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Purified, dry DNA and dry nucleoprotein were irradiated with ^{60}Co-γ-rays under a nitrogen or oxygen atmosphere. The DNA was isolated from the irradiated nucleoprotein. In the DNA the following radiation induced changes were investigated: Double strand breaks, single strand breaks and crosslinks between the DNA molecules. An oxygen effect (OER > 1) was found for all of these events except for crosslinks in irradiated DNA. In the nucleoprotein, the oxygen effect is more marked than in pure DNA.

The radiosensitivity of many kinds of living cells has been shown to be higher by a factor of 2 – 4 in oxygen as compared to nitrogen [1–3]. In some cell types, however, the sensitization of cell inactivation by oxygen is much lower and the oxygen enhancement ratio (OER) approaches 1. These cell types are radiosensitive mutants of the more radioresistant wild type of various bacterial strains [4–6]. It was suggested by ALPER [4, 5] that cell inactivation is due to two types of damage: type O and type N. According to this model the type O lesion is mainly or wholly responsible for the oxygen effect and type N is supposed to be more easily repaired or by-passed by some strains than others. In this way the model explains the experimental finding that radiosensitive strains with a low capability of repair show a low OER-value for survival.

In order to understand the mechanism leading to these two types of damage, it is, of course, necessary to find out if a corresponding oxygen effect can be detected in radiation-induced molecular changes of biological macromolecules. Investigations carried out so far do not permit unequivocal conclusions.

The radiation effect on cell membranes shows very high OER values in the order of 5 – 10 (l. c. [7]). Proteins, e. g. ribonuclease, irradiated in the dry state show an increased sensitivity under oxygen [8], whereas in dilute aqueous solution, only a small oxygen effect is measured [9, 10]. Numerous investigations have been made about the oxygen effect on DNA. Recent summaries [11, 12] show the results to be contradictory concerning the radiation effect on DNA in irradiated bacteriophages or cells. In experiments with isolated DNA [13] also no satisfactory evidence was found whether the radiation damage in DNA is enhanced by oxygen or not. Consequently, we decided to reexamine the effect of oxygen on pure dry thymus DNA irradiated in N_2 or in O_2. As criteria for the radiation effect, double and single strand breaks were measured as well as crosslinks formed between the DNA molecules. Moreover, dry nucleoprotein was irradiated. The DNA was separated after irradiation from the protein and examined for radiation induced alterations. In this way the effect of the surrounding protein coat on the radiosensitivity of DNA can, to a certain degree, be tested.

Materials and Methods

1. Isolation of DNA and preparation of nucleohistone

DNA was isolated from calf thymus with duponol according to the method of KAY et al. [14]. In addition, the DNA solutions were treated with trypsin

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[1] T. ALPER, Radiat. Res. **5**, 573 [1956].
[2] T. ALPER, Mutation Res. **4**, 15 [1967].
[3] P. HOWARD-FLANDERS, Advances biol. med. Physics **6**, 553 [1958].
[4] T. ALPER, Nature [London] **217**, 862 [1968].
[5] T. ALPER: Proc. sec. symp. on microdosimetry, ed. H. G. EBERT, p. 5, Comm. Europ. Communities, Brussels 1970.
[6] D. R. DAVIES, Nature [London] **215**, 829 [1967].
[7] D. K. WATKINS, Advances biol. med. Physics **14**, in press.
[8] W. GÜNTHER and H. JUNG, Z. Naturforsch. **22 b**, 313 [1967].
[9] T. BRUSTAD, Radiat. Res. **27**, 456 [1966].
[10] H. JUNG and H. SCHÜSSLER, Z. Naturforsch. **21 b**, 224 [1966].
[11] G. P. VAN DER SCHANS and J. BLOK, Int. J. Radiation Biol. **17**, 25 [1970].
[12] D. C. DEAN, M. G. ORMEROD, R. W. SERIANI, and P. ALEXANDER, Nature [London] **222**, 1042 [1969].
[13] U. HAGEN and H. WELLSTEIN, Strahlentherapie **128**, 565 [1965]; J. T. LETT and P. ALEXANDER, Radiat. Res. **15**, 159 [1961].
[14] E. R. M. KAY, N. S. SIMMONS, and A. L. DOUNCE, J. Amer. chem. Soc. **74**, 1724 [1952].

(Worthington BC, Code TRSF) for 24 hrs at 20 °C (0.01% in 0.08 M Tris buffer, pH 7.8). The DNA was precipitated with ethylalcohol and dried with acetone and diethylether. The protein content of the DNA fibers was less than 0.5% (determined according to LOWRY et al.[15]).

The nucleoprotein was isolated by re-precipitation of nucleohistone obtained from Worthington BC (Code NHL). To remove the salt present in the preparation, about 800 mg of nucleohistone were dissolved in 320 ml 1 M NaCl over night, homogenized and precipitated again by adding H_2O to a final concentration of 0.14 M NaCl. The nucleoprotein fibers were washed in alcohol and dried with acetone and ether. According to WEINERT[16] nucleoprotein prepared in this way contains about 35–40% DNA, 45–47% protein and about 15% water and salt. Histone amounts to three quarters of the overall protein content; the rest is the so called *acid protein*. The RNA content is negligibly small.

2. *Irradiation conditions*

Samples of 10 mg of pure DNA or 20 mg of nucleoprotein were weighed into glass ampoules and evacuated to 10^{-2} to 10^{-3} Torr over night in order to reduce the water content of the preparations as far as possible. After filling with twice purified nitrogen or oxygen the ampoules were sealed. In this way a large number of samples could be prepared simultaneously ensuring identical experimental conditions within one series of experiments. The samples were irradiated with a ^{60}Co-γ-source (Gammacell 220, Atomic Energy of Canada Ltd.) at 0 °C at a dose rate of 1.15 Mrad/h using doses from 1 to 16 Mrad for nucleoproteins and doses of 0.3 to 5 Mrad for thymus DNA. After irradiation the DNA was dissolved in 20 ml of 0.01 M NaCl over night and shaken gently for 2 hours. The DNA content of the solution was determined by measuring the absorption at 260 nm. To isolate the DNA from the irradiated nucleoprotein, the latter was dissolved in 10 ml 0.1 mM NaEDTA over night and treated with trypsin as described above. From this solution the DNA was isolated with phenol as described by COLTER et al.[17]. The protein content of the DNA obtained was between 1.17 and 5.6 percent.

3. *Determination of the molecular weight of native and denatured DNA*

The sedimentation coefficients of the native DNA samples were determined in 0.2 M NaCl + 0.025 M phosphate buffer (pH 7.3). The denaturation of the DNA was performed by the method of DAVISON et al.[18] and the denatured DNA was dissolved in 0.2 M NaCl + 0.025 M phosphate buffer, pH 7.3 + 2% CHOH. An analytical ultracentrifuge (Spinco E, Beckman Instr.) with a scanning system (17 000 and 24 000 rev/min, absorption at 265 nm) was used to investigate the DNA samples at a concentration of about 20 μg/ml. The concentration distribution of DNA within the centrifuge cell was converted into an integral distribution of the sedimentation coefficients S_i^0 (l. c.[19, 20]) and transformed into a molecular weight distribution by means of an equation given by EIGNER and DOTY[21]. In this way it was possible to calculate the weight average molecular weight and the number average molecular weight (M_w and M_n). For each radiation dose four evaluations were carried out. Details of the method have previously been described[20].

4. *Calculation of breaks and crosslinks*

4.1. Soluble DNA: The various radiation induced lesions in DNA are denoted by the following symbols:

B_1: Frequency of single strand breaks per nucleotide after dose D. p_1: Probability of single strand breaks, i. e. $B_1 \cdot \mathrm{rad}^{-1}$ assuming B_1 to increase linearly with dose:

$$B_1 = p_1 \cdot D \,. \tag{1}$$

B_2: Frequency of double strand breaks per nucleotide pair after dose D. p_2: Probability of double strand breaks, i. e. $B_2 \cdot \mathrm{rad}^{-1}$ assuming B_2 to increase linearly with dose:

$$B_2 = p_2 \cdot D \,. \tag{2}$$

C_2: Frequency of crosslinks between double-stranded molecules per nucleotide pair after dose D, i. e. number of crosslinked units per nucleotide pair. q_2: Probability of crosslinks between double,stranded molecules, i. e. $C_2 \cdot \mathrm{rad}^{-1}$ assuming C_2 to increase linearly with dose:

$$C_2 = q_2 \cdot D \,. \tag{3}$$

The frequencies of breaks and crosslinks in a DNA sample can both be determined if M_w and M_n are known. We used the formulae (4 and 5) indicated by CHARLESBY[22]:

$$1/M_n = 1/(M_n)_0 + (B - C/2)/m \,, \tag{4}$$

$$1/M_w = 1/(M_w)_0 + (B/2 - C)/m \,, \tag{5}$$

where m = molecular weight of the monomer, i. e. of an average nucleotide in a single strand or of an average nucleotide pair in a double strand. $(M_w)_0$ and $(M_n)_0$ are the average molecular weights of the unirradiated controls.

From these equations B and C are easily obtained:

$$B = 2\,m[2/M_n - 1/M_w - 2/(M_n)_0 + 1/(M_w)_0]/3 \,. \tag{6}$$

$$C = 2\,m[1/M_n - 2/M_w + 2/(M_w)_0 - 1/(M_n)_0]/3 \,. \tag{7}$$

[15] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, J. biol. Chemistry **193**, 265 [1951].
[16] H. WEINERT, Dissertationsschrift, Freiburg (Br.) 1967.
[17] J. S. COLTER, Z. A. BROWN, and K. A. O. ELLEM, Biochim. biophysica Acta [Amsterdam] **55**, 31 [1962].
[18] P. F. DAVISON, D. FREIFELDER, and B. HOLLOWAY, J. molecular Biol. **8**, 1 [1964].
[19] H. WEINERT and U. HAGEN, Strahlentherapie **136**, 204 [1968].
[20] TH. COQUERELLE, L. BOHNE, and U. HAGEN, Z. Naturforsch. **24b**, 885 [1969].
[21] J. EIGNER and P. DOTY, J. molecular Biol. **12**, 549 [1965].
[22] A. CHARLESBY: Atomic Radiation and Polymers, Pergamon Press, Oxford, London, New York 1960.

4.2. DNA samples with an insoluble gel fraction: By irradiation of dry DNA an insoluble gel is formed, i. e. when this DNA is suspended in 0.01 M NaCl only part of it is dissolved. The percentage of the insoluble fraction (gel) can be determined through the extinction at 260 nm before and after centrifugation of the DNA solutions (15 min at $8000 \times g$). The soluble fraction of the DNA, denoted by s, was determined after various doses. The possibility that the extinction observed before centrifugation might be enhanced by the scattering action of the large aggregates in the gel could be excluded. Even after high doses, the extinction of the DNA-solutions always showed the value corresponding to the weighed amount of DNA. According to CHARLESBY and PINNER [23] p_2 and q_2 can be calculated from the relation of s to the dose using the equation:

$$s+s^{1/2}=p_2/q_2+m[q_2\cdot(M_n)_0\cdot D]^{-1}. \qquad (8)$$

A straight line is obtained plotting $(s+s^{1/2})$ *versus* $1/D$, whose intersection with the ordinate indicates the ratio p_2/q_2. From the slope of the line q_2 can be calculated, p_2 results from q_2 and the ratio p_2/q_2.

Results

1. Radiation effects on pure dry DNA

1.1. Native DNA: Irradiation of dry thymus DNA resulted in the formation of an insoluble gel, i. e. the DNA molecules were so frequently crosslinked, that partly a three-dimensional insoluble structure had been formed. In this case the frequency of breaks and crosslinks in the whole fraction was determined using the method described in section 4.2. Fig. 1 a shows the dependence of gel formation on the radiation dose. The values for the soluble fraction s are plotted against the dose. Under N_2 there is more insoluble gel than under O_2. However, even after high doses a certain number of DNA molecules were soluble indicating breaks besides the crosslinks. In Fig. 1 b, $(s+s^{1/2})$ is plotted against $1/D$ and the slope of the lines in the Fig. was determined by regression analysis. The values for p_2 and q_2 as well as their standard error are calculated according to Eq. (8) from these data (Table 1). The double breaks show a marked oxygen effect (OER$\approx$2) whereas for crosslinks but a small effect of this type (OER = 1.3) was observed.

1.2. Denatured DNA: After the denaturation procedure applied to the DNA samples irradiated in the dry state even the molecules of the gel

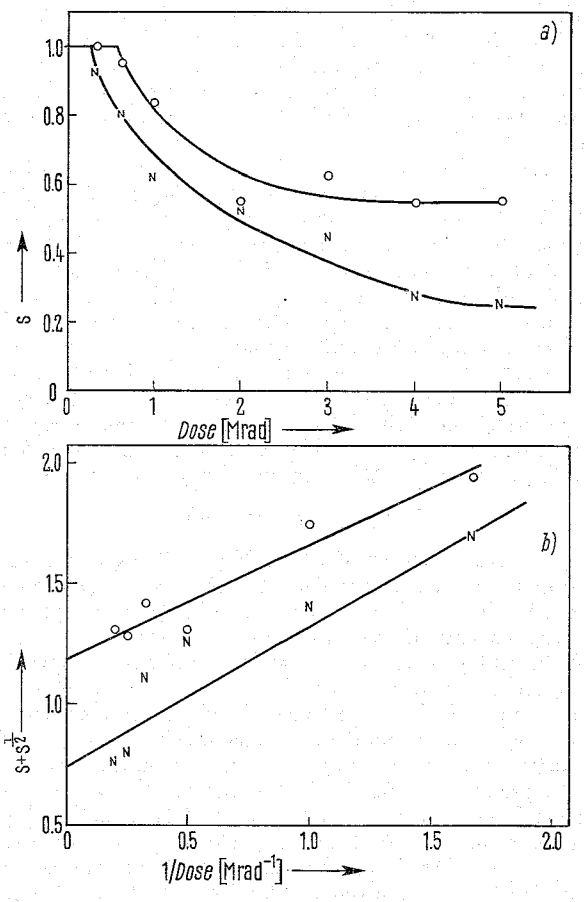


Fig. 1. a) Fraction of the soluble DNA molecules (s) after irradiation of purified dry DNA as a function of dose. N: Irradiation under nitrogen, O: Irradiation under oxygen. Average values of 2 measurements. b) Plot of $(s+s^{1/2})$ against the reciprocal dose $(1/D)$ according to CHARLESBY and PINNER [23]. The slope of the curves were calculated by regression analysis.

Sample	$p_1 \cdot 10^{11}$	$p_2 \cdot 10^{11}$	$q_2 \cdot 10^{11}$	p_2/q_2
DNA in nitrogen	23 ± 3	8.3 ± 2.2	11.2 ± 3.1	0.75
DNA in oxygen	45 ± 3	17 ± 3.3	14 ± 2.8	1.2
NP in nitrogen	51 ± 5	4.7 ± 0.3	2.1 ± 0.4	2.2
NP in oxygen	170 ± 14	24 ± 2	4.8 ± 1.2	5.0

Table 1. Probability of single strand breaks (p_1), double strand breaks (p_2) and crosslinks (q_2) in DNA generated by γ-irradiation of dry DNA and dry nucleoprotein (NP), respectively ($\pm$S.E., n=16–28).

fraction were separated into single strands and became soluble. Thus, radiation induced single strand breaks could easily be determined by sedimentation analysis (cf. methods, 4.1). B_1 ist plotted against the dose (Fig. 2) and the probability of breaks p_1 is calculated from the mean slope (Table 1). There is again a distinct oxygen effect.

[23] A. CHARLESBY and E. H. PINNER, Proc. Roy. Soc. [London] Ser. A **249**, 367 [1959].

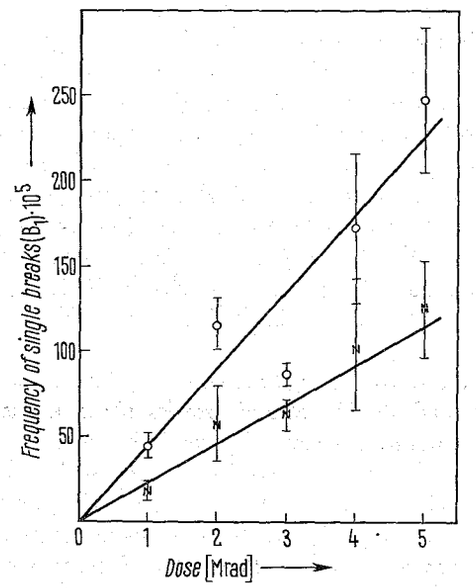
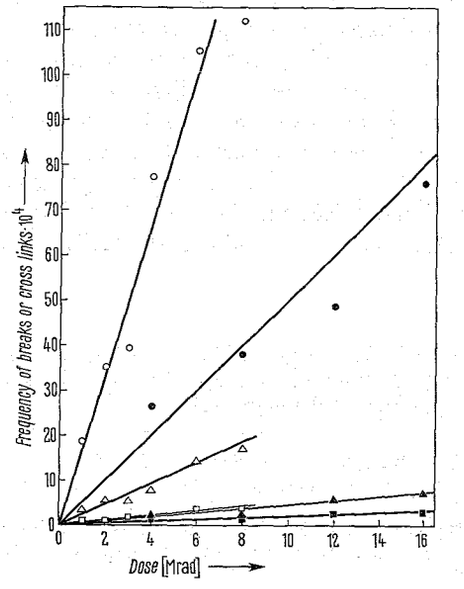


Fig. 2. Frequency of single strand breaks in DNA, irradiated in the dry state under nitrogen (N) or under oxygen (O) (± S.E., $n=4$).



Fg. 3. Frequency of breaks and intermolecular crosslinks in the DNA of irradiated nucleoprotein. Irradiation was performed under nitrogen (closed symbols) or under oxygen (open symbols). Average values of 4 measurements: ▲ Frequency of double strand breaks (B_2) under N_2; △ Frequency of double strand breaks (B_2) under O_2; ● Frequency of single strand breaks (B_1) under N_2; ○ Frequency of single strand breaks (B_1) under O_2; ■ Frequency of crosslinked units (C_2) under N_2; □ Frequency of crosslinked units (C_2) under O_2.

2. Radiation effects on DNA irradiated as nucleoprotein

Even after high doses the whole DNA of the irradiated nucleoprotein could be dissolved. The determination of the molecular weight could, therefore, be performed according to the methods outlined in 3 and 4.1. For native as well as denatured DNA the frequencies of breaks and crosslinks show a linear increase with dose (Fig. 3). Table 1 contains the values of the probability for breaks and crosslinks determined from the slope of the straight line. All radiation lesions show a marked oxygen effect.

Discussion

1. Breaks and crosslinks in irradiated DNA

From the probability for the various lesions in DNA to occur per rad (cf. Table 1) their G-values were calculated, i. e. the number of events per 100 eV absorbed energy (Table 2). In calculating the G-values in nucleoprotein we took into account the energy absorbed in the DNA only.

Sample	Single strand breaks Gp_1	Double strand breaks Gp_2	Crosslinks Gq_2
DNA in nitrogen	0.72 ± 0.09	0.13 ± 0.03	0.18 ± 0.05
DNA in oxygen	1.4 ± 0.1	0.27 ± 0.05	0.22 ± 0.04
NP in nitrogen	1.6 ± 0.2	0.07 ± 0.01	0.03 ± 0.01
NP in oxygen	5.3 ± 0.4	0.37 ± 0.03	0.08 ± 0.02

Tab. 2. G-values for various lesions in DNA generated by γ-irradiation of dry DNA and dry nucleoprotein (NP), respectively.

Single strand breaks are produced more frequently in the nucleoprotein than in pure DNA. Possibly, the energy absorbed in the protein contributes to their formation by radical site migration. Double strand breaks occur in the nucleoprotein with a similar probability as in DNA and show a linear increase with dose. This indicates, that a double strand break is produced by one single hit and not by a random coincidence of two independent single breaks, as has been observed after irradiation of DNA in dilute aqueous solution [24].

Intermolecular crosslinking of the double strand occurs much less frequently in the nucleoprotein

[24] U. HAGEN, Biochim. biophysica Acta [Amsterdam] **134**, 45 [1967].

than in pure DNA (Table 2). The calculated ratios p_2/q_2 (Table 1) are in agreement with our observations about the gel formation. According to CHARLESBY [23], an insoluble gel is only formed, if $p_2/q_2 < 2$. This is the case only in irradiated dry pure DNA, not in irradiated nucleoprotein. The G-values for crosslinks (G_q) measured in DNA are much lower than those determined in irradiated synthetic polymers or proteins, where a G_q-value of about 1 is found in most cases (CHARLESBY [25]). This high probability of crosslinking may be explained by assuming the radical sites in irradiated synthetic polymers to migrate until they recombine with another one [26]. Possibly such radical site migration is impeded in the DNA molecules.

2. *Oxygen effect*

From the probability of the various events (Table 1) the oxygen enhancement ratios (OER) and their statistical errors (cf. PARRAT [27]) were calculated (Table 3). In dry pure DNA we found a significant sensitization in the presence of oxygen for single and double strand breaks, whereas the frequency of crosslinks was not significantly altered by oxygen.

radiation effect	DNA	Nucleoprotein
single strand breaks	2.0 ± 0.3	3.3 ± 0.4
double strand breaks	2.0 ± 0.7	5.1 ± 0.6
crosslinks	1.3 ± 0.4	2.3 ± 0.7

Table 3. Oxygen enhancement ratios (OER) for the various radiation effects in DNA, irradiated as dry pure DNA or as nucleoprotein.

In dry nucleoprotein, there is a distinct oxygen effect for all radiation lesions of DNA studied. The OER-values are higher than in pure DNA, possibly because of some protective action exerted by the protein coat. This effect of the surrounding protein might be compared to experiments by HUTCHINSON [28], where the inactivation of transforming DNA was tested after X-irradiation. Irradiating DNA solutions no oxygen effect is observed; however, adding glutathion (1.4 mM final concentration) to the DNA solution leads to sensitization by oxygen (OER = 3.5). An oxygen effect is also found, when the transforming DNA is irradiated in lysed bacteria (OER = 3.0) or in fresh bacteria (OER = 3.0) (l. c. [29]).

In the DNA of irradiated nucleoprotein, not only single and double strand breaks show a distinct oxygen effect, but also the crosslinks. Although the ratio of breaks to crosslinks is higher in oxygen ($p_2/q_2 = 5.0$) than in nitrogen ($p_2/q_2 = 2.2$), the absolute amount of crosslinks increases in the presence of oxygen by a factor of about 2.5. Similar observations were made on irradiated protein. Ribonuclease was separated into denatured and aggregated molecules after γ-irradiation in vacuo or in oxygen (HASKILL and HUNT [30]). The ratio of denatured to aggregated products is higher in oxygen (2.0) than *in vacuo* (1.0), the absolute amount of aggregated products, however, is also higher in oxygen than *in vacuo*. From their data, an OER-value for the formation of denatured products of 6.4 can be calculated and for the formation of aggregated products of about 3.3. Similar results on ribonuclease were also obtained by JUNG and SCHÜSSLER [31].

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[25] A. CHARLESBY, in: Radiation Research, ed. G. SILINI, North-Holland Publ. Comp., Amsterdam 1967, p. 265.
[26] D. O. GEYMER, Makromolekulare Chem. **100**, 186 [1967].
[27] L. G. PARRAT: Probability and experimental error in science. An elementary survey, Wiley, New York 1961.
[28] F. HUTCHINSON, Radiat. Res. **14**, 721 [1961].
[29] F. HUTCHINSON and J. ARENA, Radiat. Res. **13**, 137 [1960].
[30] J. HASKILL and J. W. HUNT, Radiat. Res. **32**, 606 [1967].
[31] H. JUNG and H. SCHÜSSLER, Z. Naturforsch. **21 b**, 224 [1966].