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Production of free radicals in DNA and inactivation of its biological activity by gamma-rays

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The biological significance of damage induced in DNA by ionizing radiation is quantitatively compared with free radical concentration as measured by E.S.R. spectroscopy. Biologically-active DNA of the bacteriophage ΦX174 dried together with carrier DNA was irradiated with γ-rays under vacuum. E.S.R. spectra were recorded at room temperature with a double-cavity spectrometer. After dissolution of the samples the biological activity of the ΦX174 DNA was measured. Absolute spin concentrations were measured with an accuracy of 25 per cent. The G-values for biological inactivation and for production of free spins were 1·3 and 3·2 (±10 per cent). Inactivation curves were exponential up to at least 5 Mrads; radical concentration, however, became saturated at about 2 Mrads. Cysteamine protected to the same extent against inactivation and free spins.

1. Introduction

The quantity and nature of the radicals produced in DNA by ionizing radiation have been investigated by many authors. Since spectra of irradiated DNA may vary considerably, depending on the mode of sample preparation (Wyard 1969), a quantitative correlation between biological damage and radical content is possible only if E.S.R. measurements and biological assays are carried out with the same samples. Only recently free radical spectra and survival have been measured simultaneously after irradiation, using bacteria (Swartz and Richardson 1967) and spores (Dodd and Ebert 1969). These systems have the disadvantage that radicals are produced in both DNA and other cell constituents. The latter are probably not lethal, which makes a quantitative correlation of radical yield and survival difficult. These problems have been circumvented in this work by using the biologically-active DNA of the bacteriophage ΦX174. Since preparation of large quantities of ΦX174 DNA for E.S.R. measurements is very time-consuming, calf-thymus DNA was added as a carrier. Although it cannot be excluded with certainty that radicals induced in ΦX174 DNA differ from those in calf-thymus DNA, the similar base composition of the DNA's (Chargaff 1955, Sinsheimer 1959 b) and the similarity of spectra obtained from single- and

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double-stranded material make such a difference unlikely. Moreover, the similar radiosensitivity of ΦX174 DNA in our mixtures and that of pure dry ΦX174 DNA (Hotz and Müller 1968, Ginoza 1963) seems to exclude preferential radical transfer from the carrier to the ΦX174 DNA. It will therefore be assumed that the radical spectra obtained are good substitutes for those that will be obtained with pure ΦX174 DNA.

2. Materials and methods

Calf-thymus DNA was purchased from Mann Research Laboratories, cysteamine·HCl from EGA-Chemie KG (SH-content 96 per cent as determined iodometrically). DNA of bacteriophage ΦX174 was prepared according to Sinsheimer (1959 a,b). Neither inactivation of ΦX174 DNA nor the E.S.R. signals depended on the strandedness of the carrier DNA (see also Krsmanovic-Simic, Van der Vorst and Richir 1966). Because single-stranded DNA shows a rather strong competition in the assay of biological activity, native calf-thymus DNA was used as carrier.

A solution of ΦX174 DNA (10 μg/ml.) and calf-thymus DNA (2 mg/ml.) in 10^{-2} M phosphate buffer, pH 7·1, was heated 5 min at 60° c to ensure homogeneous mixing. Aliquots of 0·5 ml. were freeze-dried at 10^{-3} torr with the cooling trap at −80°c, transferred quantitatively to a Varian quartz tube, flushed with dry N_2, freeze-dried for another 24 hours and sealed under vacuum. Samples were irradiated at room temperature with ^{60}Co gamma-rays (Atomic Energy of Canada 'Gammacell 100', dose-rate 56 rads/sec). After irradiation, colour centres in one-half of the tube were bleached by a flame. Neither the biological activity of the DNA in the other part of the tube nor the DNA radicals were affected by this procedure.

After transferring the DNA to the bleached part of the tube, E.S.R. spectra were recorded at room temperature with the Varian V 4500 A spectrometer of the Chemical Department of the University of Technology, Eindhoven. Spectra were stable for at least one month. To ensure an accurate quantitative relation between radical yield and inactivation, the number of induced radicals was measured very carefully. For calibration a dual sample cavity TE 104 was used. The absolute number of spins was determined with a 'point' sample DPPH (diphenylpicryl-hydrazyl). Channel sensitivity, compression of the r.f. field by sample and sample holder and signal height as a function of the dimensions of the sample and its location inside the cavity were measured according to Casteleijn, Ten Bosch and Smidt (1968). Signal level settings of the spectrometer were calibrated and the power saturation behaviour of the spectra was investigated.

The maximum overall error of the absolute spin concentration calibration procedure amounted to 25 per cent. This error was further reduced to about 10 per cent by averaging over at least three measurements with different DNA preparations.

After the E.S.R. measurements, the DNA was dissolved in 10^{-2} M phosphate buffer, and the biological activity of the ΦX174 DNA was determined according to Guthrie and Sinsheimer (1963). The yield of phage was proportional to the concentration of ΦX174 DNA if the concentration of calf-thymus DNA+ ΦX174 DNA did not exceed 100 μg/ml. Concentrations higher than 10 μg/ml. were used only for very large doses.

3. Results and discussion

The survival curve of ΦX174 DNA (figure 1) was exponential down to at least 10^{-4}. The 37 per cent survival dose (D_{37}) was 550 krads, which is between the values of 800 and 380 krads reported by Hotz and Müller (1968) for ΦX174 dried in broth and for frozen solutions of ΦX174 DNA in broth by Ginoza (1963) respectively. From the D_{37} a G-value for biological activity (G_b), i.e. the number of inactivated molecules per 100 eV dissipated in the ΦX174 DNA, of 1·3 is

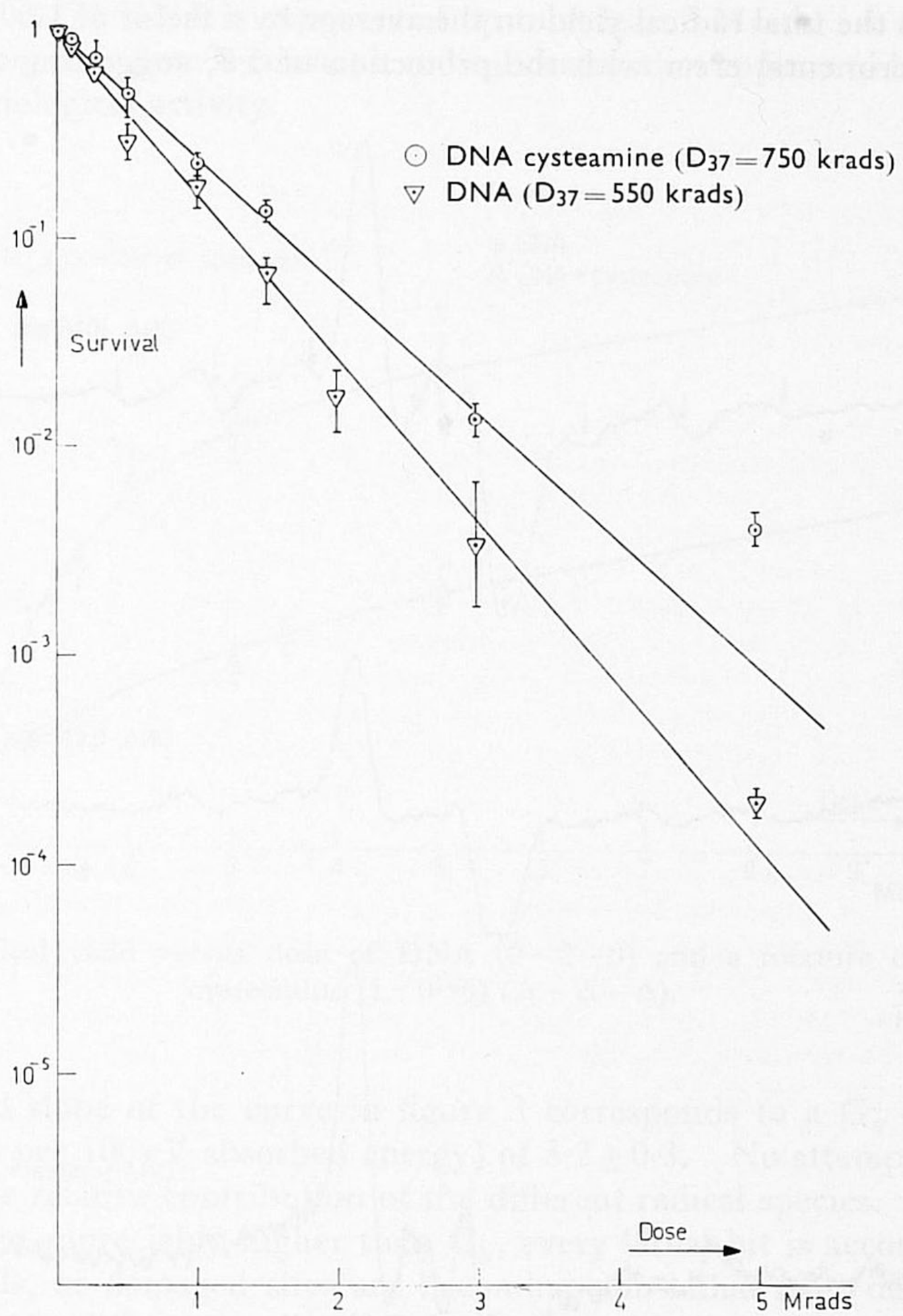

Figure 1. Inactivation of ΦX174 DNA in the absence (▽ – ▽ – ▽) and in the presence of cysteamine (⊙ – ⊙ – ⊙). Concentration of cysteamine in the DNA solutions (2 mg/ml.) before freeze-drying was 10^{-2} M, resulting in 0·56 mg cysteamine/mg DNA. The points are averages of two independent DNA assays. Errors indicate standard errors based on plaque counts.

obtained (Ginoza 1967). The deviation in the slope of the inactivation curves was smaller than 10 per cent for different sample preparations, giving an accuracy of G_b of about 0·1. Figure 2 shows that E.S.R. signals are due to at least two radical species: a weak eight-line spectrum (splitting constant 20·2 Oe) and a

strong central line (g-value $1{\cdot}988 \pm 0{\cdot}001$). The spectra were reproducible for different DNA preparations and agree well with those given in the literature (Wyard 1969 and references therein).

Samples irradiated under dry O_2 (1 atm) showed the same radiosensitivity, analogous spectra and the same radical yield. 56 per cent cysteamine in the samples protected the DNA by a factor of about 1·3 (figure 1), which equals that observed by Hotz and Müller (1968) for ΦX174 DNA dried in broth containing 10^{-1} M cysteamine. It does not affect the shape of the spectrum appreciably, but reduces the total radical yield on the average by a factor of 1·6. This agrees within experimental error with the protection of 1·3, suggesting that effects of

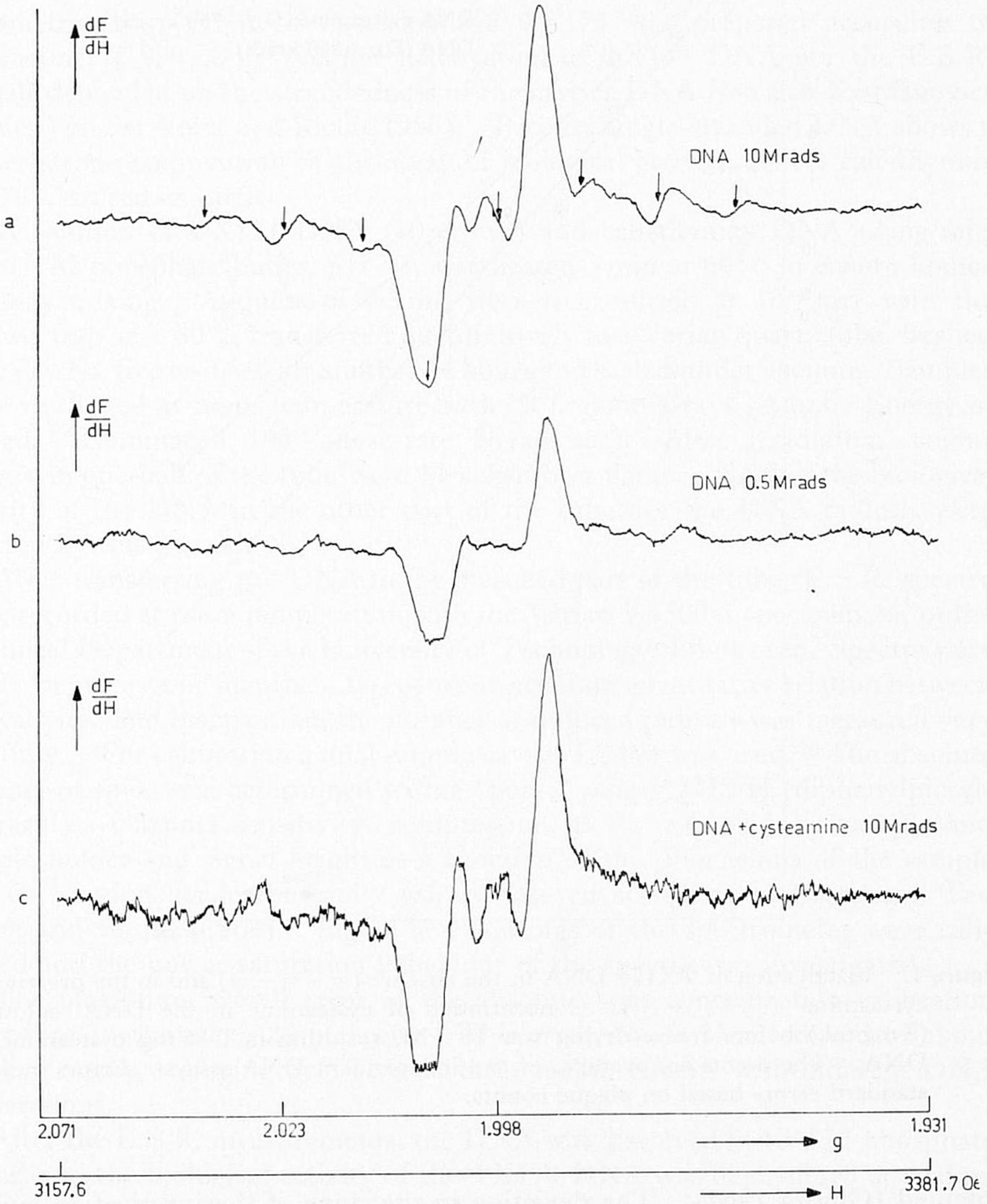

Figure 2. First derivative of E.S.R. signals of DNA. (*a*) 10 Mrads, (*b*) 0·5 Mrads, (*c*) mixture of DNA and cysteamine (1 : 0·56), 10 Mrads. Arrows indicate the positions of the lines of the thymine radical spectrum.

sulphhydryl compounds on the E.S.R. signals indeed correspond with radioprotection. Cysteamine radicals, centred at $g = 2{\cdot}023$, contributed less than 5 per cent to the total radical yield, as calculated from a spectrum of irradiated pure cysteamine.

The total radical yield begins to saturate at 2 Mrads (figure 3), a phenomenon also observed by Köhnlein (1963), Singh and Charlesby (1965) and Swartz and Richardson (1967). It has been attributed to radical destruction by hydrogen atoms formed during irradiation (Horan and Snipes 1969). Since the inactivation curve of the ΦX174 DNA is exponential up to at least 5 Mrads, i.e. the number of lethal hits per unit dose is constant, radical destruction appears to be incapable of restoring biological activity.

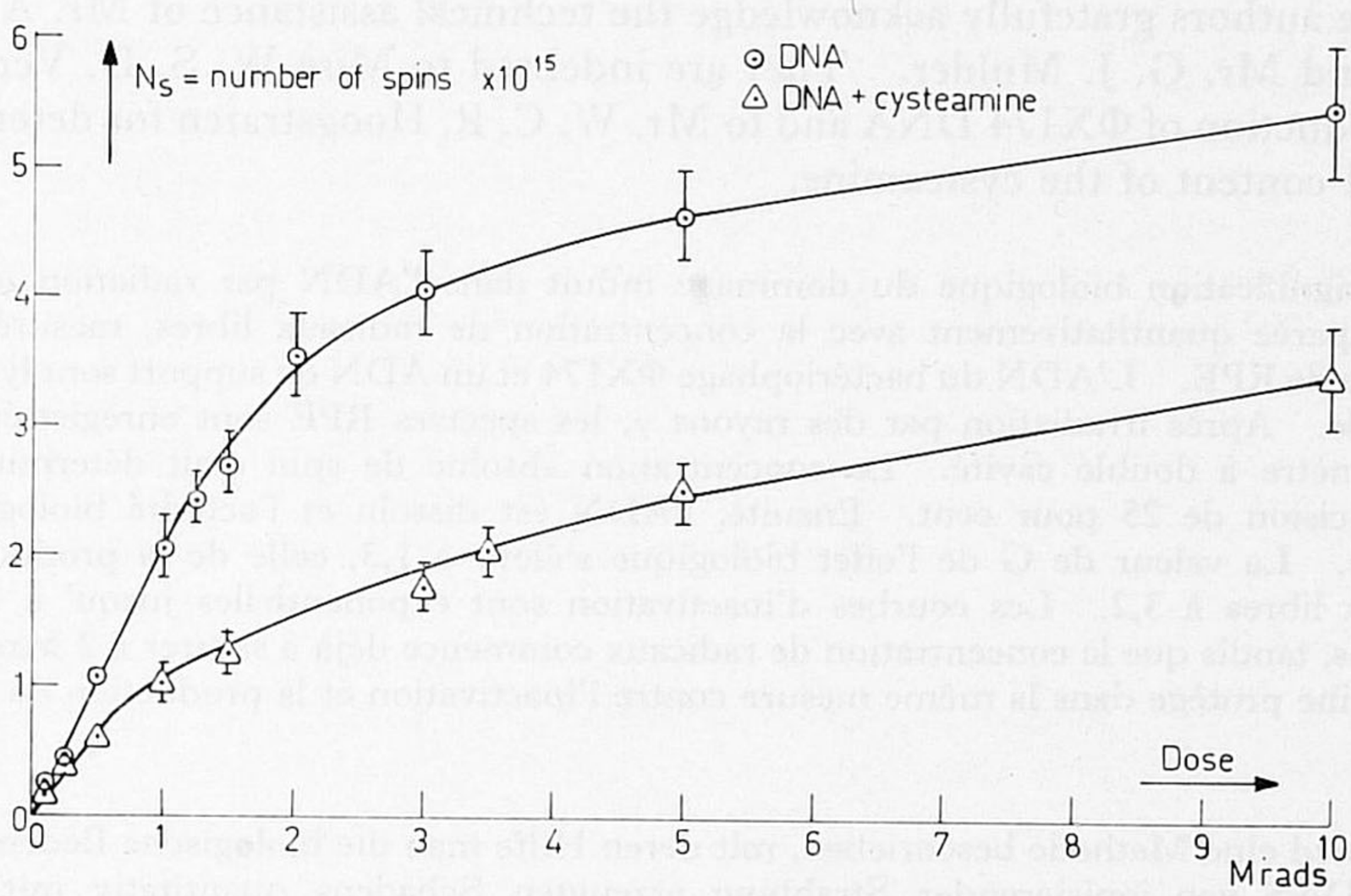

Figure 3. Radical yield versus dose of DNA (0−0−0) and a mixture of DNA and cysteamine (1 : 0·56) (Δ − Δ − Δ).

The initial slope of the curve in figure 3 corresponds to a G_s (number of induced spins per 100 eV absorbed energy) of $3{\cdot}2 \pm 0{\cdot}3$. No attempt was made to estimate the relative contribution of the different radical species.

Since G_s is appreciably higher than G_b, every lethal hit is accompanied by several radicals, or damaged sites are becoming non-lethal after dissolution of the DNA. It is difficult to discriminate between these two possibilities, but there are some arguments in favour of the former. Ion clusters along the track of the ionizing particle are a few ångströms in diameter and lie thousands of ångströms apart (Hutchinson and Pollard 1961). Since the dimensions of a dry ΦX174 DNA molecule will be 1000 Å or less (Sinsheimer 1959 b), every inactivating event will be due to one cluster. Following a primary absorption, on the average one secondary absorption event occurs (Hutchinson and Pollard 1961). Assuming that every ionization contributes a radical pair, G_s is expected to be $2 \times 2 \times 1{\cdot}3 = 5{\cdot}2$. The lower experimental value of about $2 \times G_b$ indicates that recombination of radicals occurs and that on the average only one radical pair is left per primary event. It is interesting to note that Hummel, Allen and

Watson (1966), in their work on the conductivity of insulating liquids during irradiation, arrived at the conclusion that on the average one ion pair was left from each cluster of ionizations. In the solid state these ion pairs may become stable radicals.

If on the average one ion pair survives, in a fraction of the clusters that initially consist of only one ion pair (about 60 per cent of the total number; Rauth and Simpson 1964), no ionizations are left. Since the results of the biological test suggest that every primary ionization is lethal, recombination of radicals seems not to restore biological activity, i.e. the radicals observed can account only for part of the biologically important damage.

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La signification biologique du dommage induit dans l'ADN par radiation ionisante, est comparée quantitativement avec la concentration de radicaux libres, mesurée par la méthode de RPE. L'ADN du bactériophage ΦX174 et un ADN de support sont lyophilisés ensemble. Après irradiation par des rayons γ, les spectres RPE sont enregistrés par un spectromètre à double cavité. La concentration absolue de spin était déterminée avec une précision de 25 pour cent. Ensuite, l'ADN est dissolu et l'activité biologique est mesurée. La valeur de G de l'effet biologique s'élève à 1,3, celle de la production des radicaux libres à 3,2. Les courbes d'inactivation sont exponentielles jusqu' à 5 Mrads au moins, tandis que la concentration de radicaux commence déjà à saturer à 2 Mrads. La cystéamine protège dans la même mesure contre l'inactivation et la production de radicaux libres.

Es wird eine Methode beschrieben, mit deren Hilfe man die biologische Bedeutung des in der DNS von ionisierender Strahlung erzeugten Schadens quantitativ mit der im Elektronenspinresonanz-spektrometer gemessenen Radikalkonzentration vergleichen kann. Die biologisch aktive DNS des Bakteriophagen ΦX174 wird zusammen mit einer Träger-DNS gefriergetrocknet. Nach γ-Bestrahlung werden die E.S.R.-Spektren registriert mit einem Doppelhohlraumspektrometer. Die Genauigkeit der Bestimmung der absoluten Spinkonzentration betrug 25 Prozent. Der biologische G-Wert betrug 1,3, die Radikalausbeute war 3,2. Die Inaktivierungskurven waren exponentiell bis mindestens 5 Mrads, die Radikalkonzentration zeigte jedoch schon bei 2 Mrads eine Sättigung. Der Strahlenschutzeffekt von Cysteamin war sowohl für die biologische als auch für die physikalische Meßmethode gleich.

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