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PEROXYDATION OF NUCLEIC ACIDS
BY RADIATION : BIOLOGICAL IMPLICATIONS

by

R. LATARJET, B. EKERT AND P. DEMERSEMAN

1964



Work performed by Institut Pasteur under Euratom Contract No. 005-61-1 BIOF

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In the absence of oxygen, the yield of the over-all reaction is decreased by a factor of 3. Peroxidation does not take place; the final main product is glycol.

Peroxidation is prevented by mercaptoethylamine, which acts at the level of the first two steps of the reaction. This protector acts as an antioxygen in so far as the mechanisms which it puts into play counteract those which depend on the presence of oxygen.

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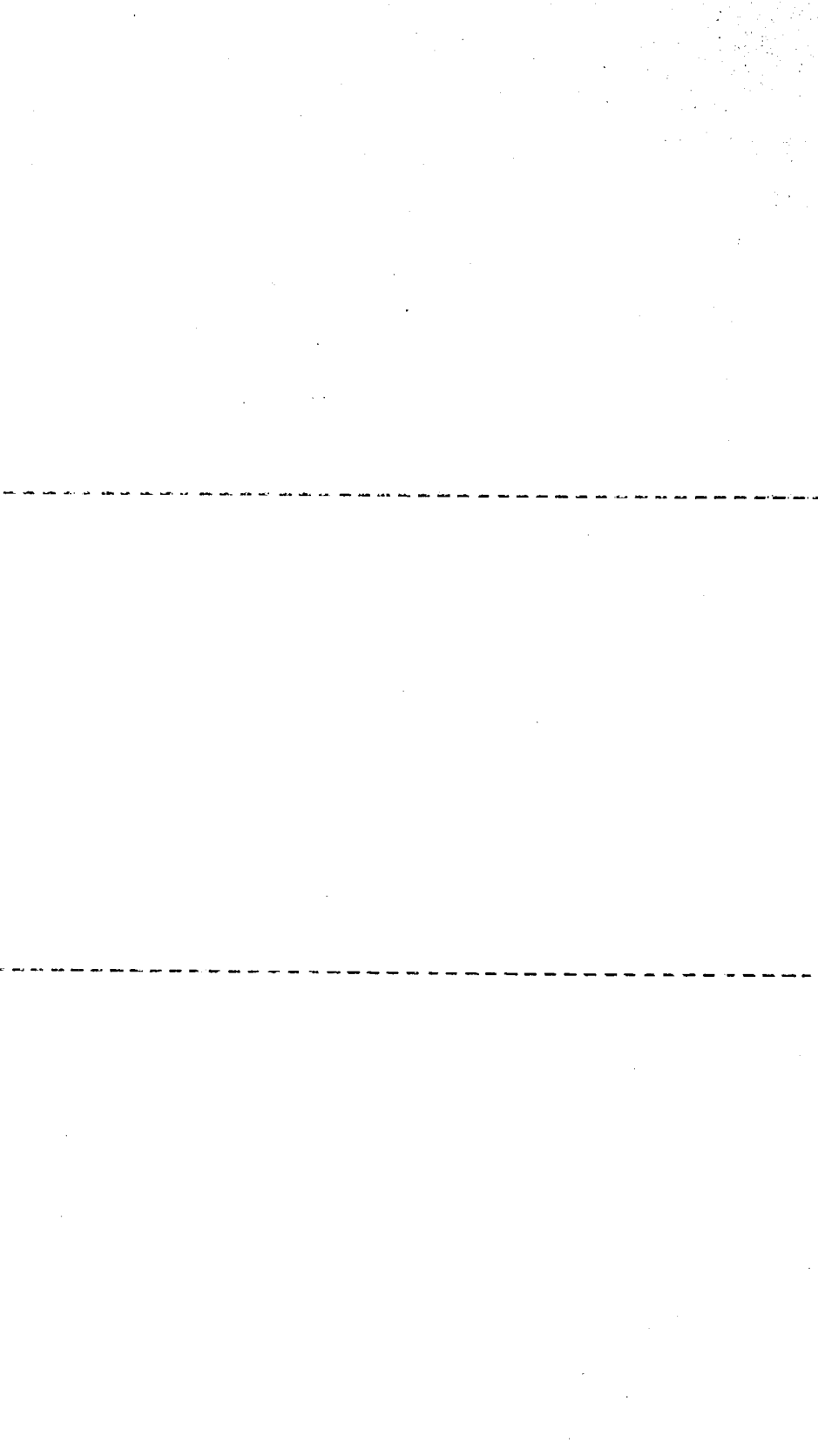
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Peroxidation of Nucleic Acids by Radiation: Biological Implications

R. LATARJET, B. EKERT, AND P. DEMERSEMAN

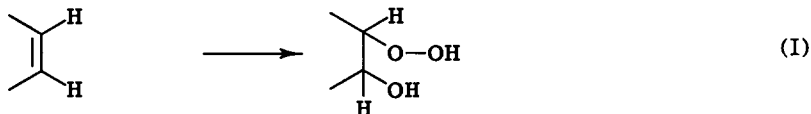
Institut du Radium, Paris, France

INTRODUCTION

In order to elucidate the mode of action of ionizing radiations on nucleic acids, radiochemical studies have been carried out since 1956 on certain constituents of these acids, namely the pyrimidine bases, in water solution. It appeared immediately that, in the presence of oxygen, irradiation sparks some important reactions of peroxidation. It is the purpose of this paper (1) to describe these reactions; (2) to utilize them as a simple system for the study of some radiochemical phenomena of biological interest, such as chemical protection and the oxygen effect; and (3) to discuss their role as primary radiolesions.

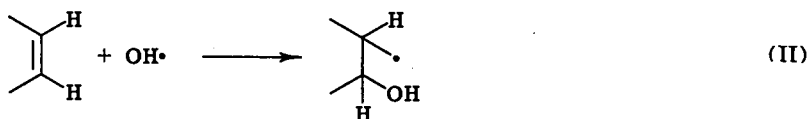
MECHANISM OF THE PEROXIDATION OF PYRIMIDINES

In 1956 and 1957 Weiss *et al.* (1, 2) demonstrated that, under the influence of X-radiation, nucleic acids in aerated water solution are peroxidized on the 5-6 bond of the pyrimidines. This fact was confirmed by Ekert and Monier (3, 4). According to the scheme suggested by Weiss *et al.*, the double bond opens and gives a hydroxyhydroperoxide (I). In fact, this reaction is more complex. On the basis of

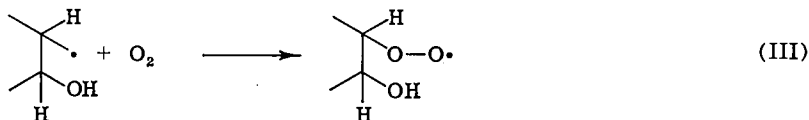


Ekert's recent work (cf. Latarjet *et al.*, 5), we consider that it follows three steps:

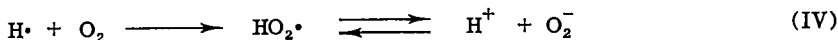
1. Irradiation produces radiolysis of water. The $\text{OH}\cdot$ radical acts on the 5-6 double bond to give the hydroxyhydropyrimidyl radical (which we may call HHP) (II):



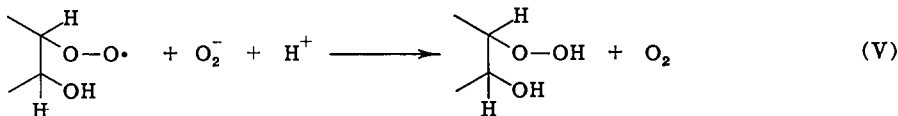
2. Molecular oxygen, present in the solution, is fixed on this radical to form the hydroxyhydroperoxy-pyrimidyl radical (HHPP) (III):



3. The O_2^- ion, which is formed by reaction IV (Scholes *et al.*, 6), transforms



that radical into a pyrimidine hydroxyhydroperoxide (V).

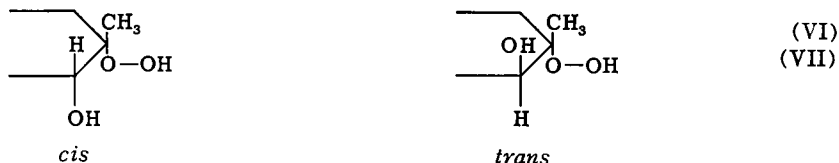


EVOLUTION OF THE PEROXIDE

The fate of this peroxide depends on the base.

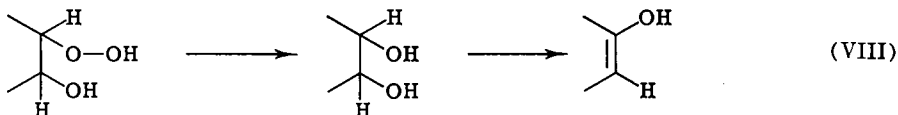
Thymine (3, 4). Thymine is destroyed according to a linear function of the dose of radiation, with a high efficiency ($G = 2.4$ for a $10^{-3} M$ solution). Peroxidation accounts for most of this destruction and is by far the main reaction induced by the radiation in aerated water solution. But the peroxide is in part destroyed by radiation, to such an extent that the G value for the peroxide found is only 0.85.

This peroxide is rather stable. It has been synthesized in crystalline form by Ekert. The two isomers *cis* and *trans* coexist, with 80% *cis* (VI) and 20% *trans* (VII).



However, at room temperature (and more rapidly at higher temperatures) the *cis*-isomer is slowly transformed into the *trans* one. Then, the latter slowly loses its peroxidic oxygen and gives the *trans*-glycol.

Uracil. The peroxide, quite unstable, is transformed into glycol by loss of its peroxidic oxygen. Then the glycol, which is also unstable, loses water, thus yielding isobarbituric acid (VIII).



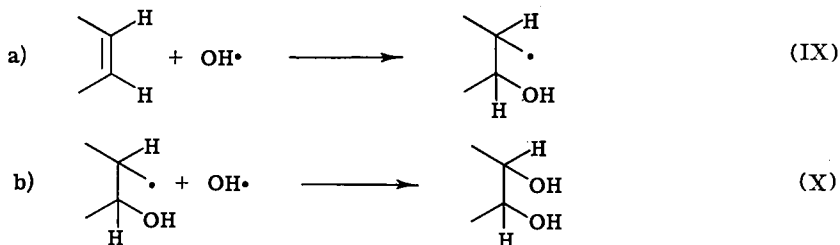
Cytosine (7). The peroxide, which is very unstable, has not been isolated. It loses its peroxidic oxygen to give the glycol, which is transformed into isobarbituric acid along two different pathways (Fig. 1): (1) loss of NH_2 , then loss of water; (2) loss of water \rightarrow 5-hydroxycytosine, then loss of NH_2 .

The production of 5-hydroxycytosine should be noticed, since this compound can be considered either as a new base or as a possible base analog.

ROLE PLAYED BY PEROXIDATION IN THE "OXYGEN EFFECT"

When a pyrimidine in aqueous solution is irradiated in the absence of oxygen, the reactions evolve differently (8). The first reaction, the attack of the base by an $\text{OH}\cdot$ radical which produces the HHP radical, remains the same, but its yield is lessened by the absence of oxygen. It is well known that, by combining with $\text{H}\cdot$ radicals ($\text{H}\cdot + \text{O}_2 \rightarrow \text{HO}\cdot_2$), oxygen diminishes the probability of the recombination $\text{H}\cdot + \text{OH}\cdot \rightarrow \text{H}_2\text{O}$, thus increasing the amount of available $\text{OH}\cdot$ radicals. Absence of oxygen decreases this amount.

Once it has been formed, the HHP, which does not find oxygen, reacts with a second $\text{OH}\cdot$ radical to form the glycol (IX, X).



This is the main reaction in the absence of oxygen. But others are possible, yielding a variety of compounds. For example, in the case of thymine, in addition to the glycol, one finds dihydrothymine and 5-hydroxymethyluracil, the latter with a G value of 0.1. This latter is a new base which recalls the well-known 5-hydroxymethylcytosine present in the DNA of the T-even phages.

In the absence of oxygen, $10^{-3} M$ thymine in aqueous solution is destroyed with a G value equal to 0.8, i.e., one-third of that in the presence of oxygen. It seems rather remarkable that this ratio is of the same magnitude as that found in most biological systems, which measures what one calls the "oxygen effect." According to our observations, the sensitization to radiation produced by oxygen has two origins in the case of a pyrimidine in water solution: (1) the enrichment of $\text{OH}\cdot$ radicals available for attacking the base; (2) in the next step, once the HHP has been formed, the possibility of a peroxidation reaction.

The constancy of the size of the oxygen effect among chemical and biological water-containing systems brings a powerful argument in favor of the hypothesis of a common, i.e., a very basic mechanism. This mechanism should exert itself at

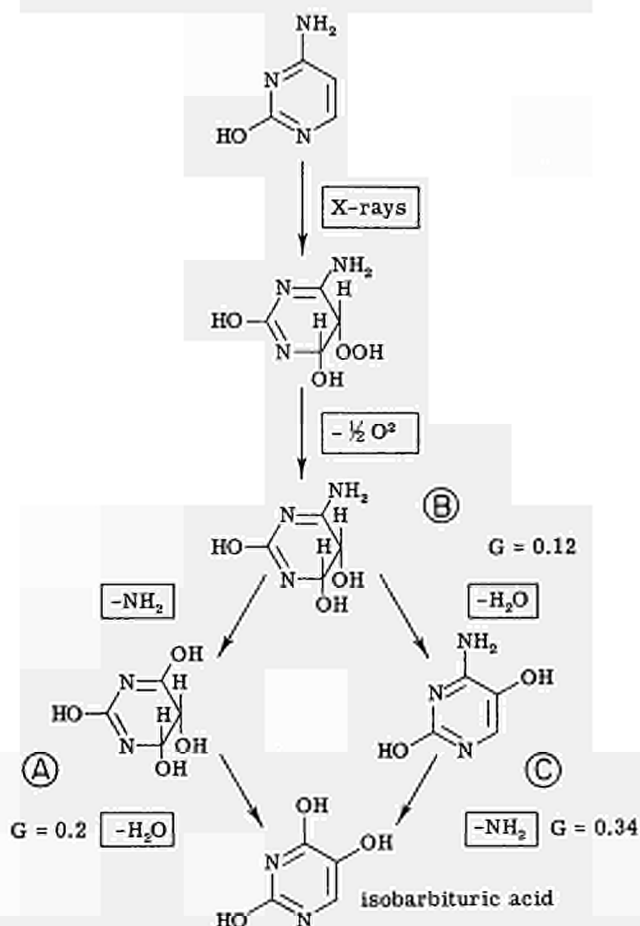


FIG. 1. Effect of X-rays on cytosine in the presence of oxygen

the level of the early radiochemical reactions and could in general be of the type just described.

CHEMICAL RADIOPROTECTION AND PEROXIDATION

Diverse mechanisms have been suggested to explain radioprotection by chemicals, the most efficient of which are sulfur compounds such as mercaptoamines and thiouronium salts. Radioperoxidation of thymine offers a simple quantitative system for the study of chemical protection.

Thymine ($10^{-3} M$) in aerated aqueous solution at $0^\circ C$ received 300 krad of X-rays (40 kv) in the presence of various amounts of mercaptoethylamine (cysteamine). In aqueous solution this substance comes, within a few minutes, into

equilibrium with nascent cystamine, its disulfide form, which in the present system participates in the protection phenomenon (9). By polarography, the thymine hydroperoxide ($E_{1/2} = -0.1$ volt) and the hydrogen peroxide ($E_{1/2} = -0.9$ volt) formed were measured. In parallel, the destruction of cystamine was determined by Lelièvre's method (9), and the residual thymine by spectrophotometry at 264 $m\mu$ after descending paper chromatography (*n*-propanol- H_2O , 10:3) in order to avoid any interaction with the radio-formed compounds. Thus, the complete result of the reaction was established. This work, carried out by Ekert and Miss A. Bouyat (unpublished observations), has disclosed the following facts:

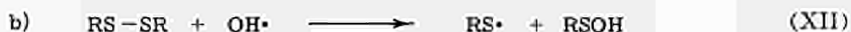
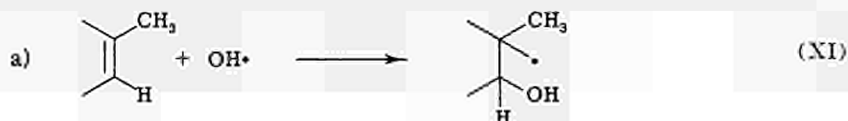
1. At high concentrations of cysteamine (5 to 10 molecules, i.e., 2.5 to 5 molecules of cystamine, for 1 molecule of thymine), protection is almost complete. Neither thymine hydroperoxide nor hydrogen peroxide appears in detectable amounts; the base is not attacked. We can say already that the latter is protected against the action of $OH\cdot$ radicals, and that cystamine has competed with the 5-6 double bond of the base for the capture of those radicals.

2. At low concentrations of cysteamine (0.25 to 1 molecule for 1 molecule of thymine), protection is about proportional to the amount of protector. At the concentration 1 for 1, half of the thymine is protected. Thymine hydroperoxide is not formed, whereas hydrogen peroxide is formed in higher amounts than normal. At the concentration of 0.25 for 1, there is twice as much H_2O_2 as in the absence of protector.

3. A detailed chromatographic analysis of the irradiated solutions reveals the presence of large amounts of thymine glycols *cis* and *trans*.

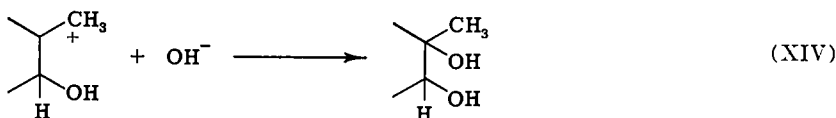
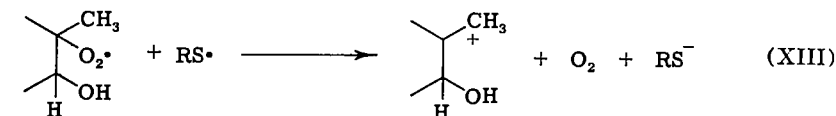
These observations lead us to consider that the protective action takes place in the first two reactions in the transformation of thymine (see section on mechanism of peroxidation):

1. The protector competes with the 5-6 double bond for the capture of $OH\cdot$ radicals, i.e., for the formation of HHP (XI, XII).



2. The protector reacts on the peroxide radical HHPP and reduces it (XIII). This leads to suppression of peroxide formation and to the appearance of the glycol (XIV) and to an increase in hydrogen peroxide (XV).¹

¹ Notice that part of the peroxidative process, which normally leads to thymine peroxide, is deviated by the protector, to form H_2O_2 . In this respect, hydrogen peroxide represents a degraded type of peroxidation.



When the protector is present in large amounts, the first mechanism is important enough to stop the reactions at the first step. When there is less protector, a part of the thymine goes through the first two steps, to form a peroxide radical. The protector may exert its second type of action at this level.

In those reactions, nothing is really characteristic of thymine. They could apply to many other organic substrates. We therefore believe that the present conclusions can, to a certain extent still undetermined, be generalized, and that the protective effect of mercaptoamines is due to a double synergistic mechanism: (1) avidity for OH^\bullet radicals which, by competition with the substrate, curb the beginning of the reactions; (2) reduction of the substrate's peroxy radical. It is remarkable that these two mechanisms are the counterparts of those two which we have considered at the origin of the oxygen effect (above); they are "anti-oxygen." By the processes which are put into play, protection by cysteamine appears to be the reverse phenomenon of sensitization by oxygen. This explains the well-known fact that protection by cysteamine is counteracted by increased oxygen pressure (10) or by oxygen bubbling—as far as oxygen and cysteamine can reach the same functional sites of the system, which is not always the case, especially in coiled DNA (11, 12). It also explains why both phenomena enjoy the same character of generality.

INDIRECT RADIOPEROXIDATION OF NUCLEIC ACIDS

It is well known that ionizing radiations provoke the peroxidation of many diverse molecules existing in living matter. Such peroxides may rise in the vicinity of nucleic acids. One may wonder whether in such a case the nucleic acid may, in its turn, be indirectly peroxidized. In fact, the actions of organic peroxides on biological substrates are little known. In certain cases there is a strong action. For example, we showed some years ago (13) that disuccinyl monoperoxide (DSP) is a powerful inhibitor of a transforming DNA of *Pneumococcus*. That study disclosed three interesting facts: (1) The activity of DSP is very high; at a concentration of $3 \times 10^{-8} M$ in distilled water, it may bring the transforming activity down to 10% within 10 minutes at room temperature. (2) This activity is not re-

producible from day to day; some days, the peroxide appears inactive. (3) The activity is not displayed by all peroxides; for example, *cu nene* hydroperoxide, at a concentration of $10^{-3} M$, is inactive, whereas hydrogen peroxide is active, but less than DSP.

In order to understand this action better, we have studied the effects of DSP and of hydrogen peroxide on a simple organic substrate, hydroquinone (14). We observed that in aqueous solution DSP leads to the expected quinone only when a suitable catalyst is present. This catalytic activity is borne by some metal ions, namely iron and copper, at concentrations as low as $10^{-11} M$. This explains the variability observed among daily experiments on DNA when the purity of water and the cleanliness of glassware were not strictly controlled. Minute traces of catalytic metals modify the speed of the reactions, the complexity of which is great. For example, in the presence of a catalyst, hydrogen peroxide does not oxidize hydroquinone into quinone. It produces an unknown substance, detected by its pink-violet color, the formation of which is a function of the concentration of the catalytic metal. That substance also appears when hydroquinone is X-irradiated (15). We are dealing here probably with a reaction of the Fenton type sparked by $\text{OH}\cdot$ radicals.

We have treated thymine with DSP. In the absence of a catalyst, nothing happens. In dilute solution and in the presence of certain metal ions, thymine is transformed into its glycol. Never were we able to detect thymine peroxide among the products of the reaction. Conversely, this peroxide is formed under the effect of concentrated H_2O_2 in the presence of a catalyst metal. However, since thymine peroxide is spontaneously degraded into glycol, direct and indirect peroxidations by ionizing radiations appear to lead to the same final derivatives, i.e., to the same type of lesion of the pyrimidine bases.

PEROXIDATION OF PYRIMIDINES AS A PRIMARY RADIOLESION OF NUCLEIC ACID

There are two ways of considering as a primary lesion the peroxidation of a base within a nucleic acid molecule, and of envisaging its biological consequences: either as a change localized at the base, or as the origin of a change in the secondary structure of the chain in its vicinity.

1. As a local change, it is unlikely that it represents a specific one endowed with particular new properties. For example, thymine peroxide does not compete with thymine in thymineless bacteria (Latarjet, unpublished data). The opening of the double bond represents too large a steric alteration to leave any chance that a pyrimidine peroxide may act as an antipyrimidine. This is also true for the glycol. It represents a nonreplicable spot along the chain, a fraudulent base. At this locus, at the time of replication, an error is likely to occur with regard to base pairing in the daughter strand.

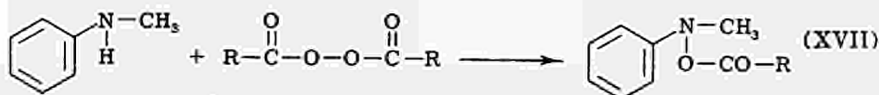
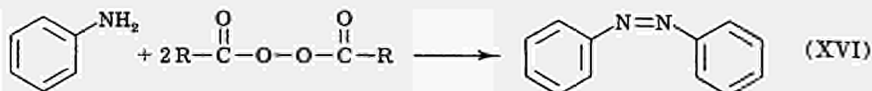
Special emphasis may be placed on 5-hydroxycytosine and 5-hydroxymethyl-

uracil among those compounds formed by radiation under the conditions outlined in the second and third section above. They are true pyrimidine bases. The second has recently been found in the DNA of a *Bacillus* bacteriophage (J. Marmur, personal communication).

In the cell nucleus, the concentration of oxygen presumably varies with time, and also from one region to another. The two sets of reactions we observed in aerated and in deaerated solutions are likely to occur simultaneously at diverse rates in diverse parts of the DNA. Among those reactions, straight conversion of a base into another one may be of particular biological significance.

2. We are still ignorant which direct or indirect consequences peroxidation may have on the secondary structure of nucleic acid. But a few observations relevant to this point may be made:

a. We have already underlined the high efficiency of DSP on a transforming double-stranded DNA. Such peroxydiacids have been shown to react on primary, secondary, and tertiary amines according to reactions such as XVI and XVII.



Adenine, cytosine, and guanine carry a nonsubstituted primary amine function which may undergo the first reaction. It would give a kind of "dimer" reminiscent of that produced by ultraviolet radiation (17). Such dimerization could be revealed by a decrease in the thermal fusion of the double helix (18). Moreover, the N heteroatom of purines and pyrimidines has a behavior somewhat similar to that of the N of secondary or tertiary amines. In that case, the second reaction, of the oxidative type, could lead to alterations in the secondary structure of the nucleic acid by degradation of the valence bonds or of bonds of the van der Waals type.

b. Contrary to what happens with ultraviolet radiation, irreversible bridging between the two helices seems rare or absent in DNA irradiated with X-rays. In our laboratory, N. Rebeyrotte (unpublished data) has observed that thermal fusion is not diminished in a transforming DNA of *Pneumococcus* irradiated with X-rays, whereas it is diminished when the same degree of inactivation of the transforming ability is achieved by ultraviolet.

A recent observation by Ekert and Masson (unpublished data) fits in the same direction. They irradiated with γ -rays a double-helix polymer, poly AU, obtained by the coupling of a polyadenine with a polyuracil under the action of Mg^{++} and Mn^{++} ions at pH 7. At doses up to 126 kr, which alter many bases and break the phosphodiester chains at several points, the structure of the double helix is not

markedly altered, since the fusion temperature and the thermal hyperchromic effect remain unchanged.

SUMMARY

Radioperoxidation is an efficient process on nucleic acids in aerated water solution. At the level of the pyrimidines, it accounts for the bulk of the reaction induced by ionizing radiation.

In the absence of oxygen, the yield of the over-all reaction is decreased by a factor of 3. Peroxidation does not take place; the final main product is glycol.

Peroxidation is prevented by mercaptoethylamine, which acts at the level of the first two steps of the reaction. This protector acts as an antioxygen in so far as the mechanisms which it puts into play counteract those which depend on the presence of oxygen.

Indirect peroxidation by peroxides formed by radiation in the vicinity of DNA is a possible event. It depends highly on the presence of trace metal catalysts. It may yield the same final compounds as direct peroxidation.

These alterations can play a role as primary lesions in some radiobiological processes.

ACKNOWLEDGMENTS

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