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Base liberation and concomitant reactions in irradiated DNA solutions

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(Dedicated to K. G. Zimmer on the occasion of his sixtieth birthday)

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Irradiation of aqueous solutions of DNA causes liberation of free nucleobases and of compounds which behave chromatographically like nucleosides. These components can be separated from irradiated DNA by DEAE-Sephadex chromatography. Their amount increases linearly with dose, resulting in a *G*-value of 0·16 to 0·18. Further separation of the liberated products by ion-exchange chromatography on Dowex 50 × 4 and by thin-layer chromatography on cellulose plates shows that about 60 per cent of the liberated material consists of nucleosides, part of which carries a damaged sugar molecule. Different methods used to determine the formation of malonic dialdehyde in irradiated DNA lead to the conclusion that this type of sugar damage does not contribute very much to the breakage of the DNA backbone. Several mechanisms leading to single strand breaks in DNA are discussed.

1. Introduction

It has been reported by Hems (1960) that nucleotide bases are liberated from DNA after exposure to ionizing radiation. The number of bases released is approximately one-quarter of the bases destroyed. It can be assumed that, concomitantly with the splitting of the N-glycosidic bond, some chemical reactions take place in the sugar moiety. These changes of the deoxyribose may lead to breakage of the nucleotide chain. Accordingly, Krushinskaja and Shal'nov (1967) proposed a mechanism of chain breakage by which malonic dialdehyde is formed and a base is liberated. In agreement with the mechanism of Krushinskaja and Shal'nov, Bopp and Hagen (1970) observed recently that most of the 5′-termini in the radiation-induced breaks carry phosphate groups. Moreover, it was shown by Simon (1969) that not only free bases are liberated from irradiated DNA, but also compounds which behave chromatographically like nucleosides.

To obtain more information about the relationship between base liberation and chain breakage, a quantitative determination of the bases and nucleosides released was performed, together with some experiments about the sugar damage in irradiated DNA. In analysing the results, we also took into account that some of the single strand breaks in irradiated DNA are induced only by the alkaline treatment in the denaturation procedure (Bopp and Hagen 1970).

2. Materials and methods

2.1. *DNA and irradiation conditions*

Calf-thymus DNA was isolated by alcohol precipitation according to the method of Kay, Simmons, and Dounce (1952). The protein content was

0·5 per cent. Aqueous DNA solutions of 500 μg/ml. were irradiated by ^{60}Co γ-rays (Gammacell 220, Atomic Energy of Canada Ltd.) after saturation with N_2. Solutions of 40 ml. were concentrated to about 5 ml. in a rotating evaporator prior to chromatography. Alkaline treatment of the irradiated DNA solution was performed by adding NH_4OH to 1 M final concentration. After 30 min incubation at 37°c the solution was neutralized with HCl.

2.2. *Chromatographic methods*

2.2.1. *Ion exchange on DEAE-Sephadex*

DEAE-Sephadex A 25 was purchased from Deutsche Pharmacia. The columns were prepared according to Rushizky, Bartos and Sober (1964) as modified by Simon (1969), and 20 mg of irradiated DNA applied to a 1·45 × 25 cm column. Elution of free bases and nucleosides was achieved with 0·02 M *tris* pH 7·6, omitting urea, because we found no difference in the amount of U.V.-absorbing effluent using 7 M urea with the *tris* buffer or not. The eluted material was monitored by its absorption at 270 mμ.

2.2.2. *Ion exchange on Dowex*

For the separation of the nucleosides and bases, the effluent from the DEAE-Sephadex column was concentrated and desalted by chromatography on an ion retardation resin AG 11 A 8 (Bio Rad, Richmond). The solution was adjusted to pH 4 and readsorbed to a 1·35 × 4 cm column of Dowex 50 × 4 (50–100 mesh) H^+ form after Cohn (1961). This ion exchange resin, purchased from Serva (Heidelberg), was prepared by washing with 2 N NaOH, stirred cautiously for 30 min and treated thereafter with 2 N HCl for 30 min. The resin was filled into the column and rinsed with water until neutral.

2.2.3. *Thin-layer chromatography (TLC)*

Qualitative estimates were made on TLC-cellulose F 0·1 mm (Merck, Darmstadt). For quantitative measurements we preferred cellulose plates without fluorescence indicator because it would interfere with the U.V.-absorption of the eluate in 0·1 N HCl. As ascending solvent, we used 80 per cent *n*-propanol + 20 per cent water; developing time was 6 hours. For elution we scraped cellulose off the spots into test-tubes, added 1 ml. of 0·1 N HCl and heated it in a water-bath at 100°c for 10 min. After centrifugation we measured the absorption at 260 mμ. 96 per cent recovery of the total material was obtained. As reference, we scraped blanks of the same R_F value. To compare our results with the results of other authors, descending paper chromatography was performed on Whatman No. 1 with the same solvent for 12 hours. For the identification of the bases and nucleosides, the U.V.-spectra and the ratios of absorption at 250, 260, 280, and 290 mμ (Harbers 1964) were measured in comparison with authentic solutions.

2.3. *Chemical procedures*

2.3.1. *Identification of deoxyribose*

The test for deoxyribose with diphenylamine was carried out according to the method of Burton (1956). The reagent was composed of 1·5 g diphenylamine, 100 ml. acetic acid, and 1·5 ml. concentrated H_2SO_4. Shortly before use 0·5 ml. of acetaldehyde (16 mg/ml.) was added. To 1 ml. of test solution

2 ml. of the reagent was mixed, incubated for 17 hours at 30°c and the colour development measured photometrically at 600 mμ. To calibrate the diphenyl-reaction we used a 10^{-3} molar mixture of purine and pyrimidine nucleosides with the same quantitative composition as in calf-thymus DNA.

2.3.2. *Tests for malonic dialdehyde*

Malonic dialdehyde was estimated with 2-methylindole at room temperature as well as with 2-thiobarbituric acid in a boiling-water bath. The methylindole reagent according to Scherz, Stehlik, Bancher and Kaindl (1967) was made up as follows: 0·1 g 2-methylindole was dissolved in 100 ml. ethanol, then 25 ml. of concentrated HCl was added. To 1 ml. of the DNA samples 2 ml. of reagent was added and thoroughly mixed. After 20 min the spectrum was taken in a Beckman recording spectrophotometer and the absorbance measured at its maximum at 555 mμ [$\epsilon_{555} = 8{\cdot}6 \times 10^4$ l. mole^{-1} cm^{-1}].

Thiobarbituric acid (TBA) develops its colour reaction with malonic dialdehyde only after heating at 100°c. The reagent was prepared of 0·6 g 2-thiobarbituric acid dissolved completely in a minimum of 0·1 N NaOH and filled up to 100 ml. with distilled water (the final pH value was 2).

The test solution was mixed with this TBA reagent in the volume-ratio of 1 : 2 and the absorption measured at 532 mμ after 20 min of heating and subsequent cooling in normal tapwater for 2 min [$\epsilon_{532} = 1{\cdot}46 \times 10^5$ l. mol^{-1} cm^{-1}].

The calibration curves for both reactions were prepared with 1,1,3,3,-tetra-ethoxypropan. According to the method of Scherz *et al.* (1967), 10 mg of this tetraacetal and 1 ml. of concentrated HCl were adjusted to 500 ml. with distilled water. With this procedure a quantitative conversion into malonic dialdehyde is achieved. 1 μg tetraethoxypropan (mol. wt 220·3) corresponds to 0·327 μg malonic dialdehyde.

3. Results

3.1. *Release of bases and nucleosides*

To determine the release of bases and nucleosides, the irradiated DNA solutions were chromatographed on a DEAE-Sephadex column. With this technique, oligonucleotides are separated with increasing NaCl concentration according to their charge or their chain length, respectively (Rushizky *et al.* 1964). Compounds without charge, i.e. free bases and nucleosides, are eluted first with low concentrated *tris* buffer or simply with water. The amount of these uncharged compounds, released from irradiated DNA, increases linearly with dose (figure 1). From the slope of this curve, the G-value, i.e. the number of events per 100 eV absorbed energy, is calculated by regression analysis. It amounts to $0{\cdot}16 \pm 0{\cdot}01$ when the DNA solutions are kept neutral and to $0{\cdot}18 \pm 0{\cdot}015$ when the DNA solutions are treated with alkali after irradiation.

From the calculated curves in figure 1, there is a small release at dose zero, whereas chromatographic elution of non-irradiated DNA was difficult to obtain because the material clogs the column. This observation was also made by Hems (1960) chromatographing irradiated DNA on paper. The total material, eluted from the DEAE-Sephadex column with 0·02 M *tris* buffer, pH 7·6 is referred to as ' effluent ' in the following sections. Analysing the ' effluent '

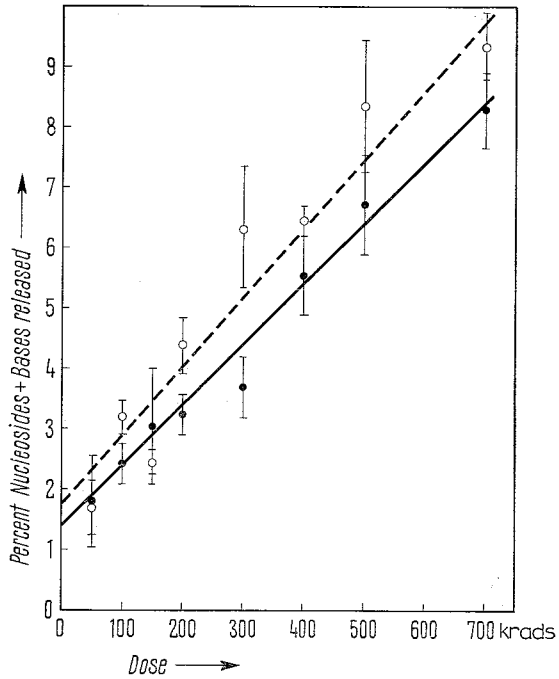

Figure 1. Chromatography of irradiated DNA on DEAE-Sephadex. Elution of charge-free components (bases and nucleosides) from irradiated DNA. Percentage of total U.V.-absorption in the 'effluent' against dose. Mean values ± S.E. ($n=2$–7). ● DNA samples were kept neutral before chromatography. ○ DNA samples were treated with alkali before chromatography. Irradiation conditions and alkaline treatment as indicated in the methods, § 1.

by the diphenylamine reaction to detect sugar molecules, we found that 57 per cent of the total U.V.-absorption are compounds giving positive deoxyribose reaction.

3.2. *Separation of the components in the DEAE-Sephadex effluent*

3.2.1. *Ion exchange chromatography*

Further separation of the 'effluent' was achieved by chromatography on Dowex 50 × 4. The elution profile from this column is shown in figure 2. Analysing various samples in this way, we found in fractions 1–14 (eluted with 0·5 N HCl) 60 ± 10 per cent of the U.V.-absorbing material, when the DNA solutions were kept neutral, and 70 ± 8 per cent when the DNA solutions were treated with alkali. This relationship was independent of the irradiation dose. The diphenylamine reaction, tested in both cases (neutral and alkali), showed that in fractions 1–14 nearly all of the material consists of nucleosides. It should be kept in mind, however, that this reaction is not specific for intact deoxyribose (see Ashwell 1957). Hence a damaged sugar linked to a base might be also recognized. Elution with 2 N HCl (fractions 15–25) releases free bases from the Dowex column; the diphenylamine reaction in these fractions was negative.

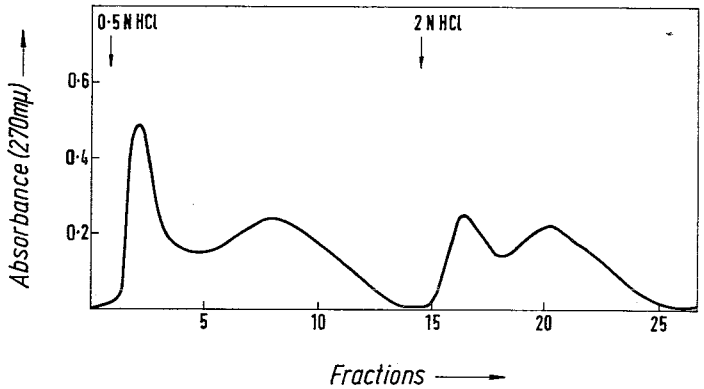

Figure 2. Ion-exchange chromatography on Dowex 50 × 4 of the 'effluent' from the DEAE-Sephadex column. Fractions 1–14 nucleosides. Fractions 15–25 bases. Flowrate 0·75 ml./min, 6 ml./fraction. In this experiment, 61 per cent of the applied material was eluted with 0·5 N HCl and 30·8 per cent with 2 N HCl.

3.2.2. *Thin-layer chromatography*

Thin-layer chromatography (TLC) on cellulose plates made a more detailed study possible of the components in the effluent from the DEAE-Sephadex column. The propanol–water solvent system was the same as used by Hems

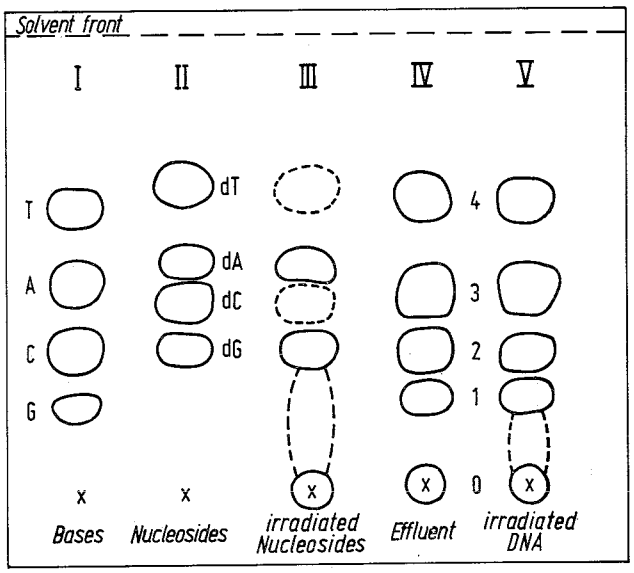

Figure 3. Thin-layer chromatography of DNA components and of irradiated DNA. I. Column: separation of four bases (90 mμmoles applied). G: guanine; C: cytosine; A: adenine; T: thymine. II. Column: separation of four nucleosides (90 mμmoles). dG: deoxyguanosine; dC: deoxycytidine; dA: deoxyadenosine; dT: thymidine. III. Column: separation of irradiated nucleosides (90 mμmoles). Radiation dose 500 krads, concn. 10^{-3} M. IV. Column: separation of the 'effluent' components eluted from the DEAE-Sephadex (80 μl. with an absorbance at 270 mμ = 13·8). V. Column: separation of irradiated DNA. Radiation dose 500 krads at 500 μg/ml. (450 μg applied). The spots were outlined under a U.V.-lamp (254 mμ). Fluorescence (350 mμ) and very weak spots are surrounded by an interrupted line.

(1960), who separated irradiated DNA solutions on paper. Applying aliquots of test solutions of bases and of nucleosides separately to a 0·1 mm cellulose layer, separation of the four components is achieved in unidimensional chromatography (figure 3, columns I and II). Since the nucleosides have very similar R_F values as the corresponding bases, mixtures of bases and nucleosides would not well be separated.

Testing the DEAE-Sephadex effluent (figure 3, column IV), four components migrate (spots 1–4), whereas some of the material remains on the starting point (spot 0). Spots 1 and 4 have the position of guanine and thymine, whereby spots 2 and 3 cover a larger area. Nearly the same chromatographic behaviour as with the ' effluent ' was seen with irradiated DNA solutions (figure 3, column V). In this case a fluorescing tail is observed between spots 0 and 1. Further, spot 0 in column V is much more intensive in U.V.-absorption than in column IV, as all the phosphate containing material in irradiated DNA remains at the origin. Corresponding chromatograms were obtained by paper chromatography according to the method of Hems (1960).

To characterize spot 0; 2 and 3 of the ' effluent ' (figure 3, column IV), the following experiments were performed. To find out if the fixation of material at the origin (spot 0) is due to an irradiation effect, a mixture of nucleosides was irradiated and chromatographed on TLC (figure 3, column III). Exactly as in the case of the ' effluent ', some of the U.V.-absorbing material sticks to the starting point. Further, there appears a fluorescing tail from the starting point to spot 1 as well as a drastic fading of the pyrimidine nucleosides. When spot 0 was tested by diphenylamine reaction, this test was positive in columns III, IV and V: we assume that in irradiated nucleosides as well as in the ' effluent ' there are destroyed sugar molecules bound to an intact base which cannot move with the solvent. Free deoxyribose itself moves under these conditions to about a level of thymidine, whereas irradiated sugar partly sticks to the origin and partly moves with a broad tailing.

For further identification, the spots of the ' effluent ' were scraped out and extracted as described in § 2.2.3. The U.V.-absorption of the various spots is shown in table 1. 36·5 per cent was recovered from the starting point (spot 0), which we would refer to as ' damaged nucleosides '. In this fraction a strong reaction with diphenylamine is also observed. Spots 1–4 were characterized by their U.V.-absorption spectrum and by dyphenylamine. The ratio of the absorption at different wave-lengths indicates that spot 1 is guanine and spot 4 is thymine, whereas spot 2 is a mixture of cytosine and deoxyguanosine and spot 3

Spot	E_{260}	Percentage of total	Diphenylamine reaction	Identification
0	0·35	36·5	+ + +	Damaged nucleosides
1	0·125	13·0	−	Guanine
2	0·065	6·7	+	Cytosine + deoxyguanosine
3	0·375	39·1	+	Adenine + deoxyadenosine
4	0·045	4·7	−	Thymine

Table 1. U.V.-absorption and characterization of the TLC chromatogram of the DEAE-Sephadex ' effluent '.

a mixture of adenine and deoxyadenosine. This is in agreement with the corresponding R_F values of the spots.

Analysing in the same way the spots from irradiated DNA (figure 3, column V), the ratios of migrating bases and nucleosides found were about equal to those in the ' effluent '.

3.3. *Damage to the deoxyribose moiety*

In connection with the radiation-induced sugar damage, we have been interested in the formation of malonic dialdehyde under our irradiation conditions. The absorption spectra of thiobarbituric acid was determined with free malonic dialdehyde, with unirradiated DNA, and with irradiated DNA. We obtained the same spectra as published by Kapp and Smith (1970), with a peak at 532 mμ in the case of free malonic dialdehyde and two additional peaks at 457 mμ and 496 mμ in the case of DNA. Applying a dose of 20 krads to a DNA solution (1 mg/ml.), 4·42 mμmoles malonic dialdehyde was produced per ml. corresponding to a G-value of 0·21, which agrees with the value obtained by Kapp and Smith (1970).

This method requires the DNA-solution to be boiled for 20 min in acidic conditions. Since it is known that heating DNA gives rise to breaks in the phosphoester-backbone as well as to an increasing purine release, we decided to analyse for malonic dialdehyde in addition at room temperature, using the method introduced by Scherz *et al.* (1967) with methylindole. Figure 4 shows the spectrum of methylindole after reaction with tetraethoxypropan for calibration as well as after reaction with DNA solutions. Maximum absorption was measured at 555 mμ. Not until 2–3 hours after reaction did an additional peak

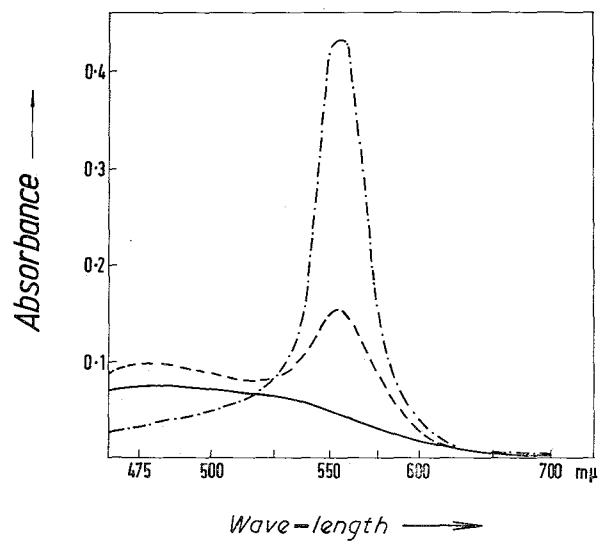

Figure 4. Absorption spectra obtained from the methylindole reaction. ——— unirradiated DNA (500 μg/ml.); – – – irradiated DNA (500 μg/ml., 1 Mrad); –·–·–·– Malonic dialdehyde (13·3 mμmoles in the reaction mixture) $\epsilon = 8{\cdot}6 \times 10^4$ l. mole^{-1} cm^{-1}. The spectrum was taken 20 min after mixing with the reagent. For quantitative measurements of malonic dialdehyde in irradiated DNA the absorption was read at 555 mμ, unirradiated DNA was taken as basis.

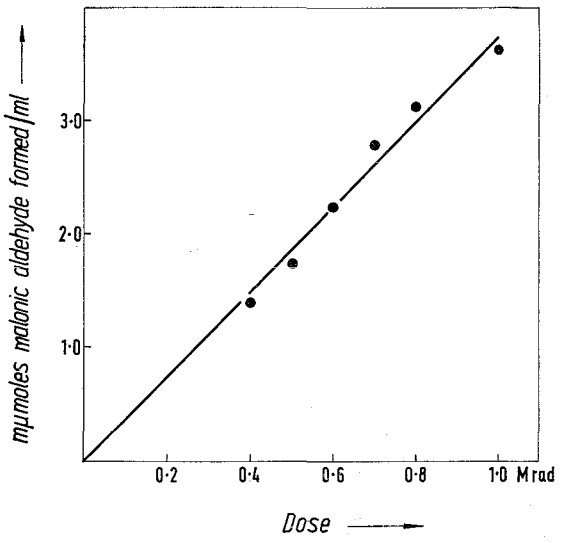

Figure 5. Formation of malonic dialdehyde in irradiated DNA, determined with methylindole according to Scherz *et al.* (1967).

at 475 mμ appear. Quantitative recording at 555 mμ resulted in the linear dose-response curve of figure 5. From the slope of this line a *G*-value of 0·004 for malonic dialdehyde formation (G_{MA}) is calculated.

4. Discussion

The results presented contribute to our knowledge of the nature of the chemical reactions leading to radiation-induced breakage in the DNA nucleotide chain. Free bases as well as nucleosides are liberated from the irradiated DNA. These nucleosides, however, show partly chromatographic behaviour differing from that of intact nucleosides. As the same behaviour, i.e. sticking to the starting point, is seen also with irradiated nucleosides or irradiated deoxyribose, we assume that in the nucleoside fraction obtained from the irradiated DNA the sugar is damaged, possibly by an oxidation reaction. These altered nucleosides represent about one third of the total amount of charge-free compounds. In table 2 the *G*-values for these radiation-induced events in DNA are summarized.

Treating the irradiated DNA with alkali, a slightly higher amount of charge-free compounds is eluted resulting in a *G*-value of 0·18. The difference to the corresponding *G*-value in neutral conditions, however, is not significant. In table 2 the *G*-values too are summarized as recently obtained for the number of single-strand breaks as well as for the frequency of 5′-P-endgroups (Bopp and Hagen 1970). These data allow discussion of some mechanisms leading to chain breakage in DNA:

1. Loss of one nucleoside clearly leads to a splitting of the polynucleotide chain. This is independent of whether the nucleoside contains a damaged sugar or not. From the *G*-value of 0·12 after alkaline treatment one can deduce that 30 per cent of the total number of breaks ($G_{ssb}=0{\cdot}4$) can be explained by this mechanism, whereby also 5′-phosphate groups are formed.

	In neutral conditions	In alkaline conditions
Total amount of U.V.-absorbing material released	0·16	0·18
Free bases	0·06	0·06
Total amount of nucleosides	0·10	0·12
Altered nucleosides	0·06	
Intact nucleosides	0·04	
Malonic dialdehyde	0·004	
Breakage of single strands (*a*,*b*,*c*)	0·25	0·37–0·40
5′-phosphate groups (*a*)	0·20	0·35
5′-OH groups (*a*)	0·02	0·04

(*a*) Bopp and Hagen (1970. (*b*) Hagen (1967). (*c*) Kapp and Smith (1970).

Table 2. *G*-values of some radiation-induced events in DNA.

2. The loss of nucleosides is not sufficient to account for all of the 5′-phosphate groups, detected in the irradiated DNA. We should like to propose, therefore, another mechanism, whereby the sugar is primarily attacked on the carbon 3′ and a subsequent splitting of the phosphoester bond leads to 5′-phosphate groups. 25–30 per cent of the total number of breaks may be formed by this way. We have no information, however, about the nature of the deoxyribose damage.

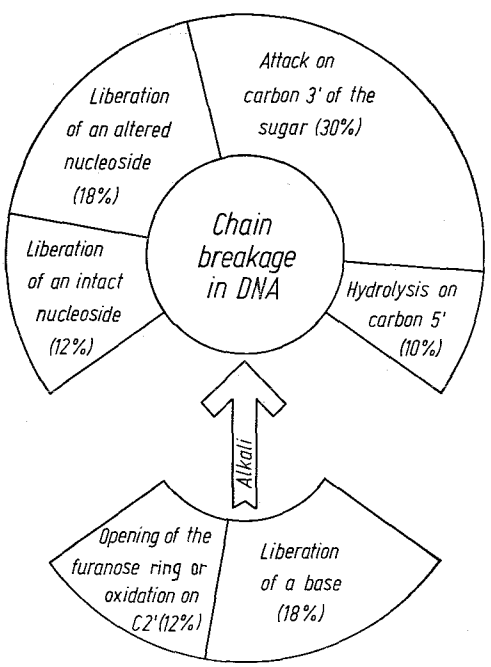

Figure 6. Diagram of the possible reactions involved in the radiation-induced chain breakage in DNA. About 30 per cent of the breaks are induced only after treatment with alkali: breakage occurs mostly by β-elimination. The per cent values are only approximate ones.

3. As 5′-OH groups are rarely formed in irradiated DNA, the hydrolysis of the phosphoester bond on the 5′-end accounts only for about 10 per cent of the chain breakage.

4. About 30 per cent of the total number of breaks are formed by subsequent alkaline treatment of DNA. The liberation of the base may lead to an opening of the furanose ring by a mechanism proposed by Rhaese and Freese (1968). After alkaline treatment, there is a splitting of the phosphoester bond by β-elimination (Bayley, Brammer and Jones 1961), whereby 5′-phosphate groups are also formed. This mechanism, however, explains only partly the alkali induced chain breakage, as the G-value of base liberation is lower than the increase in breaks after alkaline treatment (table 2). There may be an opening of the furanose ring without base liberation or an oxidation of the carbon 2′, which originates an alkali labile phosphoester bond. A summary of the possible reactions involved in the chain breakage is given in figure 6.

5. Testing for formation of malonic dialdehyde with methylindole avoiding any heating shows only 1 per cent of the total number of breaks to occur in conjunction with this product. This is in agreement with the results of Hartmann, von Sonntag and Schulte-Frohlinde (1970), who found in the radiolytic products of deoxyribose only negligible quantities of malonic dialdehyde. Heating in the presence of thiobarbituric acid leads to the formation of new products reacting like malonic dialdehyde, which are still bound to the DNA (Kapp and Smith 1970). These compounds cannot, however, explain the primary reactions leading to chain breakage in DNA.

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L'irradiation de l'ADN en solution aqueuse entraîne la libération de bases et d'autres composés réagissant comme des nucléosides. Ces produits peuvent être séparés de l'ADN irradié par chromatographie sur DEAE-Sephadex. Leur taux augmente linéairement avec la dose; on en déduit une valeur de G entre 0,16 et 0,18. La séparation ultérieure de ces produits par chromatographie sur résine échangeuse d'ions (Dowex 50×4) et sur couche mince de cellulose montre que 60 pour cent d'entre eux sont des nucléosides dont une partie contient des lésions du sucre. Le dialdéhyde malonique dans l'ADN irradié a été déterminé par différentes méthodes. On peut en conclure que ce type de lésion du sucre contribue peu à la scission de la chaîne polydésoxyribonucléotidique. Plusieurs mécanismes de formation des cassures monofilaires dans l'ADN sont discutés.

Bei der Bestrahlung wässriger Lösungen von DNS werden Basen und andere Verbindungen freigesetzt, die eine ähnliche Reaktion zeigen wie Nucleoside. Diese Produkte können von der bestrahlten DNS mittels Chromatographie an DEAE-Sephadex getrennt werden. Ihre Menge nimmt mit steigender Dosis linear zu. Der errechnete G-Wert beträgt 0,16 bis 0,18. Eine weitere Auftrennung der Komponenten durch Ionenaustausch- und Dünnschichtchromatographie zeigt, daß 60 Prozent Nucleoside sind, von denen ein Teil am Zucker geschädigt ist. Mit verschiedenen Methoden wurde Malonaldehyd in bestrahlter DNS-Lösung nachgewiesen mit der Schlußfolgerung, daß diese Form des Zuckerschadens nicht wesentlich zur Bruchentstehung beiträgt. Verschiedene Mechanismen zur Entstehung des Einzelstrangbruches in der DNS werden diskutiert.

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