. The average number of single-strand breaks per molecule (p_{ss}) in PM2 DNA as a function of dose. Data derived from the same experiment as in figure 35.

The effect of cysteamine on double-strand breaks has also been examined. It has been shown by Van der Schans et al. (1973) , that irradiation of DNA in solution leads to double-strand breaks resulting from two processes. Some breaks arise from a one hit process

and these breaks are formed linearly with dose. Double-strand breaks also result from two separate single-strand breaks lying close together on opposite strands. The number of such breaks increase proportional to the square of the dose. In figure 37 the effect of cysteamine on the number of double-strand breaks formed by irradiation in a rapid-mixing experiment is shown.

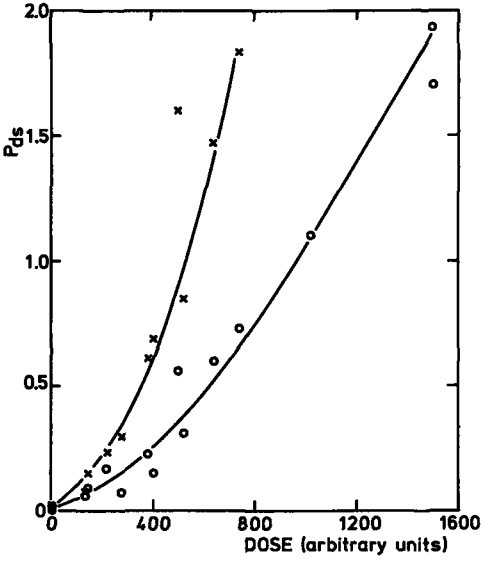


Figure 37. Production of double-strand breaks in PM2 DNA (5 μg/ml) in phosphate buffer pH 7.1 in the rapid-mixing device. Two solutions saturated with N_2O were irradiated simultaneously. One of them was mixed with 5×10^{-3} M cysteamine in buffer, 1.5 msec after irradiation (o). The other solution was left unmixed (x).

In the rapid-mixing apparatus, the DNA solution flows with an average velocity of about 10 m/sec through capillary tubes and this means that significant shear forces occur in the liquid. Therefore one might suspect that at least part of the double-strand breaks are due to shear.

Evidence for the occurrence of shear-induced double-strand breaks in irradiated DNA is shown in table XI. The number of single-strand breaks appeared not to be affected by the shear forces , whereas the number of double-strand breaks was increased significantly. The presence of cysteamine in the solution during the shear experiment did not influence the number of shear-induced breaks. No breaks were introduced in unirradiated DNA by the shear forces.

Table XI

Effects of shear in the rapid-mixing apparatus upon the number of single-strand breaks (P_{ss}) and the number of double-strand breaks (P_{ds}) in irradiated PM2 DNA

Number of breaks in irradiated DNA			Number of breaks in irradiated DNA after subjecting it to shear			
			DNA solution mixed with buffer		DNA solution mixed with 10^{-2}M cysteamine	
Dose (rads)	P_{ss}	P_{ds}	P_{ss}	P_{ds}	P_{ss}	P_{ds}
0	0.3	-	0.3	-	0.3	-
30	1.3	-	1.4	-	1.5	-
60	2.6	-	2.5	-	2.6	-
120	4.7	-	4.9	-	4.3	-
400	-	0.26	-	0.77	-	0.69
800	-	0.49	-	1.21	-	1.28

Irradiation of a solution of DNA (5 µg/ml) in 10^{-2} M phosphate buffer pH 7.1 , with γ-rays under N_2O.

Since it is impossible to distinguish,in the present experiments,between shear-induced and radiation-induced double-strand breaks , the effect of cysteamine on radiation-induced double-strand breaks

cannot be evaluated. If the biological activity of the DNA is corrected mathematically for all double-strand breaks (shear-induced and radiation-induced) , which have been shown to be lethal with an efficiency of 100 per cent (Van der Schans et al. 1973) , the survival is no longer dependent on the addition of cysteamine immediately after irradiation (figure 38).

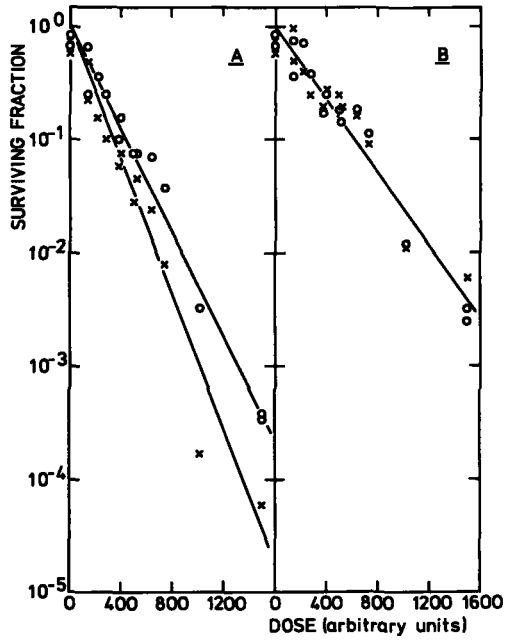


Figure 38. Inactivation of PM2 DNA (5 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 in the rapid-mixing device. Two solutions saturated with N_2O were irradiated simultaneously. One of these was mixed with 5 x 10^{-3} M cysteamine , 1.5 msec after irradiation. The mixed (o) and the unmixed (x) samples were assayed on spheroplasts of Pseudomonas BAL31/PM2.
B : The same experiment as in A , but after correction for all double-strand breaks.

The inactivation of the DNA plotted in this way is only determined by single-strand breaks and nucleotide damage. According to table IV , single-strand breaks introduced by γ-rays in PM2 DNA contribute very little to lethality. Assuming that the contribution of single-strand breaks to lethality is also negligible under the conditions used in the present experiments , it can be concluded that lethal nucleotide damage is not affected significantly by cysteamine , when added 1.5 msec after irradiation to the DNA.

VII.2 Experiments with ΦX174 DNA

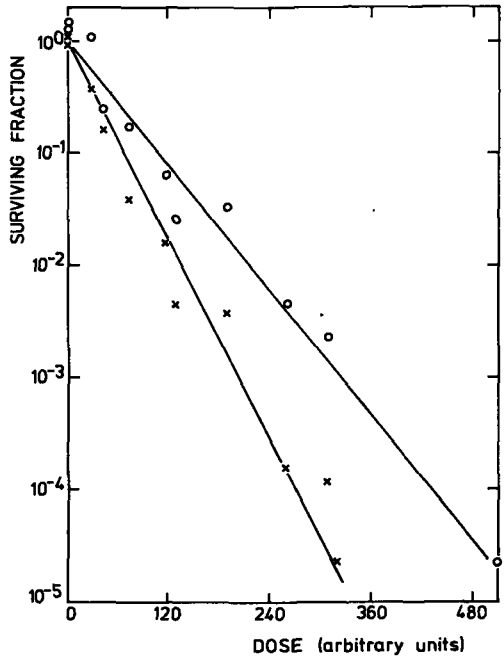


Figure 39. Inactivation of ΦX174 DNA (3 μg/ml) in 10^{-2} M phosphate buffer pH 7.1 in the rapid-mixing device. Two solutions were irradiated simultaneously under N_2O. One of these (o) was mixed with 10^{-2} M cysteamine in buffer. The other solution (x) was left unmixed. The DNA was assayed on spheroplasts of *E.coli* K12S *uvr*$^+$.

The problems concerning the occurrence of double-strand breaks by shear forces in the rapid-mixing device are circumvented by using DNA of the bacteriophage ΦX174. This single-stranded DNA is inactivated by ionizing radiation through the introduction of (single-strand) breaks and nucleotide damage (Blok 1967). The effects of mixing the DNA immediately after irradiation with cysteamine on the biological activity of the DNA are shown in figure 39. Just as PM2 DNA the single-strand DNA is protected by the addition of cysteamine when the DNA is irradiated in the absence of oxygen.
Figure 40 shows that the number of breaks is decreased by adding cysteamine immediately after irradiation of an anoxic DNA solution.

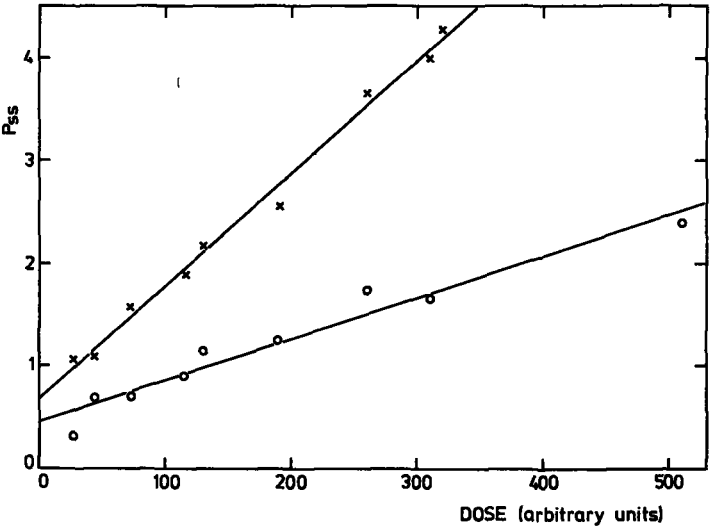


Figure 40. The average number of single-strand breaks per molecule (p_{ss}) in ΦX174 DNA as a function of dose. Data derived from the same experiment as in figure 39.

From data such as shown in figure 39 and 40 the dose needed to produce an average of one lethal hit per molecule (D_{37}) and the dose corres-

ponding to an average of one single-strand break per molecule (D_{ss}) can be calculated. From the relation

$$1/D_{37} = 1/D_{ss} + 1/D_{nd}$$

the dose needed for the production of an average of one lethal nucleotide damage per molecule (D_{nd}) can be calculated. It is shown in table XII that the amount of lethal nucleotide damage is not significantly affected by the addition of cysteamine. This observation is consistent with the results obtained with PM2 DNA.

Table XII

Effects of cysteamine on irradiated ΦX174 DNA in rapid-mixing experiments

	Unmixed control	Mixed with 10^{-2} M cysteamine
D_{37}	34 ± 3	49 ± 3
D_{ss}	92 ± 3	250 ± 20
D_{nd}	54 ± 7	62 ± 6

Irradiation of a solution of ΦX174 DNA (3 μg/ml) in phosphate buffer pH 7.1 in the rapid-mixing apparatus. The values are in arbitrary units ± standard deviation.

VII.3 Concluding remarks

The correlation between survival and the number of radiation-induced breaks in DNA , the probable target for cell killing has been examined in phages , bacteria and in mammalian cells. It is not known , however , whether only breaks are involved in killing of the cell. Most organisms have the capacity to repair the majority of the single-strand breaks induced by radiation , and at least some radioresistent organisms seem to be capable to repair double-strand breaks. Nothing is known about the contribution of nucleotide damage to

lethality in cells. Only inactivation of biologically active DNA , isolated from bacteriophages , has been shown to be due mainly to nucleotide damage.

In this chapter it is shown that even a few msec after irradiation of single-stranded and double-stranded bacteriophage DNA in the absence of oxygen , cysteamine is capable to prevent the formation of breaks at least partly , but it does not have any effect on the formation of lethal nucleotide damage. However it should be born in mind that the radicals in DNA which are intermediates in the formation of nucleotide damage may be quite different from the radicals (possibly located at the sugar moieties) which will form breaks. The lifetime of the former may be too short to modify them after 1.5 msec by addition of cysteamine. Even if these radicals were sufficiently long-lived , it would not be warrented to apply the conclusion that cysteamine does not protect against nucleotide damage in cellular systems , because in cells the DNA is surrounded by many different organic molecules , the presence of which will influence undoubtedly the nature of the radicals generated in DNA by radiation.

References

Adams,G.E., 1968 , Radiation Chemistry of Aqueous Systems , edited by G.Stein , The Weizmann Science Press of Israel , p. 241

Adams,G.E., 1972[a] , Advances in Radiation Chemistry , Vol. III , edited by M.Burton and J.L.Magee , Wiley-Interscience , p. 125

Adams,G.E., 1972[b] , Brit.Medical Bull., 29 , 48

Adams,G.E.,Armstrong,R.C.,Charlesby,A.,Michael,B.D. and Willson,R.L., 1969 , Trans.Far.Soc., 65 , 732

Adams,G.E.,Asquith,J.C.,Dewey,D.L.,Foster,J.L.,Michael,B.D. and Willson,R.L., 1971 , Int.J.Radiat.Biol., 19 , 575

Adams,G.E.,Boag,J.W.,Currant,J. and Michael,B.D., 1965 , Pulse Radiolysis , edited by M.Ebert,J.P.Keene,A.J.Swallow and J.H.Baxendale , Academic Press , p. 117

Adams,G.E.,Boag,J.W. and Michael,B.D., 1965 , Trans.Far.Soc., 61 , 492

Adams,G.E. and Cooke,M.S., 1969 , Int.J.Radiat.Biol., 15 , 457

Adams,G.E.,Cooke,M.S. and Michael,B.D., 1968 , Nature , 219 , 1368

Adams,G.E. and Dewey,D.L., 1963 , Biochem.Biophys.Res.Comm., 12 , 473

Adams,G.E.,Greenstock,C.L.,Hemmen,J.J.van and Willson,R.L., 1972 , Radiat.Res., 49 , 85

Adams,G.E.,McNaughton,G.S. and Michael,B.D., 1968 , Trans.Far.Soc., 64 , 902

Alexander,P.,Dean,C.J.,Lehman,A.R.,Ormerod,M.G.,Feldschreiber,P. and Serianni,R.W., 1970 , Radiation Protection and Sensitization , edited by H.L.Moroson and M.Quintiliani , Taylor and Francis , p. 15

Allen,A.O., 1954 , Radiat.Res., 1 , 85

Allen,A.O., 1961 , The Radiation Chemistry of Water and Aqueous Solutions , D.van Nostrand Company

Allen,A.O.,Hochnadel,C.J.,Ghormley,J.A. and Davies,T.W., 1952 , J.Phys.Chem., 56 , 575

Alper,T., 1971 , Biophysical Aspects of Radiation Quality , International Atomic Energy Agency , p. 171

Anbar,M. and Neta,P., 1967 , Int.J.Appl.Radiat.Isotopes , 18 , 493

Bacq,Z.M., 1965 , Chemical Protection against Ionizing Radiation , Thomas

Barszcz,D. and Shugar,D., 1972 , Acta Biochem.Pol., 19 , 25

Benzinger,R.,Kleber,I. and Huskey,R., 1971 , J.Virology , 7 , 646

Bleichrodt,J.F.,Verheij,W.S.D. and Jong,J.de, 1972 , Int.J.Radiat. Biol., 22 , 325

Blok,Joh., 1967 , Proceedings of the Third International Congress of Radiation Research , edited by G.Silini , North Holland Publishing Company , p. 423

Blok,Joh. and Loman,H., 1973 , Current Topics in Radiation Research Quart., in press

Blok,Joh.,Luthjens,L.H. and Roos,A.L.M., 1967 , Radiat.Res., 30 , 468

Blok,Joh. and Verheij,W.S.D., 1968 , Radiat.Res., 34 , 689

Bridges,B.A., 1961 , J.Gen.Microbiol., 26 , 467

Bridges,B.A., 1969 , Advances in Radiation Biology , Vol. III , edited by L.G.Augenstein , R.Mason and M.Zelle , Academic Press , p. 123

Brière,R.,Lemaire,H. and Rassat,A., 1965 , Bull.Soc.Chim.France , 32 , 3273

Brustad,T.,Bugge,H.,Jones,W.B.G. and Wold,E., 1972 , Int.J. Radiat.Biol., 22 , 115

Brustad,T.,Jones,W.B.G. and Nakken,K.F., 1971 , Int. J.Radiat. Phys.Chem., 3 , 55

Brustad,T.,Jones,W.B.G. and Wold,E., 1973 , Int.J.Radiat.Biol., 24 , 33

Cadet,J. and Teoule,R., 1971[a] , Biochim.Biophys.Acta , 238 , 8

Cadet,J. and Teoule,R., 1971[b] , Int.J.Appl.Radiat.Isotopes, 22 , 273

Cavalieri,L.F and Bendich,A., 1950 , J.Amer.Chem.Soc., 72 , 2587

Cavalieri,L.F.,Tinker,J.F. and Bendich,A., 1949 , J.Amer.Chem.Soc., 71 , 533

Conlay,J.J., 1963 , Nature , 197 , 555

Czapski,G., 1971 , Annual Review of Physical Chemistry , Vol. 22 , edited by H.Eyring , C.J.Christensen and H.S.Johnston , Ann.Reviews Inc., p.171

Daniels,M. and Schweibert,M.C., 1967 , Biochim.Biophys.Acta , 134 , 481

Dewey,D.L., 1965 , Progr.Biochem.Pharm., 1 , 59

Dewey,D.L. and Michael,B.D., 1965 , Biochem.Biophys.Res.Comm., 21 , 392

Draganić,I.G. and Draganić,Z.D., 1971 , The Radiation Chemistry of Water , Academic Press

Dugle,D.L. and Chapman,J.D., 1971 , Radiat.Res., 47 , 316

Emmerson,P.T., 1968 , Radiat.Res., 36 , 410

Emmerson,P.T., 1972 , Advances in Radiation Chemistry , Vol. III , edited by M.Burton and J.L.Magee , Wiley-Interscience , p. 209

Fahr,E., 1968 , Biophysik , 5 , 2

Fregene,O.A., 1967 , Radiat.Res., 31 , 256

Fricke,H. and Thomas,J.K., 1964 , Radiat. Res.Suppl., 4 , 35

Ginoza,W., 1963 , Nature , 199 , 453

Ginoza,W., 1967 , Annual Review of Microbiology , Vol. 21 , edited by C.E.Clifton, S.Raffel and M.P.Star , Ann.Reviews Inc., p. 325

Goedbloed,J.P. and Hemmen,J.J.van, 1968 , Int.J.Radiat.Biol., 14 , 351

Gordon,S.,Hart,E.J.,Matheson,M.S.,Rabani,J. and Thomas,J.K., 1963 , Disc.Far.Soc., 36 , 193

Gray,L.H., 1957 , Brit.J.Radiology , 30 , 403

Gregoli,J.,Taverna,C. and Bertinchamps,A., 1970 , Int.J.Radiat.Biol., 18 , 577

Hart,E.J.,Gordon,S. and Fielden,E.M., 1966 , J.Phys.Chem., 70 , 150

Hayon,E., 1969 , J.Chem.Phys., 51 , 4881

Hemmen,J.J.van, 1971 , Nature New Biol., 231 , 79

Hemmen,J.J.van and Bleichrodt,J.F., 1971 , Radiat.Res., 46 , 444

Hemmen,J.J.van and Meuling,W.J.A., 1973 , Current Topics in Radiation Research Quart., in press

Hemmen,J.J.van,Meuling,W.J.A.,Schans,G.P.van der and Bleichrodt,J.F., 1973 , submitted for publication

Hemmen,J.J.van,Meuling,W.J.A. and Bleichrodt,J.F., 1973 , Int.J. Radiat.Biol., in press

Hemmen,J.J.van,Meuling,W.J.A.,Jong,J.de and Luthjens,L.H., 1973 submitted for publication

Hems,G., 1958 , Nature , 181 , 1721

Hems,G., 1960[a], Nature , 186 , 710

Hems,G., 1960[b], Radiat.Res., 13 , 777

Henglein,A.,Schnabel,W. and Wendenburg,J., 1969 , Einführung in die Strahlenchemie , Verlag Chemie

Hersch,P.A., 1960 , Analyt.Chem., 32 , 1030

Hoff,A.J. and Koningsberger,D.C., 1970 , Int.J.Radiat.Biol., 17 , 459

Holian,J. and Garrison,W.M., 1967[a] , Chem.Comm., 14 , 676

Holian,J. and Garrison,W.M., 1967[b] , J.Phys.Chem., 71 , 462

Hotz,G. and Müller,A., 1968 , Proc.Natl.Acad.Sc.USA , 60 , 251

Howard-Flanders,P., 1960 , Nature , 186 , 485

Hutchinson,F., 1966 , Cancer Res., 26 , 2045

Jansz,H.S.,Pouwels,P.H. and Schiphorst,J., 1966 , Biochim.Biophys. Acta , 123 , 626

Johansen,I.,Ward,J.F.,Siegel,K. and Sletten,A., 1968 , Biochem. Biophys.Res.Comm., 33 , 949

Jong,J.de, 1972 , Thesis , Amsterdam

Jong,J.de,Loman,H. and Blok,Joh., 1972[a] , Int.J.Radiat.Biol., 22 , 11

Jong,J.de,Loman,H. and Blok,Joh., 1972[b] , Int.J.Radiat.Biol., 22 , 579

Kaiser,A.D. and Hogness,D.S., 1960 , J.Molec.Biol., 2 , 392

Keene,J.P., 1965 , Pulse Radiolysis , edited by M.Ebert , J.P.Keene , A.J.Swallow and J.H.Baxendale , Academic Press , p. 1

Keck,K., 1968 , Z.Naturforsch., 23B , 1034

Khattak,H.N. and Green,J.H., 1966[a] , Int.J.Radiat.Biol., 11 , 131

Khattak,H.N. and Green,J.H., 1966[b] , Int.J.Radiat.Biol., 11 , 577
Lampe,F.W.,Field,F.H. and Franklin,J.L., 1957 , J.Amer.Chem.Soc., 79 , 6132
Le Roux,Y.,Boulanger,J.P. and Arnaud,R., 1971 , C.R.Acad.Sc. Ser.C, Paris , 272 , 1757
Loman,H. and Blok,Joh., 1968 , Radiat.Res., 36 , 1
Loman,H. and Ebert,M., 1970 , Int.J.Radiat.Biol., 18 , 369
Luthjens,L.H., 1973 , Rev.Sci.Instrum., in press
Luthjens,L.H. and Schmidt,A.M., 1973 , Rev.Sci.Instrum., 44 , 567
Matheson,M.S. and Dorfman,L.M., 1969 , Pulse Radiolysis , M.I.T. Press
Matheson,M.S. and Rabani,J., 1965 , J.Phys.Chem., 69 , 1324
Michael,B.D., 1969 , Thesis , London
Morley,T.G., 1971[a] , Thesis , Cambridge
Morley,T.G., 1971[b] , Stud.Biophys., 28 , 117
Mullenger,L. and Ormerod,M.G., 1969 , Int.J.Radiat.Biol., 15 , 259
Myers,L.S., 1973 , The Radiation Chemistry of Macromolecules , Vol. II edited by M.Dole , Academic Press , p. 323
Myers,L.S.,Hollis,M.L. and Theard,L.M., 1968 , Advances in Chemistry Series , 81 , 345
Nakken,K.F.,Sikkeland,T. and Brustad,T., 1970 , FEBS Letters , 8 , 33
Nicolau,C.,Körner,O. and Cristea,A., 1966 , Stud.Biophys., 1 , 59
Nicolau,C.,Körner,O. and Cristea,A., 1967 , Revue Roum.Phys., 12 , 95
Neta,P., 1972 , Chemical Reviews , 72 , 533
Neta,P. and Schuler,R.H., 1971 , Radiat.Res., 47 , 612
Nofre,C.,Lefier,A. and Cier,A., 1961 , C.R.Acad.Sc.,Paris , 253 , 687
Ponnamperuma,C.,Lemmon,R.M. and Calvin,M., 1963 , Radiat.Res., 18 , 540
Pullman,B. and Pullman,A., 1961 , Nature , 189 , 725
Redpath,J.L. and Willson,R.L., 1973 , Int.J.Radiat.Biol., 23 , 51
Rhaese,H.J., 1968 , Biochim.Biophys.Acta , 166 , 311
Roberts,P.B. and Fielden,E.M., 1971 , Int.J.Radiat.Biol., 20 , 363
Sauerbier,W., 1964 , Biochem.Biophys.Res.Comm., 17 , 46
Schans,G.P.van der, 1969 , Thesis , Amsterdam
Schans,G.P.van der,Aten,J.B.T. and Blok,Joh., 1969 , Anal.Biochem.,

32 , 14

Schans,G.P.van der,Bleichrodt,J.F. and Blok,Joh., 1973 , Int. J. Radiat.Biol., 23 , 133

Schans,G.P.van der,Weyermans,J.P. and Bleichrodt,J.F., 1971 , Molec. Gen.Genetics , 110 , 263

Scholes,G., 1963 , Progress in Biophysics and Biophysical Chemistry , Vol. 13 , edited by J.A.V.Butler , Pergamon Press , p. 59

Scholes,G., 1968 , Radiation Chemistry Of Aqueous Systems , edited by G.Stein , The Weizmann Science Press of Israel , p. 259

Scholes,G.,Shaw,P.,Willson,R.L. and Ebert,M., 1965 , Pulse Radiolysis , edited by M.Ebert,J.P.Keene, A.J.Swallow and J.H.Baxendale , Academice Press , p. 151

Scholes,G.,Ward,J.F. and Weiss,J., 1960 , J.Molec.Biol., 2 , 379

Scholes,G. and Weiss,J., 1952 , Exptl.Cell Res.Suppl., 2 , 219

Scholes,G.,Weiss,J. and Wheeler,C.M., 1956 , Nature , 178 , 157

Scholes,G.,Willson,R.L. and Ebert,M., 1969 , Chem.Comm., 17

Schwarz,H.A., 1962 , J.Phys.Chem., 66 , 255

Schweibert,M.C. and Daniels,M., 1971 , Int.J.Radiat.Phys.Chem., 3 , 353

Shragge,P.C.,Michaels,H.B. and Hunt,J.W., 1971 , Radiat.Res., 47 , 598

Simic,M.,Neta,P. and Hayon,E., 1969 , J.Phys.Chem., 73 , 3794

Sweet,J.P. and Thomas,J.K., 1964 , J.Phys.Chem., 68 , 1363

Theard,L.M.,Peterson,F.C. and Myers,L.S., 1971 , J.Phys.Chem., 75 , 3815

Ward,J.F., 1971 , Int.J.Radiat.Phys.Chem., 3 , 239

Willson,R.L., 1966 , Thesis , Newcastle upon Tyne

Willson,R.L., 1970 , Int.J.Radiat.Biol., 17 , 349

Willson,R.L.,Greenstock,C.L.,Adams,G.E.,Wageman,R. and Dorfman,L.M., 1971 , Int.J.Radiat.Phys.Chem., 3 , 211

Young,E.T. and Sinsheimer,R.L., 1967 , J.Molec.Biol., 30 , 147

Summary

In the work described in this thesis it was attempted to get more information on the radiation chemistry of DNA and its possible role in radiosensitization and radioprotection.

The main part of the present work is devoted to the effects of ionizing radiation on aqueous solutions of adenine , one of the four bases in DNA. With the aid of the method of pulse radiolysis some reactions of the adenine radicals were studied. The hydroxyl adduct of adenine shows a rapid first order decay and this probably reflects a unimolecular rearrangement of the radical. The electron adduct of adenine reacts rapidly with the radiosensitizer oxygen with a rate constant $k = 3.7 \times 10^9\ M^{-1} sec^{-1}$. Electron transfer reactions were shown to occur between the electron adduct of adenine and various electron-affinic compounds like paranitroacetophenone. The rate constants for these reactions have been determined and lie between 10^9 and $10^{10}\ M^{-1} sec^{-1}$.

The main products in the steady state radiolysis of deaerated adenine solutions have been identified as 4,6-diamino-5-formamido-pyrimidine , 6-amino-8-hydroxy-7,8-dihydropurine and 8-hydroxy-adenine. In addition a product was found which was only detectable at high radiation dose ; this possibly is 4,6-diamino-5-hydroxy-pyrimidine.

It could be shown using ^{14}C- and ^{3}H-adenine that in the radiolysis of adenine reconstitution reactions occur , both in the absence and in the presence of oxygen. This followed from the finding that during irradiation adenine loses its ^{3}H-label by formation of water , and from the observation that the ultimate yield of adenine destruction is considerably lower than expected from the primary yields of the water radicals.

The effects of radiosensitizers have also been studied in the radiation chemistry of purified biologically active DNA. It was found that

paranitroacetophenone or triacetoneamine-N-oxyl when present in freeze-dried DNA samples during irradiation did not sensitize the DNA , indicating that the 'direct action model' of sensitization is not relevant to pure DNA.

In rapid-mixing experiments in which DNA solutions were irradiated and mixed thoroughly within 1.5 msecond after irradiation with oxygen , paranitroacetophenone or triacetoneamine-N-oxyl , it was observed that reactions between long-lived DNA radicals and these sensitizers do not produce extra lethal lesions in the DNA , although radiation-induced reactions between DNA and triacetoneamine-N-oxyl do occur when these compounds are irradiated as a mixture.

The effects of the radioprotective compound cysteamine have also been studied in rapid-mixing experiments. It was shown that this compound reacts with DNA radicals resulting in a decrease of the number of single-strand breaks per molecule. No effects of cysteamine on the number of directly formed double-strand breaks could be detected with the used technique. It is shown that the amount of lethal nucleotide damage is not affected by cysteamine , added 1.5 msecond after irradiation of the DNA.

Samenvatting

Het werk dat in dit proefschrift wordt beschreven , is uitgevoerd met het oogmerk meer kennis te vergaren over de invloed van ioniserende straling op DNA. Bovendien werd nagegaan of beschermende en sensibiliserende verbindingen de stralingsschade in DNA beïnvloeden.
Het grootste gedeelte van dit proefschrift is gewijd aan de effekten van γ-straling in oplossingen van adenine , één van de vier basen in DNA. Met behulp van de methode der pulsradiolyse werden een aantal reakties van adenine radikalen bestudeerd. Het hydroxyl-adduct van adenine vertoont een eerste orde reaktie die waarschijnlijk een unimolekulaire omlegging van het radikaal vertegenwoordigt.
Het electron-adduct van adenine ondergaat elektronoverdrachtsreakties met verschillende verbindingen. Paranitroacetofenon is hiervan een voorbeeld. De bimolekulaire reaktiesnelheidsconstanten zijn voor een aantal van deze reakties bepaald en liggen tussen 10^9 en 10^{10} $M^{-1}sec^{-1}$.
De belangrijkste produkten die worden gevonden na bestraling van een zuurstofvrije oplossing van adenine zijn geïdentificeerd als 4,6-diamino-5-formamido-pyrimidine , 8-hydroxy-adenine en 6-amino-8-hydroxy-7,8-dihydropurine. Bij hoge stralingsdoses wordt nog een ander produkt gevonden , mogelijk is dit 4,6-diamino-5-hydroxy-pyrimidine.
Met gebruikmaking van ^3H- en ^{14}C-adenine werd gevonden dat reconstitutiereakties optreden zowel in aanwezigheid als in afwezigheid van zuurstof. Het optreden van deze reakties werd geconcludeerd uit het verlies van tritium uit adenine door bestraling , waarbij water wordt gevormd , en uit het feit dat de destructie van adenine veel kleiner is dan verwacht mag worden op grond van het aantal water radikalen dat met adenine reageert.

De effekten van stralingssensibilisatoren zijn bestudeerd met behulp van gezuiverde biologisch aktieve DNA's. Paranitroacetofenon of triacetonamine-N-oxyl , indien aanwezig in gevriesdroogde DNA

preparaten bleken het DNA bij bestraling niet te sensibiliseren. Hieruit wordt geconcludeerd dat het model van Adams , dat opgesteld is voor sensibilisatoren in het geval van direkte stralingswerking , althans voor DNA niet juist is.

In snelmeng-experimenten waarbij DNA oplossingen worden bestraald en waaraan vervolgens binnen 1,5 msec een oplossing van paranitroacetofenon , triacetonamine-N-oxyl of zuurstof wordt toegevoegd , kon geen invloed van deze verbindingen op de overleving van het DNA worden waargenomen. Bij bestraling van mengsels van DNA en triacetonamine-N-oxyl in oplossing bleken echter wel radikaalreakties tussen deze componenten op te treden.

Met behulp van de snelmeng-techniek werden ook de effekten van een beschermende verbinding , cysteamine , onderzocht. Cysteamine reageert met DNA radikalen en deze reaktie leidt tot een vermindering van het aantal enkelstrengsbreuken. Effekten van cysteamine op het aantal dubbelstrengsbreuken dat met één treffer wordt gevormd konden met de gebruikte techniek niet worden waargenomen. Wel kon worden aangetoond dat de hoeveelheid letale nucleotideschade niet wordt veranderd door cysteamine , wanneer het 1,5 msec na bestraling van het DNA wordt toegevoegd.

Nawoord

Het onderzoek dat in dit proefschrift is beschreven maakt deel uit van een projekt van het Medisch Biologisch Laboratorium TNO dat gericht is op een beter begrip van de effekten van fysische en chemische agentia op desoxyribonucleïnezuur. Nochtans ben ik zelf verantwoordelijk voor de wetenschappelijke en andere onachtzaamheden in dit proefschrift. Voor het overige heeft een groot aantal mensen hun stempel op het voorgaande gedrukt. Enkele van hen wil ik met name hiervoor bedanken.

In de eerste plaats mijn promotor Henk Loman , die mijn belangstelling voor de stralingschemie opwekte en die in hoge mate bijdroeg aan de totstandkoming van dit proefschrift.
Mijn copromotor Johan Blok ben ik zeer erkentelijk voor de suggestie om als dienstplichtig militair in het MBL te gaan werken , waardoor voor mij het nuttige met het dienstplichtige werd verenigd. Bovendien dank ik hem als afdelingshoofd en later als voorzitter van de werkgroep 'primaire lesie en SH' voor de vele suggesties bij het werk in de afdeling stralingsbiofysica.

I like to thank you Ged Adams and Robin Willson for the introduction you gave me to rapid-mixing techniques as well as pulse radiolysis. During my stay in the Gray Laboratory of the Cancer Research Campaign I got intrigued by radiosensitization and I am gratefull for the opportunity you gave me to get that motivation for further investigations.

Een gedeelte van de beschreven experimenten waren alleen mogelijk door de hulp van de sektie Stralingschemie van het Interuniversitair Reactor Instituut , waar Andries Hummel , Lee Luthjens en Marinus Hom mij de mogelijkheden gaven om zinvol met de Van de Graaff versneller te werken.

Met veel genoegen noem ik hier ook de afdeling Stralingsbiofysica en haar medewerkers.
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De constructie van de snelmeng apparatuur werd uitstekend verzorgd door J.Engelen , J.Visser , J.van der Hoek en H.van Houten.

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