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Quantitative E.S.R.-measurements of radiation-induced radicals in nucleic-acid bases and pentoses

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Cytosine, 5-methylcytosine, 5-hydroxymethylcytosine, thymine, uracil, 5-bromouracil, adenine, hypoxanthine, guanine, xanthine, D-ribose and D-2-deoxyribose have been irradiated as dry crystalline powders at about 100°K and 300°K and investigated by electron-spin-resonance spectrometry. First derivative spectra of each substance were recorded at 9·4 kMc/sec after X- and gamma-irradiation with a whole range of doses up to 10 Mr. Qualitative results include new structural details of spectra confirming the richness of hyperfine structure in methyl containing compounds, but emphasis has been placed on quantitative determinations. *G*-values obtained from the initial linear parts of dose effect curves range from 0·1–1·0 for bases and 4–6 for pentoses after irradiation at room temperature. Although somewhat higher radical yields are often found if irradiations and measurements are executed at low temperature, these yields always decrease on warming-up to room temperature below the values given above.

After irradiation at room temperature, the radical-concentrations in all bases lacking a methyl group deviate from a linear dependence on radiation dose above 0·3 Mr and attain a constant level, although linearity up to at least 1 Mr is observed with bases containing a methyl group and with pentoses. At low temperature linearity is found up to 1 Mr for all bases and pentoses except 5-bromouracil, for which the corresponding value is 0·5 Mr

1. INTRODUCTION

As has already been outlined in the preceding papers (Muller 1964, Müller and Köhnlein 1964), a series of E.S.R.-measurements on irradiated nucleic-acid components was initiated with the emphasis on quantitative investigations on the induced radicals. Nevertheless, there were qualitative results which have not been described in early work on these substances (Shields and Gordy 1959, Shen Pei-Gen, Blyumenfeld, Kalmanson and Pasynskii 1959).

2. EXPERIMENTAL

The experimental procedures used were the same as were described in the preceding paper (Müller and Köhnlein 1964). All bases were obtained as crystalline powders of the highest purity available from the California Corporation for Biochemical Research. In addition cytosine, thymine, adenine, hypoxanthine and the pentoses D-ribose and D-2-deoxyribose (supplied by Nutritional Biochemicals

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Corporation) and thymine (supplied by Fluka, Switzerland and by Roth, Germany) were investigated. Generally, the materials were used without further processing, but thymine was dissolved in water and recrystallized for some of the experiments. All nucleic-acid bases were irradiated in air and in evacuated quartz tubes for investigations at room temperature: irradiations at low temperature were always done *in vacuo*. Pentoses were irradiated in evacuated tubes only.

3. Results and discussion

3.1. *Qualitative observations*

The spectra obtained after irradiation of nucleic-acid bases at room temperature are shown in figures 1 and 2. No differences were found between samples irradiated in air and *in vacuo*. The spectra are generally similar to those published by Shields and Gordy (1959), but display more hyperfine structure, in agreement with the results on nucleotides. Shields and Gordy also failed to observe spectra from irradiated purines, probably because of a lack of sensitivity. Obviously, the purine spectra are always single lines occasionally accompanied by two weak satellites. Results on amino acids suggest that the occurrence of rather narrow single lines and the low yield discussed later are due to the aromatic character of the purines, as expressed for example in the resonant energy of the π-electrons. Aromatic amino acids also show similar singlet spectra and low yields, in contrast

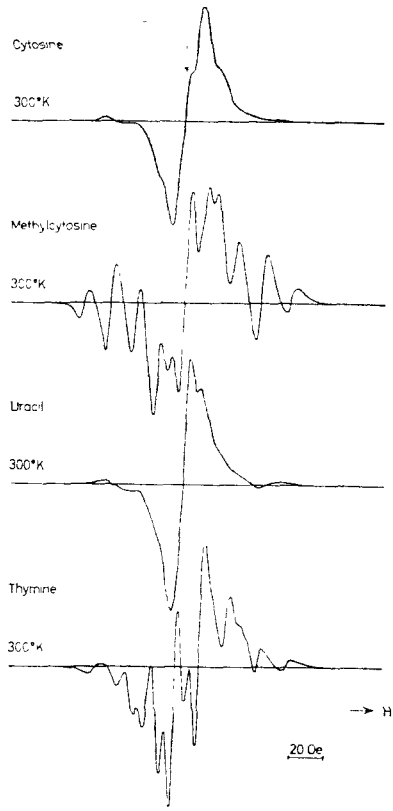

Figure 1. First derivative spectra of pyrimidine bases at room temperature. Arrow indicates $g = 2{\cdot}0036$.

to aliphatic amino acids (Müller, Schambra and Pietsch 1964). Although the spectra of cytosine and uracil contain some hyperfine structure, which is not, however, well resolved, methylcytosine and thymine abound in lines. The obvious reason is the occurrence of a methyl group in the latter compounds. Actually, the hyperfine structure of thymine and methylcytosine had already been attributed to interaction with the methyl group by Shields and Gordy (1959) and by Köhnlein (1963). Nucleic-acid bases obtained from different sources yielded identical results, with the exception of thymine. The spectra obtained from different preparations of thymine are given in figure 3. The spectra are composed of at least two sets of hyperfine lines. One group consists of eight

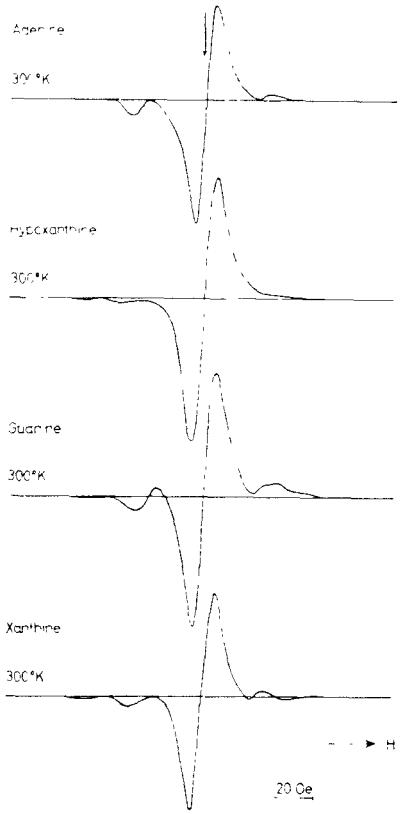

Figure 2. First derivative spectra of purine bases at room temperature. Arrow indicates $g = 2{\cdot}0036$.

main lines plus seven satellites, and the other one of essentially a doublet superimposed on the former. The octet is represented in its purest form in spectrum IV, whereas the doublet is superimposed with variable relative intensity on the other spectra. It was thought that the observed differences might be due to impurities or to different mean sizes of crystals. Spectrum III was in fact obtained from thymine recrystallized from an aqueous solution of thymine, which yielded spectrum II when taken from the bottle. Clearly, the spectra are somewhat different, but no definite association of the differences with particular treatments can be made at present. To exclude the possibility that the different

spectra were due to impurities, samples from all thymine preparations were tested by thin-layer chromatography and subsequent fluoroscopic analysis, but no traces of impurities were found. However, remarkable parallelisms exist between the spectra of thymine, thymidine (Müller and Köhnlein 1964) and thymidine-5'-monophosphate (Müller 1964). Although spectrum IV closely resembles the spectrum of thymidine, a contribution of the doublet spectrum which varies with time after irradiation is observed with the nucleotide.

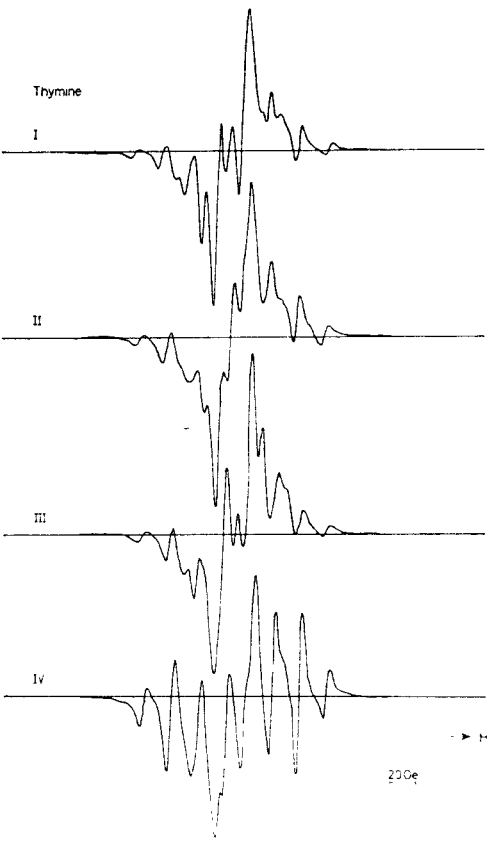

Figure 3. First derivative spectra of different polycrystalline preparations of thymine at room temperature. Samples were supplied by NBC (I), Roth (II and III) and Fluka (IV). Sample III was recrystallized, the others were used without processing.

The spectra recorded after irradiation at low temperature fit well into the pattern described (figures 4–8). Generally, less structure is observed at 100 °K immediately after irradiation at this temperature than under other conditions. After warming up to room temperature the spectra are similar to those found after irradiation at room temperature, xanthine being the only purine which gives a spectrum exhibiting the two satellites in addition to the main singlet (figure 8). A peculiar effect is seen in the cytosine spectrum (figure 4). Initially, and after warming up, a poorly-resolved doublet is observed. However, on repeated cooling at 100 °K, this is converted to a spectrum containing six well-resolved lines and in addition some poorly-resolved lines. This conversion is reversible,

the doublet appearing again at room temperature. So far cytosine is the only substance which yields a better-resolved spectrum at reduced temperature. Unusual behaviour is also observed with 5-methylcytosine. No spectrum could be detected at low temperature with this substance even after high doses of radiation given at 100°K. Moreover, the violet coloration produced on irradiation at room temperature was absent. 5-hydroxymethylcytosine was also used for experiments at low temperature, because this is a constituent base of phage

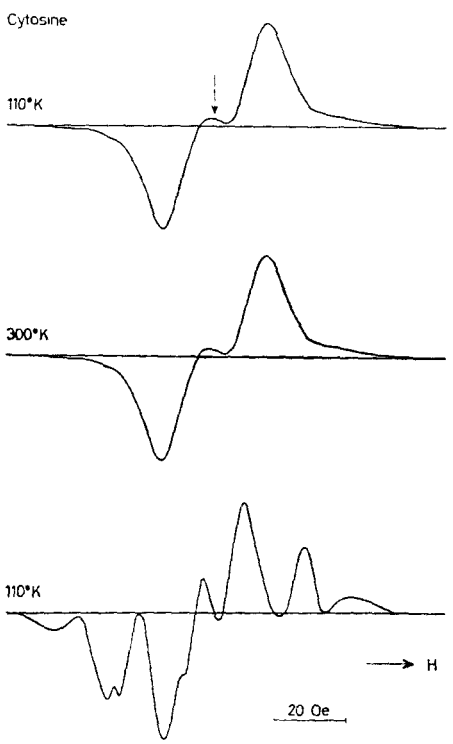

Figure 4. First derivative spectra of cytosine *in vacuo* at about 100°K immediately after irradiation at low temperature, after warming up to room temperature and after cooling again to 100°K. Arrow indicates $g = 2{\cdot}0036$.

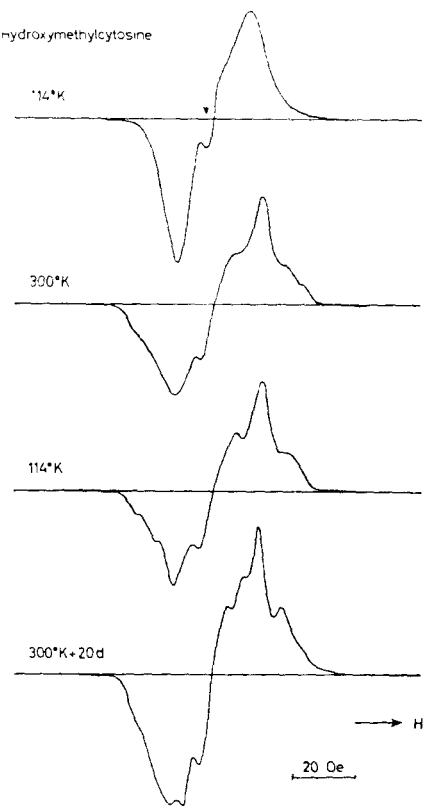

Figure 5. First derivative spectra of 5-hydroxymethylcytosine *in vacuo* at about 100°K immediately after irradiation at low temperature, after warming up to room temperature, after cooling again to 100°K and after 30 days of storage at room temperature. Arrow indicates $g = 2{\cdot}0036$.

deoxyribonucleic acid and because it supplements the observations on the other compounds (figure 5). Not much structure was visible at low temperature, but at least six lines appeared at room temperature, for which the hydroxymethyl group is again probably responsible. The thymine spectrum at low temperature is composed of two well-resolved lines superimposed on a small multiline spectrum corresponding to the two groups of lines observed after irradiation at room

temperature. On warming up to room temperature the doublet decreases relative to the multiplet, in close correspondence with the findings on thymidine-5′-monophosphate (Müller 1964). For these experiments at low temperature, only thymine supplied by the Nutritional Biochemicals Corporation was used, and at room temperature this material gave rise to spectrum I.

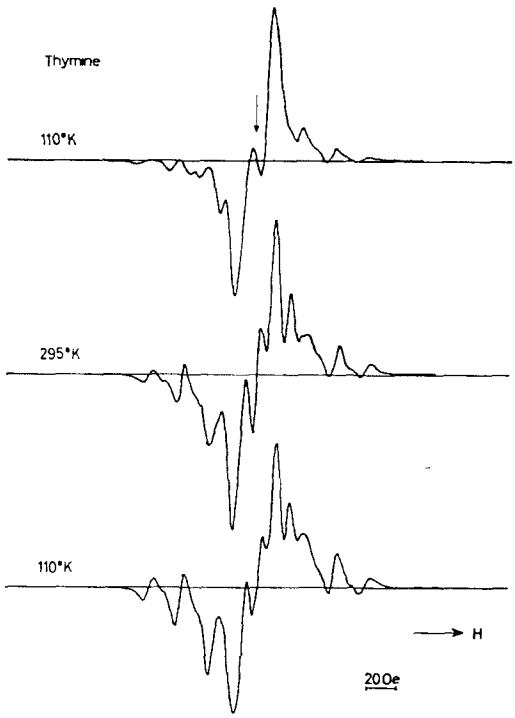

Figure 6. First derivative spectra of thymine *in vacuo* at about 100°K immediately after irradiation at low temperature, after warming up to room temperature and after cooling again to 100°K. Arrow indicates $g = 2{\cdot}0036$.

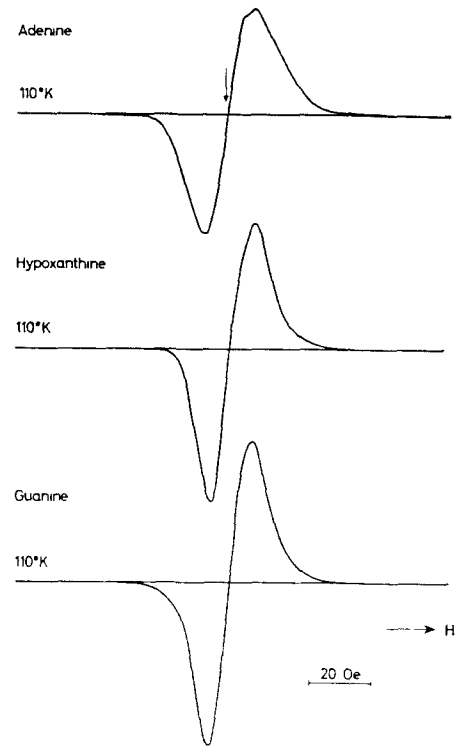

Figure 7. First derivative spectra of adenine, hypoxanthine and guanine *in vacuo* at about 100°K immediately after irradiation at low temperature. Arrow indicates $g = 2{\cdot}0036$.

The spectra of the pentoses D-ribose and D-2-deoxyribose after irradiation at room temperature are shown in figures 9 and 10. The latter spectrum is composed of five prominent lines, but the spectrum of D-ribose changes from a poorly-resolved quintet into an even less-resolved quartet in the course of 30 days of storage *in vacuo* at room temperature. This transformation can be regarded as a loss of some lines, including the central line. After irradiation at low temperature the usual loss of hyperfine structure is observed in the spectra of both pentoses (figures 11 and 12). Three prominent lines are recorded with D-ribose and two prominent lines with D-2-deoxyribose. On warming up to room temperature, the former spectrum is transformed into the quintet which is also found after irradiation at room temperature. D-2-deoxyribose, similarly, on warming up yields a well-resolved quintet spectrum.

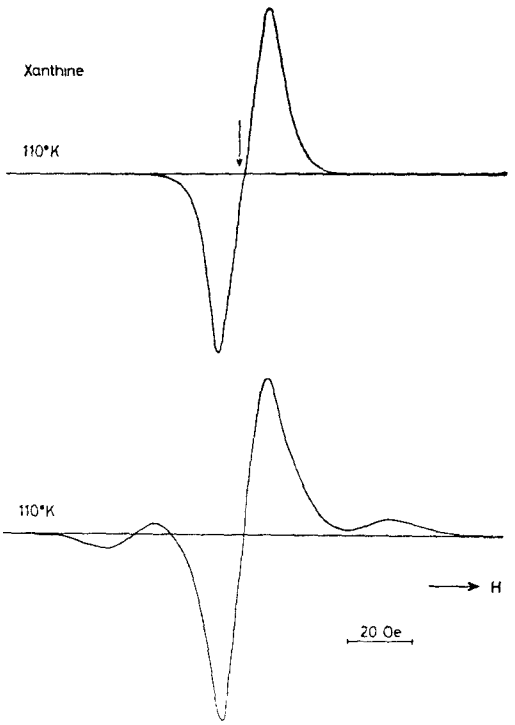

Figure 8. First derivative spectra of xanthine *in vacuo* at about 100°K immediately after irradiation at low temperature and after intermediate warming up to room temperature. Arrow indicates $g = 2{\cdot}0036$.

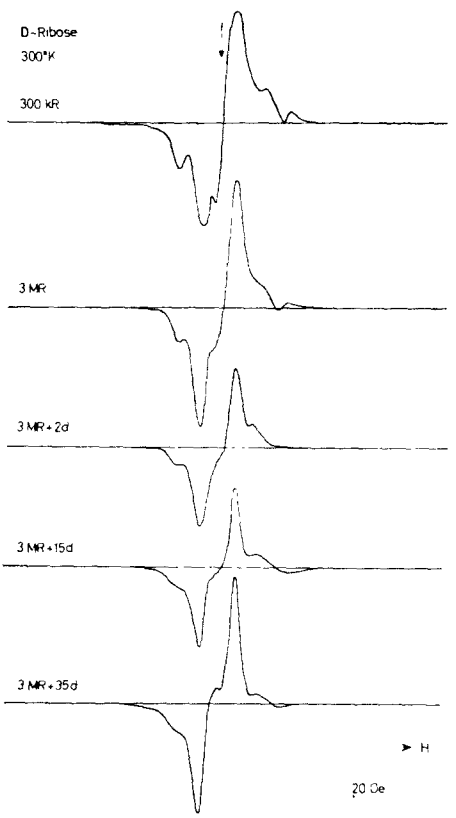

Figure 9. First derivative spectra of D-ribose *in vacuo* at room temperature after various doses of radiation and subsequent storage. Arrow indicates $g = 2{\cdot}0036$.

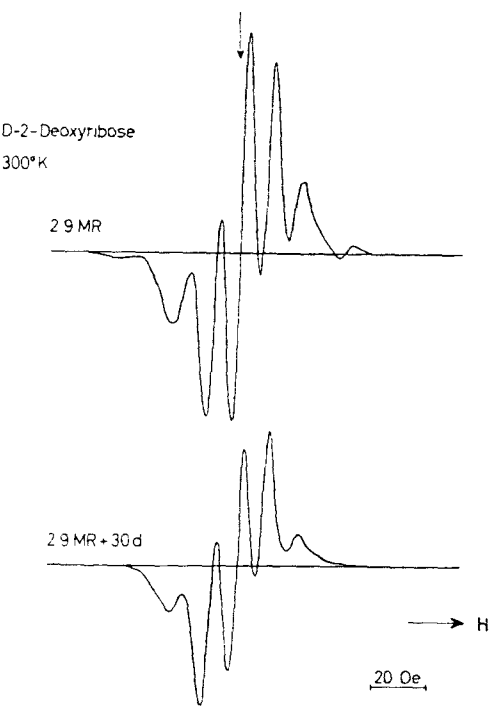

Figure 10. First derivative spectra of D-2-deoxyribose *in vacuo* at room temperature immediately after irradiation and after storage for 30 days. Arrow indicates $g = 2{\cdot}0036$.

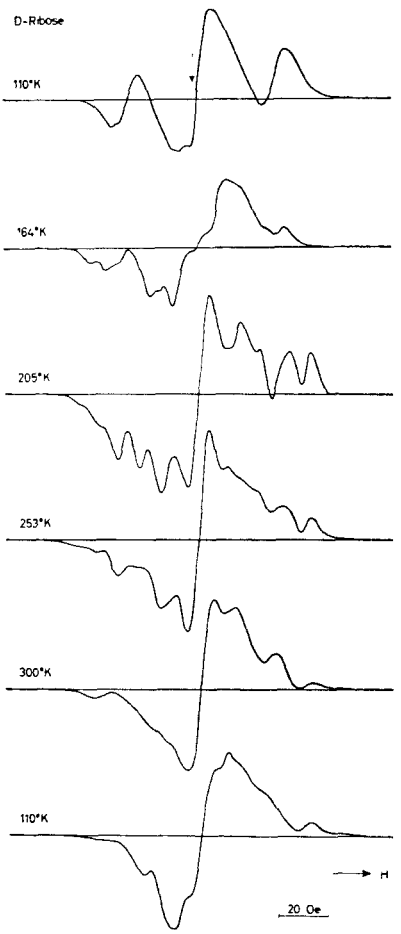

Figure 11. First derivative spectra of D-ribose *in vacuo* at about 110°K after irradiation at low temperature, at successively increased temperatures and after cooling again to 110°K. Arrow indicates $g = 2{\cdot}0036$.

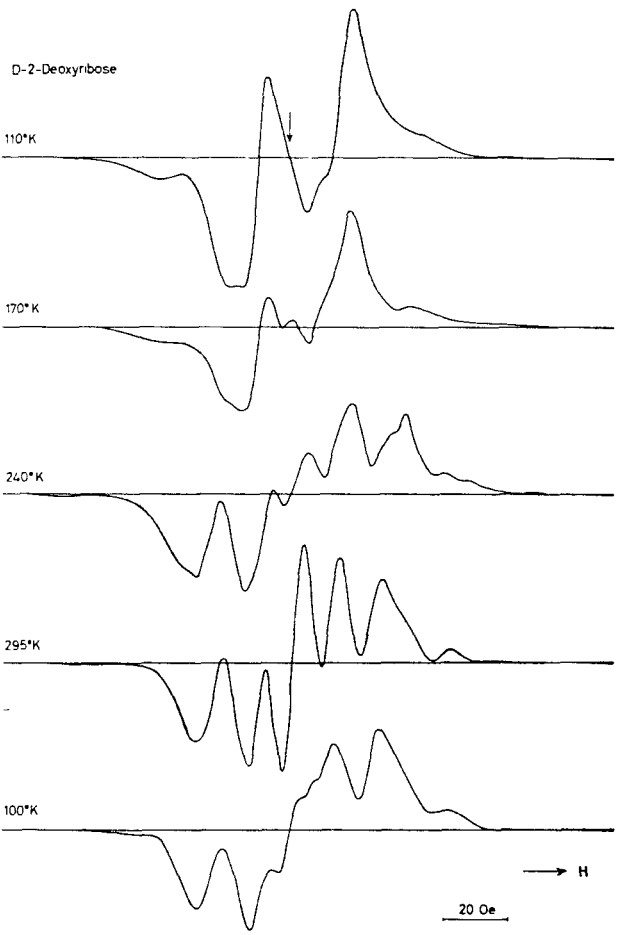

Figure 12. First derivative spectra of D-2-deoxyribose at about 110°K after irradiation at low temperature, at successively increased temperatures and after cooling again to 100°K. Arrow indicates $g = 2{\cdot}0036$.

3.2. *Quantitative observations*

As described in greater detail in connection with the results on nucleotides (Müller 1964) and nucleosides (Müller and Köhnlein 1964) the dependence of radical-concentration on radiation dose can be described by a simple exponential function:

$$C = C_\infty[1 - \exp(-D/D_{37})]. \qquad (1)$$

Experimental points are plotted in figures 13–18 together with curves representing the exponential dependence. It is seen from figures 13 and 14 that results obtained in air and *in vacuo* at room temperature show no quantitative differences, except for 5-methylcytosine which yields about twice as many readicals *in vacuo* as in air. Saturation of radical-concentration occurs slightly above 10^{17} radicals/g for

pyrimidines lacking a methyl group, but those containing a methyl group saturate at concentrations somewhat higher than 10^{18} radicals/g. Dose-effect curves of purines saturate at concentrations similar to those of pyrimidines lacking a methyl group, with the exception of adenine which saturates at the low value 3×10^{16} radicals/g. At low temperature all nucleic-acid bases except adenine show saturation of radical-concentration at about 10^{18} radicals/g, adenine showing saturation at about 2×10^{17} radicals/g (figures 15 and 16).

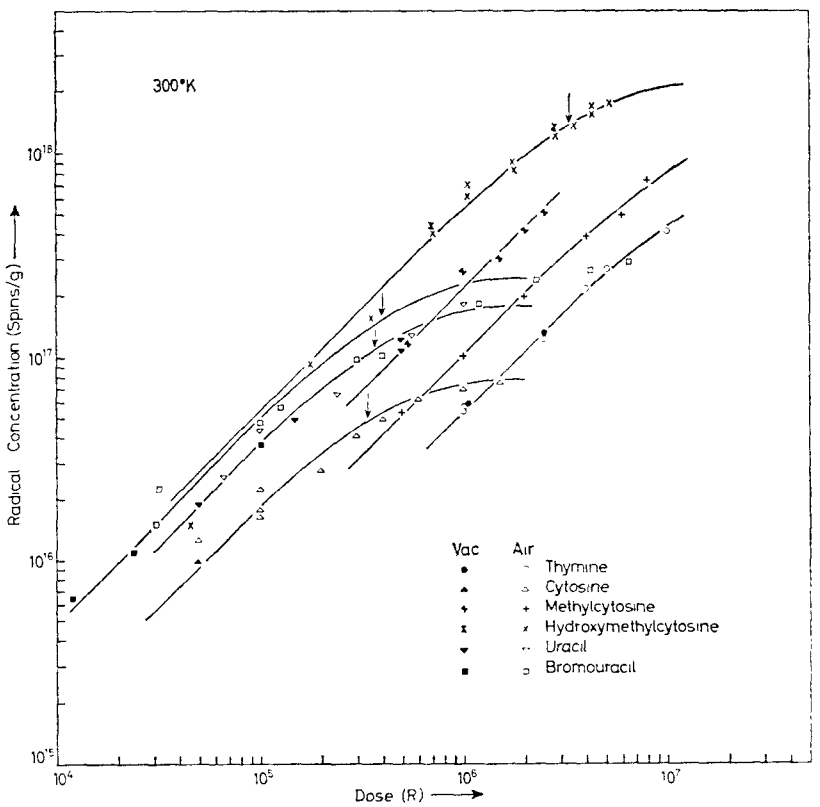

Figure 13. Radical concentrations in pyrimidine bases a room temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

Dose-effect curves of radical-production in the pentoses D-ribose and D-2-deoxyribose are presented in figures 17 and 18. Only D-2-deoxyribose at room temperature shows saturation of radical-concentration below 10^{19} radicals/g, though all other curves approach or exceed this value. From the initial linear parts of these dose-effect curves, G-values are derived which are compiled in table 1, together with D_{37}-values (defined in the preceding paper).

Table 1 shows that G-values of nucleic-acid bases do not exceed 1 at room temperature or 2·3 at low temperature, but the G-values for pentoses are significantly higher. Exceptionally low radical yields are obtained from thymine and adenine at room temperature (Müller, Köhnlein and Zimmer 1963), and from 5-methylcytosine and adenine at low temperature. In fact, the radical-concentration from 5-methylcytosine at 100°K was so small that only an upper

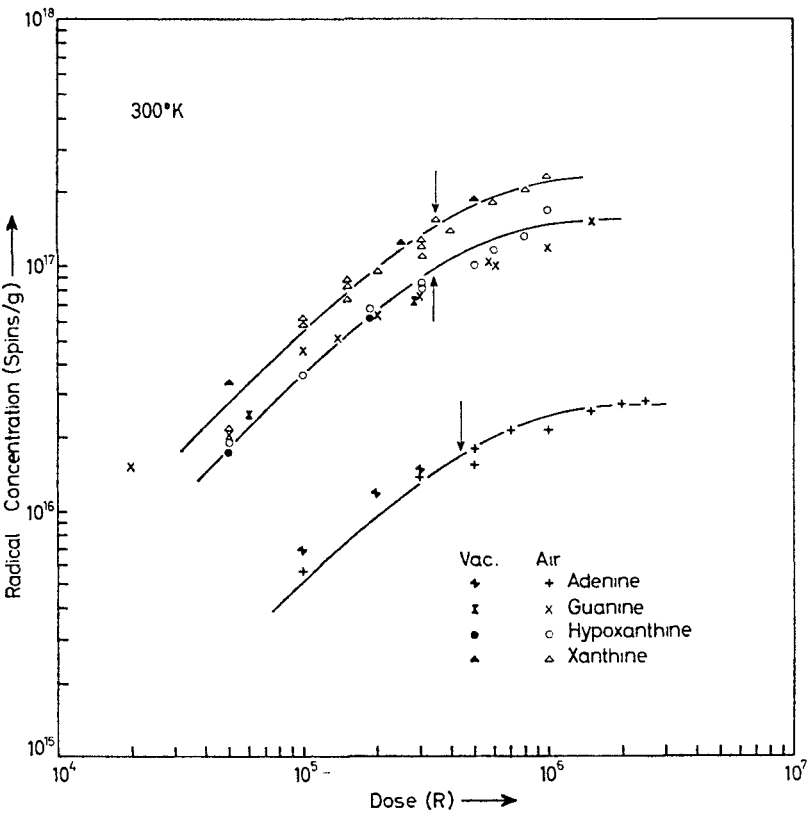

Figure 14. Radical concentrations in purine bases at room temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

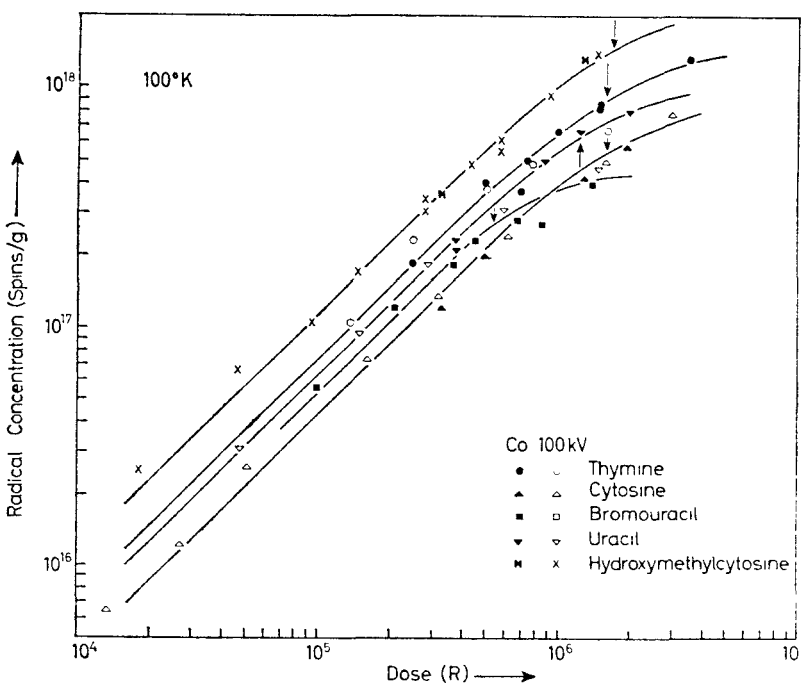

Figure 15. Radical concentrations in pyrimidine bases *in vacuo* at low temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

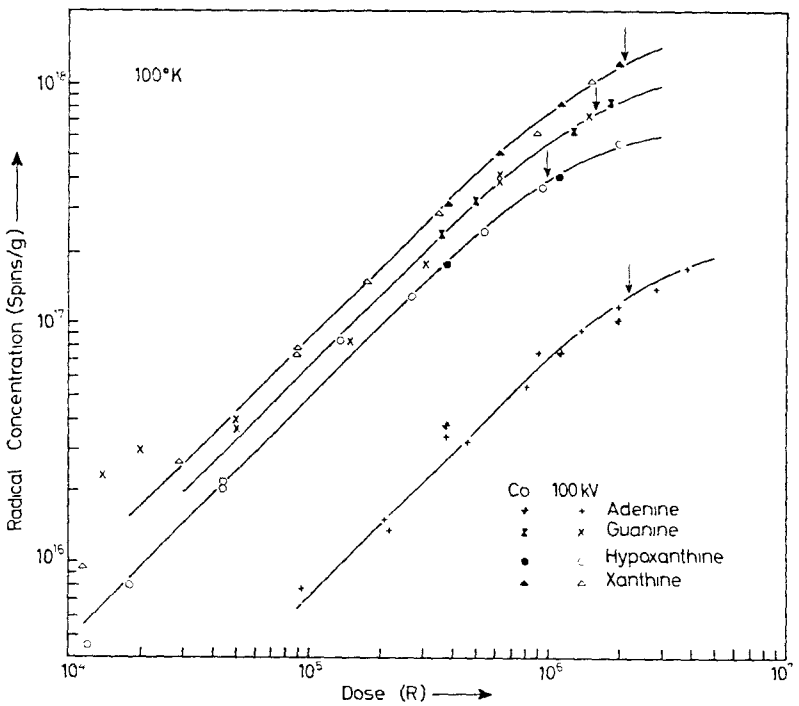

Figure 16. Radical concentrations in purine bases *in vacuo* at low temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

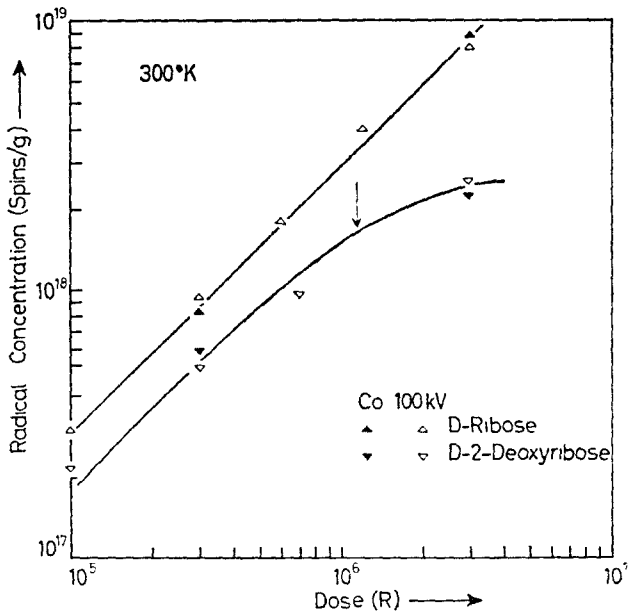

Figure 17. Radical concentrations in pentoses *in vacuo* at room temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

limit for the G-value could be given. G-values at 100°K are usually higher than those determined at room temperature. When the 100°K samples are warmed up to room temperature, however, the radical-concentrations are lower than those measured after room temperature irradiation in all bases and pentoses except thymine. The G-value for thymine after room temperature irradiation is much lower. The implications of these observations are further discussed in connection with the results on nucleotides (Müller 1964).

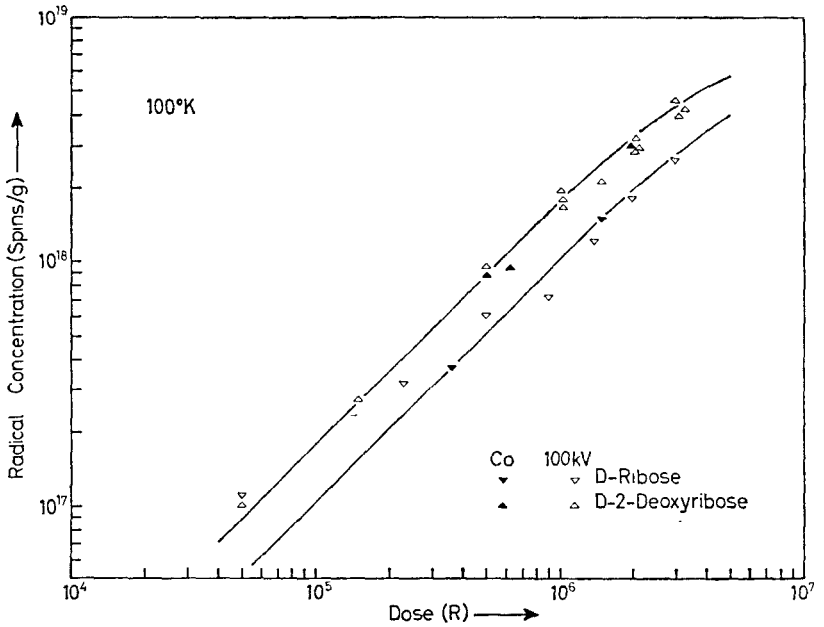

Figure 18. Radical concentrations in pentoses *in vacuo* at low temperature against dose of radiation. Drawn lines represent equation (1). Arrows indicate D_{37}-values.

The D_{37}-values reflect the observation made earlier on the saturation concentrations: considerably higher values are shown by substances containing a methyl group. Conversely one may speak of exceptionally low values for bases lacking a methyl group, since the pentoses, amino acids (Müller *et al.* 1964) and other aliphatic compounds yield values similar to those of the methyl-containing bases. In other words, only one methyl group confers aliphatic character on a nucleic-acid base with respect to saturation of radical concentration and to hyperfine structure of the spectrum. The obvious conclusion is that radicals are stabilized in or near the methyl group.

It remains to compare the results given in table 1 with those of other workers. G-values can be derived from the radical-concentrations given by Shen Pei-Gen *et al.* (1959) after a dose of 20 Mr. Initial G-values were reported by van de Vorst (1963) and van de Vorst and Williams-Dorlet (1963). Henriksen (1963) published G-values measured at room and at low temperature and after warming up samples irradiated at low temperature. The results are listed with our own results in table 2. The low values obtained by Shen Pei-Gen *et al.* are compatible with our results, if saturation of radical-concentration, as represented in figures 13–18, is taken into account. Obviously the discrepancy found with D-ribose is

small, as this substance shows saturation only above 3 Mr. The *G*-values reported by van de Vorst are also relatively small, which is again attributed to the rather high doses used. The results obtained by Henriksen are in much better agreement with our own figures, especially after irradiations at room temperature. At low temperature our results are generally higher. As Henriksen used an even lower temperature, the discrepancies may be due to residual saturation of microwave power in his work. Special care was taken to avoid this in our experiments. However, other still unknown factors may also cause the divergences at low temperature. Certainly, it is very unlikely that the values measured at room temperature after irradiation at low temperature are affected by microwave

Compound	*G*-values			D_{37}	
Irradiated at	300°K	100°K	100°K	300°K	100°K
Observed at	300°K	100°K	300°K	300°K	100°K
Cytosine	0·4	0·8	0·3	0·3 Mr	1·6 Mr
5-Methylcytosine	0·4	< 0·002	—	> 2 Mr	—
5-Methylcytosine (in air)	0·2	—	—	> 5 Mr	—
5-Hydroxymethylcytosine	1·0	2·3	0·7	3 Mr	1·7 Mr
Thymine	0·1	1·5	0·7	> 8 Mr	1·6 Mr
Uracil	0·8	1·2	0·6	0·4 Mr	1·2 Mr
5-Bromouracil	1·0	1·0	—	0·4 Mr	0·5 Mr
Adenine	0·1	0·14	0·03	0·4 Mr	2·0 Mr
Hypoxanthine	0·8	1·0	0·5	0·3 Mr	1·0 Mr
Guanine	0·8	1·3	0·3	0·3 Mr	1·6 Mr
Xanthine	1·0	1·7	0·4	0·3 Mr	2·0 Mr
D-Ribose	6	4	2·4	> 2 Mr	> 3 Mr
D-2-Deoxyribose	4	2	1·0	1·2 Mr	> 3 Mr

Table 1. Quantitative results on nucleic acid bases and pentoses.

saturation. Systematic investigations on the dependence of the final radical-concentration on the conditions prevailing at irradiation or warming up are, however, lacking. Hence, no further conclusions on the causes of the discrepancies can be drawn at present. Notwithstanding these uncertainties, all the general conclusions derived from our results obtained after irradiation at low temperatures are still valid.

ACKNOWLEDGMENTS

We should like to thank Professor Dr. K. G. Zimmer and Dr. J. W. Boag for advice and helpful criticisms on this manuscript and the accompanying ones.

We are grateful to Dr. H. Reuschl for carrying out the chromatographic tests of thymine and to Mrs. U. -B. Jung and Miss E. Weber for efficient technical assistance.

Compound	Shen Pei-Gen *et al.* (1959)	van de Vorst (1963)	Henriksen (1963)			Present work		
Irradiated at	300°K	300°K	295°K	77°K	77°K	300°K	100°K	100°K
Observed at	300°K	300°K	295°K	77°K	295°K	300°K	100°K	300°K
Cytosine	0·017	0·026	0·4	0·7	0·2	0·4	0·8	0·3
5-Methylcytosine						0·4	< 0·002	
5-Hydroxymethylcytosine						0·2	2·3	0·7
Thymine		0·02	< 0·1	0·1	< 0·1	0·1	1·5	0·7
Uracil	0·086		0·1	0·6	0·2	0·8	1·2	0·6
5-Bromouracil	0·014					1·0	1·0	
Adenine		0·04	0·1	0·3	0·1	0·1	0·14	0·03
Hypoxanthine						0·8	1·0	0·5
Guanine	0·0073	0·01		0·6	0·1	0·8	1·3	0·3
Xanthine						1·0	1·7	0·4
D-Ribose	1·5	0·7	3·6	1·1	0·9	6	4	2·4
D-2-Deoxyribose		0·9 (in air)				4	2	1·0

Table 2. Comparison of *G*-values obtained by different authors.

Cytosine, 5-méthylcytosine, 5-hydroxyméthylcytosine, thymine, uracil, 5-bromouracil, adénine, hypoxanthine, guanine, xanthine, D-ribose et D-2-déoxyribose ont été irradiés à l'état de poudres cristallines sèches aux températures voisines de 100°K et 300°K, puis examinés par résonance de spin électronique. Chaque substance est soumise à irradiation X et gamma (doses allant jusqu'à 10 Mr) et son spectre dérivée première enregistré à 9,4 kMc/sec. Tandis que ces spectres montrent de nouveaux détails dans la structure hyperfine rappelant celle des composés méthylés (richesse en lignes), on s'est surtout attaché à l'étude quantitative des résultats. Les valeurs G obtenues à partir de la portion linéaire initiale des courbes dose-effet, sont comprises entre 0,1 et 1,0 pour les bases et entre 4 et 6 pour les pentoses après irradiation à température ambiante. Quoique souvent le rendement radiochimique G soit trouvé peu supérieur à ces valeurs si l'irradiation et la mesure sont effectuées à basse température, il décroît toujours par réchauffement à température ambiante jusqu'à devenir inférieur aux valeurs ci-dessus indiquées. Cette particularité a été observée aussi bien pour les bases que pour les pentoses.

Après irradiation à température ambiante la courbe dose-effet de toutes les bases ne contenant pas le groupement méthyl, n'est plus linéaire audessus de 0,3 Mr et atteint une valeur constante, alors qu'elle le reste au moins jusqu'à 1 Mr pour les bases méthylées et pour les pentoses A basse température la linéarité est observée jusqu'à 1 Mr pour toutes les bases et les pentoses, exception faite pour le 5-bromouracil, pour lequel la valeur correspondante est 0,5 Mr.

Cytosin, 5-Methylcytosin, 5-Oxymethylcytosin, Thymin, Uracil, 5-Bromuracil, Adenin, Hypoxanthin, Guanin, Xanthin, D-Ribose und D-2-Deoxyribose wurden in Form trockener, kristalliner Pulver bei etwa 100°K und 300°K bestrahlt und durch Elektronenspin-Resonanz-Spektrometrie untersucht. Erste Ableitungen der Spektren jeder Substanz wurden nach Röntgen- und Gammabestrahlung mit einer ganzen Serie von Dosen bis zu 10 Mr bei 9,4 GHz aufgenommen. Während die qualitativen Ergebnisse neue strukturelle Einzelheiten der Spektren enthalten, durch die der Linienreichtum methylhaltiger Verbindungen bestätigt wird, wurde das Hauptgewicht der Untersuchungen auf quantitative Messungen gelegt. Die G-Werte, die aus den anfänglichen linearen Teilen der Dosis-Effektkurven abgeleitet wurden, liegen zwischen 0,1 und 1,0 bei Basen und zwischen 4 und 6 für Pentosen nach Bestrahlung bei Zimmertemperatur. Obgleich oft etwas höhere Radikalausbeuten gefunden werden, wenn Bestrahlung und Messung bei tiefer Temperatur durchgeführt werden, nehmen diese Ausbeuten bei Erwärmung auf Zimmertemperatur immer ab bis unter die oben angegebenen Werte.

Nach Bestrahlung bei Zimmertemperatur entfernen sich die Dosis-Effektkurven aller Basen, die keine Methylgruppe enthalten, oberhalb 0,3 Mr von der linearen Dosisabhängigkeit und erreichen einen konstanten Wert, während Linearität bis mindestens 1 Mr bei den Basen, die eine Methylgruppe enthalten, und bei Pentosen gefunden wird. Bei tiefer Temperatur wird Linearität bis zu 1 Mr bei allen Basen und Pentosen beobachtet mit Ausnahme von 5-Bromuracil, für das der entsprechende Wert 0,5 Mr ist.

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