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CORRESPONDENCE

Transcription on irradiated DNA

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As found previously, the amount of mRNA synthesized on DNA by RNA polymerase (RNA nucleotidyltransferase, E.C. 2.7.7.6.) is reduced by U.V. light or by ionizing radiation (Zimmermann, Kröger, Hagen and Keck 1964, Harrington 1964, Weiss and Wheeler 1964, Hagen, Keck, Kröger, Zimmermann and Lücking 1965). More recent experiments have shown that the overall number of RNA molecules synthesized on γ-irradiated DNA is nearly the same as on untreated DNA; whereas the decrease in chain-length of RNA, observed after irradiation of the DNA primer, almost equals the loss of total RNA synthesis (Hagen, Ullrich, Petersen, Werner and Kröger 1969). Furthermore, it was shown that the capacity of DNA to bind RNA polymerase was almost unaffected by irradiation. Only high radiation doses lead to the formation of new binding sites which may be ineffective for RNA synthesis, but this mechanism contributes to less than 10 per cent of the overall inactivation of priming activity of DNA (Hagen *et al.* 1969). As the dose–response curve for the loss of priming activity shows an upward curvature, it is not possible to determine the yield of inactivated molecules per unit of dose. It is the purpose of this communication to describe a procedure by which the number of critical lesions which stop the RNA polymerase synthesizing mRNA along the DNA molecule may be calculated.

From the experimental findings cited above, a model is derived, the characteristics of which are depicted on figure 1. RNA synthesis starts at special binding sites for the RNA polymerase and proceeds up, but not beyond, the nearest lesion induced by radiation in one of the two polynucleotide strands. At the site of this critical lesion, the RNA polymerase gets stuck to the DNA molecule. Thus, the decreasing amount of RNA synthesized after higher doses of radiation reflects the reduction in length of the DNA sections between the

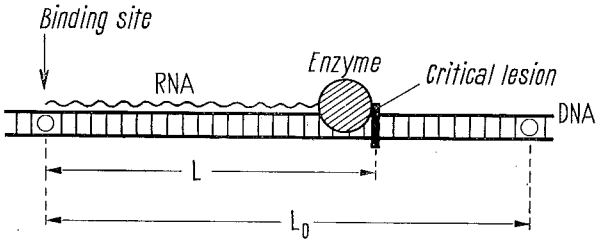

Figure 1. Scheme of RNA synthesis on γ-irradiated thymus DNA: the RNA polymerase molecule is blocked at the site of a critical lesion induced by ionizing radiation in one of the two DNA strands.

starting point for transcription and the nearest critical lesion. This model, however, is valid only under the specific experimental conditions applied, i.e. at low ionic concentration, where the various DNA sections are transcribed only once by one polymerase molecule (cf. Richardson 1969).

It is possible to express the shortening of the DNA sections transcribed by RNA polymerase as a function of the number of lesions induced per DNA section. For a first approach, we assume that all sections transcribed on unirradiated DNA have the same length L_0 (see figure 1). This length is reduced if lesions are produced within the interval L_0. The length of the interval L_z between the binding site and the nearest critical lesion is described by the expression:

$$L_z = L_0[1 - \exp(-z)]/z, \quad (1)$$

where z is the average number of critical lesions on the two DNA strands within the length L_0. The derivation of equation (1) is given in detail by Bresler, Kalinin and Peromov (1964), who have used it to calculate the reduction in recombination length of a transforming DNA molecule with a bacterial chromosome. Since the amount of RNA synthesized is proportional to the length of the DNA interval transcribed, L_z/L_0 represents the relative priming activity A_z/A_0 as a function of z:

$$A_z/A_0 = [1 - \exp(-z)]/z. \quad (2)$$

A similar formula was obtained by Michalke and Bremer (1969), describing the RNA synthesis in U.V.-irradiated *E. coli* bacteria.

Equation (2) is valid only if all DNA sections transcribed, and consequently all RNA molecules synthesized, have the same length L_0. To apply equation (2)

Relative mass fraction m_i	Sedimentation coefficient $S^0_{20,w}$	Molecular weight $M_i \times 10^{-5}$	Relative size in relation to M_w/a_i	Relative number of molecules n_i ($\sum n_i = 1$)
0·032	2·2	0·160	0·052	0·2780
0·068	4·4	0·545	0·177	0·1725
0·180	6·6	1·12	0·363	0·2225
0·202	8·8	1·88	0·610	0·1485
0·167	11·0	2·79	0·905	0·0830
0·126	13·2	3·86	1·250	0·0455
0·095	15·4	5·08	1·645	0·0259
0·062	17·6	6·45	2·09	0·0132
0·043	19·7	7·97	2·58	0·0075
0·017	22·0	9·58	3·105	0·0024
0·008	24·2	11·46	3·715	0·0010

$M_w = 308\,000$, $P_w = 1000$ nucleotides,
$M_n = 139\,000$, $P_n = 450$ nucleotides.

The S^0 values of the various RNA fractions are determined by sucrose-gradient centrifugation, the relative mass fraction m_i is obtained from the radioactivity of [8-^{14}C] AMP, incorporated into the RNA. Details of this procedure were described previously (Hagen *et al.* 1969). M_w, weight average molecular weight; M_n, number average molecular weight; P_w and P_n, chain-length in nucleotides, corresponding to M_w and M_n.

Chain-length distribution of RNA synthesized on unirradiated thymus DNA.

to describe the experimental results obtained previously for the loss of priming activity (Hagen *et al.* 1969), we determined the length of the transcribed DNA sections by measuring the molecular weight of RNA synthesized on unirradiated calf-thymus DNA. This was done by ultra-centrifugation in a sucrose gradient after dissolving the DNA–RNA–enzyme complexes. The molecular weight of the various RNA fractions was calculated from the sedimentation coefficients, according to Kurland (1960) and Coquerelle, Bohne, Hagen and Merkwitz (1969). As shown in the table there is a broad distribution in size. The lengths of the RNA molecules are given as fractions α_i of the weight average molecular weight M_w which was determined to be 308 000 corresponding to an average length of $\bar{L}_0 = 1000$ nucleotides. The last column of the table shows that more than two-thirds of the RNA molecules have a molecular weight smaller than 150 000. Their number decreases with increasing molecular weight.

To account for the inhomogeneous length distribution of the DNA intervals transcribed, equation (2) has to be applied separately to the various sections with the length $\alpha_i \bar{L}_0$, where $\bar{L}_0$ is the mean length of L_0, and α_i is the relative length of the interval under consideration with respect to $\bar{L}_0$. The relative priming activity of the ith interval after irradiation is given by the equation:

$$A_{z,i}/A_{0,i} = [1 - \exp(-\alpha_i \bar{z})]/\alpha_i \bar{z}, \tag{3}$$

where $\bar{z}$ is the average number of critical lesions on the interval of the DNA strand with the length $\bar{L}_0$. The loss of the total activity of a given DNA sample can be obtained by summing over the $A_{z,i}/A_{0,i}$ of the various intervals according to their relative number n_i and their relative lengths α_i:

$$\frac{A_z}{A_0} = \frac{\sum \alpha_i n_i (A_{z,i}/A_{0,i})}{\sum \alpha_i n_i} = \frac{\sum \alpha_i n_i [1 - \exp(-\alpha_i \bar{z})]/\alpha_i \bar{z}}{\sum \alpha_i n_i}. \tag{4}$$

A comparison of the inactivation curve to be expected on the basis of equation (4) with our recent experimental data (Hagen *et al.* 1969) is shown on figure 2. When the reciprocal relative priming activity, A_0/A_z, is plotted against dose, the experimental points fall on a straight line. Several values of the dose–effect curve represented by equation (4) were calculated for various numbers of $\bar{z}$, using the molecular weight distribution n_i/α_i of RNA as shown in the table. In figure 2 the scale for the average number of lesions ($\bar{z}$) within $\bar{L}_0$ was chosen so that the calculated points for A_0/A_z fit with the experimental points. Thus a ratio between $\bar{z}$ and the dose in rads is obtained. Since $\bar{z}$ is proportional to dose ($\bar{z} = kD$) it follows that $k = 7{\cdot}4 \times 10^{-7}$ critical lesions are formed in DNA per nucleotide pair and per rad. In these experiments the DNA was irradiated at a concentration of 500μg/ml. (0·01 M NaCl, pH 7): consequently 0·6 critical lesions are generated per 100 eV of energy absorbed.

This G-value of 0·6 should be regarded as an upper limit. The molecular weight of the RNA may be higher than computed (see Richardson 1966), or the mechanism discussed here may not account for all the inactivation of priming activity. As either reason would lead to a decreased number of changes, the G-value for the generation of a critical lesion may with caution be said to lie in the range from 0·4 to 0·6.

The G-value obtained gives us some hints as to the physico-chemical nature of the critical lesions. In principle, breaks of the polynucleotide strands or damage to the bases may be considered. In aqueous solution, alterations of the DNA bases occur with G-values of 1·0 to 1·7 (Weiss 1964), therefore only very specific

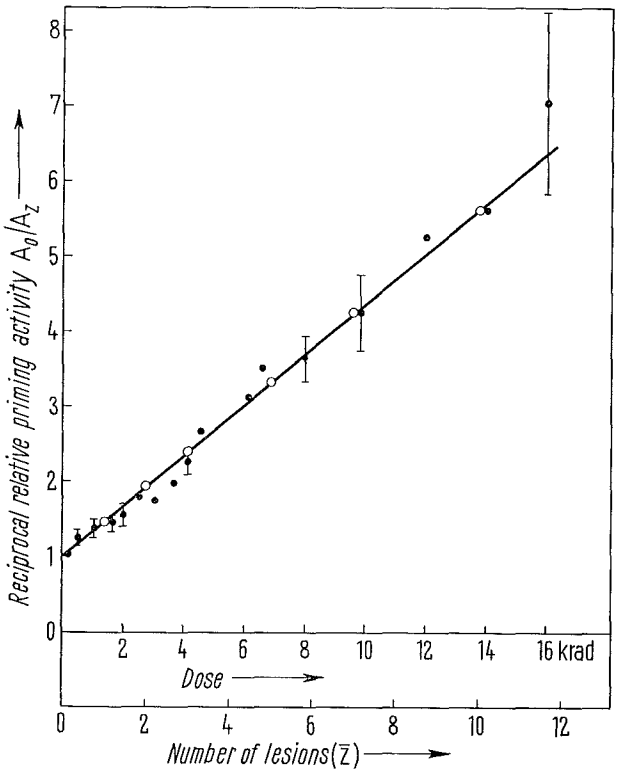

Figure 2. Plot of the reciprocal relative priming activity (A_0/A_z) versus radiation dose. ●, $\overset{\top}{\underset{\perp}{\bullet}}$, experimental data (single values and average values ± S.E., $n = 5-7$) of the loss of priming activity. Irradiation was performed with $^{60}Co + \gamma$-rays at a concentration of 500 μg/ml. in 0·01 M NaCl in N_2, the priming activity of a given DNA sample was determined as described previously (Zimmermann *et al.* 1964). —o———o—, theoretical inactivation curve calculated according to equation (4). $\bar{z}$, average number of lesions on the interval of the DNA strand with the length $\bar{L}_0$.

types of base changes could be responsible for the loss of priming activity. On the other hand, the G-value for the induction of single-strand breaks in DNA of 0·4 (Hagen 1967) is quite close to the figure derived from the calculations given above, indicating that breaks formed by radical attack in one of the DNA strands may represent the critical event to block transcription. This conclusion is supported by the recent findings that, in DNA solutions treated with hydrogen atoms, the ratio of single-strand breaks to loss of priming activity is the same as in γ-irradiated samples (Jung, Hagen, Ullrich and Petersen 1969), whereas after exposure to H atoms, base alterations are 12·5 times more frequent than after γ-irradiation. Single-strand breaks formed by endonuclease (DNase I) do not affect the priming activity of DNA to a large extent (Hagen *et al.* 1969). But, by this enzymatic degradation one single phosphoester bond is hydrolysed, whereas at the site of a radiation-induced break, either the nucleotide base bound to the broken sugar residue, or the whole nucleoside, is lost (Simon 1969). This deficient base in connection with a break in a DNA strand may actually be responsible for stopping the transcription of the genetic information.

References

Bresler, J. E., Kalinin, V. L., and Peromov, D. A., 1964, *Biopolymers*, **2**, 135.

Coquerelle, Th., Bohne, L., Hagen, U., and Merkwitz, J., 1969, *Z. naturf.* B, **24**, 885.

Hagen, U., 1967, *Biochim. biophys. Acta*, **134**, 45.

Hagen, U., Keck, K., Kröger, H., Zimmermann, F., and Lücking, Th., 1965, *Biochim. biophys. Acta*, **95**, 418.

Hagen, U., Ullrich, M., Petersen, E. E., Werner, E., and Kröger, H., 1969, *Biochim. biophys. Acta* (in the press).

Harrington, H., 1964, *Proc. natn. Acad. Sci. U.S.A.*, **51**, 59.

Jung, H., Hagen, U., Ullrich, M., and Petersen, E., 1969, *Z. Naturf.* (in the press).

Kurland, C. G., 1960, *J. molec. Biol.*, **2**, 83.

Michalke, H., and Bremer, H., 1969, *J. molec. Biol.*, **41**, 1.

Richardson, J. P., 1966, *Proc. natn. Acad. Sci. U.S.A.*, **55**, 1616; 1969, *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 9 (New York: Academic Press), pp. 75, 90.

Simon, M., 1969, *Int. J. Radiat. Biol.*, **16**, 167.

Weiss, J. J., 1964, *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 3 (New York: Academic Press), p. 103.

Weiss, J. J., and Wheeler, C. M., 1964, *Nature, Lond.*, **203**, 291.

Zimmermann, F., Kröger, H., Hagen, U., and Keck, K., 1964, *Biochim. biophys. Acta*, **87**, 160.

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ENZYMATIC RNA SYNTHESIS ON IRRADIATED DNA

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SUMMARY

The enzymatic RNA synthesis primed by γ- and ultraviolet-irradiated DNA was studied.

1. After γ-irradiation of primer DNA the total amount of synthesized RNA was measured as well as the number and the length of RNA chains. Further, the number of binding sites for RNA polymerase was determined. The results of the experiments lead to the conclusion, that the radiation induced loss of priming activity is mainly due to the formation of a specific lesion in the DNA which stops the process of RNA synthesis along the strand. The nature of this lesion is discussed.

2. High doses of γ-irradiation create new binding sites for the enzyme which may be ineffective in RNA synthesis.

3. Some experiments concerning the mechanism of action of ultraviolet light were performed. The formation of new binding sites, ineffective for RNA synthesis as well as the formation of specific lesions, stopping the process of transcription may be responsible for the reduced priming activity of ultraviolet-irradiated DNA.

INTRODUCTION

As shown previously[1-4] the priming activity of DNA for RNA synthesis is reduced by ultraviolet light or by ionizing radiation. This inhibition of the DNA-dependent RNA synthesis might, in principle, be due to various radiation-induced alterations in the DNA structure. The binding of the enzyme to the DNA can be affected[5] or the process of synthesizing RNA by the enzyme molecule could be stopped somewhere along the DNA strand by a specific lesion. To get information about the relative contribution of the various possible mechanisms involved in the inhibition of the priming activity, the lengths and the numbers of RNA chains were measured as well as the amount of synthesized RNA. In addition the binding capacity of irradiated DNA was studied.

MATERIALS AND METHODS

Calf-thymus DNA was isolated with phenol as described earlier[6]. [8-^{14}C]ATP was obtained from Schwarz Bioresearch, [γ-^{32}P]ATP from Radiochemical Centre,

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Amersham, and [2-^{14}C]thymine from Gesellschaft für Kernforschung, Karlsruhe. RNA polymerase (RNA-nucleotidyltransferase EC 2.7.7.6) was isolated from *Escherichia coli* according to the method of CHAMBERLIN AND BERG[7]. Fractions III and IV were used. The purified enzyme (Fraction IV) had a specific activity of 3500–4500 units (ref. 7) per mg protein. The priming activity of a DNA sample was determined as described previously[2,8]. Irradiation of DNA solutions (500 μg/ml or 200 μg/ml in 0.01 M NaCl (pH 7) was performed after saturation with N_2 with ^{60}Co γ-rays (Gammacell 200 or Gammacell 220, Atomic Energy of Canada) or with ultraviolet light (low-pressure mercury lamp, dose rate $2.76 \cdot 10^6$ or $14 \cdot 7 \cdot 10^6$ ergs$\cdot$cm$^{-3}\cdot$min^{-1}). The number-average molecular weight M_n' of a given DNA sample was determined by analysis of the boundary in the analytical ultracentrifuge. Details of this procedure are given in a paper by COQUERELLE *et al.*[9].

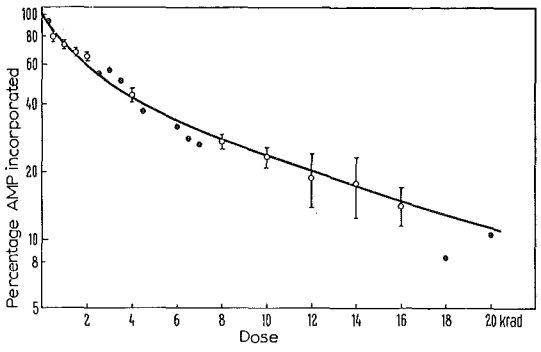
Ultrasonic degradation of DNA was performed with a Branson sonifier. For enzymic degradation DNA (250 μg/ml) was incubated with 2 μg/ml pancreatic deoxyribonuclease I (EC 3.1.4.5) (Worthington, Code DPFF) in 0.01 M NaCl+50 μM $MgCl_2$+50 μM $MnCl_2$ at 20°, the viscosity of the DNA solution being measured simultaneously in a Couette viscosimeter[6]. When a desired degradation was reached the reaction was stopped by increasing the NaCl concentration to 1 M and by adding equal amounts of water-saturated phenol. The phenol was removed by diethyl ether extraction and dialysis.

The hydrodynamic properties of the complex RNA polymerase and DNA were studied in a suitable binding buffer according to RICHARDSON[10], except that albumin was omitted. Calf-thymus DNA and enzyme were incubated in binding buffer for 30 min at 20° before physical measurements were taken. Furthermore the binding of the enzyme to DNA was studied using the nitrocellulose membrane filter technique of JONES AND BERG[11]. According to this method, free DNA and RNA polymerase separately pass through the filters, the DNA–enzyme complex, however, is retained quantitatively. For these binding experiments [2-^{14}C]thymine-labeled DNA and unlabeled DNA were isolated from bacteriophage T4. As host for labeled T4 phages we used a thymine-, leucine- and threonine-*minus* mutant of *E. coli*: CR34. To a modified minimal medium (M 9)[12] of 2.5 l we added 50 mg of each threonine, leucine and [2-^{14}C]thymine (specific activity 2.16 mC/mmole). When a titre of 10^8 cells per per ml was reached, the culture was inocculated with T4 phages at a multiplicity of 0.1. The lysate was purified by repeated centrifugation alternately at 4000 rev./min and 12 000 rev./min. The concentrated lysate with a titre of $1.3 \cdot 10^{12}$ to $2.0 \cdot 10^{12}$ phages per ml was used for DNA extractions according to the method described by COQUERELLE *et al.*[9].

RESULTS

Inactivation curve

The priming activity of a given DNA sample (A) can be expressed by the amount of synthesized RNA as determined by the incorporation of [8-^{14}C]AMP into the acid-insoluble fraction. The dose-effect curve of the relative priming activity of irradiated DNA to that of unirradiated DNA is shown in Fig. 1, summarizing a number of experiments with various DNA and enzyme preparations.



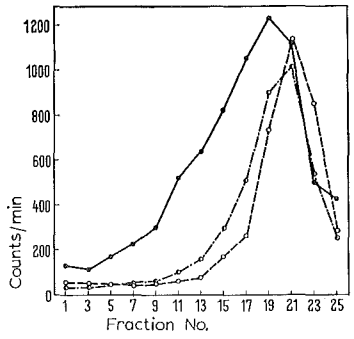


Fig. 1. Dose-effect curve of the relative priming activity of DNA, irradiated at 500 μg/ml. For determining the priming activity samples of 0.25 ml each contained: 10 μmoles Tris buffer (pH 7.9); 1.0 μmole $MgCl_2$; 0.25 μmole $MnCl_2$; 0.1 μmole [8-^{14}C]ATP (120–150 counts/min per nmole), nonlabeled CTP, GTP, UTP each; 0.2 μmole β-mercaptoethanol; 0.1 mg protein; 10 μg DNA. Incubation at 37° for 20 min. AMP incorporation on irradiated DNA was related to the AMP incorporation on unirradiated DNA. ●, single values; $\dot{\dot{\text{p}}}$, average values ±S.E. (n = 5–7).

Fig. 2. Sedimentation patterns of synthesized RNA in the sucrose gradient. Radioactivity: [8-^{14}C]-AMP. ●–●, unirradiated DNA as primer; ○– – –○, γ-irradiated DNA as primer (5 krad at 500 μg/ml); ◐–·–◐, ultraviolet-irradiated DNA as primer (10 sec = 46 · 10^4 ergs · min^{-1} · cm^{-3} at 200 μg/ml). Samples of 0.5 ml were incubated for 20 min at 37°. They contained: 20 μmoles Tris buffer (pH 7.9); 2 μmoles $MgCl_2$; 0.5 μmole $MnCl_2$; 4.0 μmoles β-mercaptoethanol; 0.2 μmole [8-^{14}C]ATP (3100 counts/min per μmole), GTP, UTP, CTP each; 0.2 mg protein, 20 μg DNA. The incubation was terminated adding EDTA (0.01 M final concn.) and sodium dodecylsulfate (0.2 % final concn.). 0.2 ml was layered on a 5-ml linear sucrose gradient (15–30 %) containing 0.01 M Tris buffer (pH 7.5) and 0.1 M NaCl. Centrifugation was performed at 25° for 5 h in a SW-39 rotor (Spinco L 2 65 ultracentrifuge) at 39 000 rev./min. Fractions of 8 drops were collected and 0.03 ml of 0.3 % serum albumin solution was added. The RNA and the protein was precipitated by 5 ml of cold 5 % trichloroacetic acid. The pellet was collected on a membrane filter (Sartorius MF 50), dried and counted in a liquid scintillation spectrometer (Tricarb, 40 mg of 2,5-diphenyloxazole and 1 mg 1,4-bis-(5-phenyloxazolyl-2)benzene per 10 ml of toluene).

Chain length of synthesized RNA

The length of the RNA chain synthesized can be determined by sucrose gradient centrifugation after dissolving the DNA–enzyme–RNA complex. The sedimentation distribution of RNA is given in Fig. 2. RNA formed on nonirradiated DNA shows a broad distribution. Using irradiated DNA as primer, there is a distinct reduction of the size of RNA chains; the long RNA chains disappear. The molecular weight distribution of RNA was calculated according to the methods described by COQUERELLE *et al.*[9] and KURLAND[13] (*cf.* Table I).

TABLE I

CHAIN LENGTH OF SYNTHESIZED RNA

Primer DNA	$s^\circ_{20,w}$ *at the peak* $\times 10^{13}$ *(sec)*	M_w $\times 10^{-5}$	M_n $\times 10^{-5}$	*Ratio* M_{w_0}/M_w
Unirradiated DNA	8.8	3.06	1.39	
γ-Irradiated DNA (5 krad at 500 μg/ml)	6.6	1.22	0.72*	2.75
Ultraviolet-irradiated DNA (10 sec)	6.6	1.77	0.91*	1.55

* These values represent upper limits as the very short RNA chains cannot be recognized with the sucrose sedimentation technique.

Information about the length of synthesized RNA can also be obtained by incorporating [γ-^{32}P]ATP into RNA in addition to [8-^{14}C]AMP[14]. The relation of incorporated [8-^{14}C]AMP to incorporated [γ-^{32}P]ATP can be used as criterion for the chain length. As purine nucleotides are preferentially found at the triphosphate end[14], only a relative value for the size of RNA is obtained. The results of these experiments are shown in Fig. 3.

Irradiating the DNA primer with γ-rays or ultraviolet light leads to a distinct decrease in the chain length of synthesized RNA, particularly in the low dose range (Fig. 3A). The amount of incorporated [γ-^{32}P]ATP can also be used to determine the relative number of RNA chains (Fig. 3B). At variance with the length the number of RNA chains is much less affected by γ-irradiation of the primer. Low doses lead to a small increase in the number of RNA chains, followed by a decrease at higher doses. After ultraviolet irradiation of the DNA primer there is, however, a noticeable reduction of the number of RNA chains especially at low doses. The increase in the

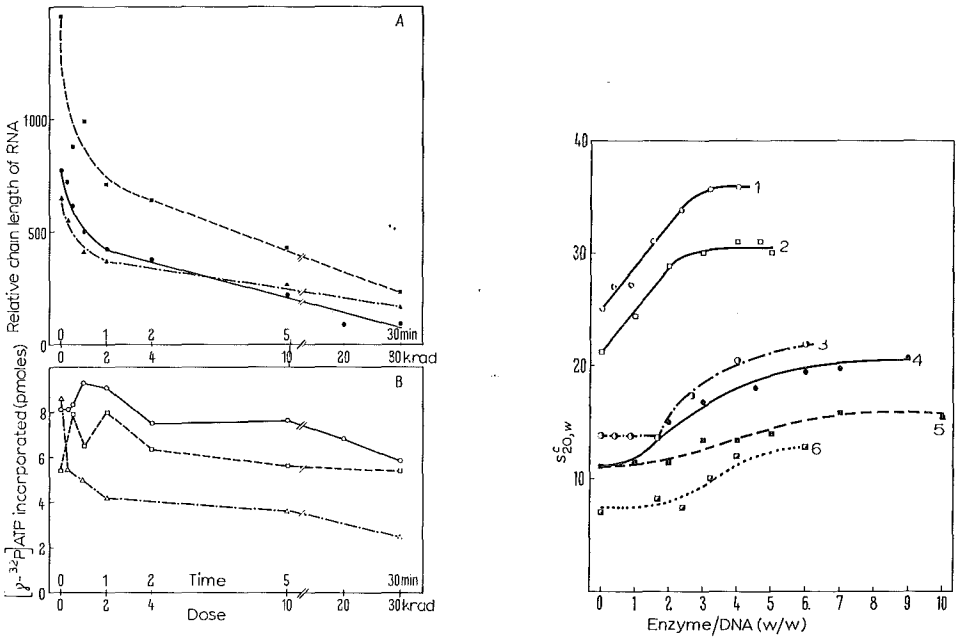


Fig. 3. Chain length and number of RNA chains after synthesis on irradiated DNA. Samples of 0.25 ml were incubated for 20 min at 37°. They contained: 10 μmoles Tris buffer (pH 7.9); 1 μmole $MgCl_2$; 0.25 μmole $MnCl_2$; 2 μmoles 2-mercaptoethanol; 0.1 μmole ATP ([8-^{14}C]ATP: 240 counts/min per nmole and [γ-^{32}P]ATP: 15 000 counts/min per nmole); 0.08 μmole ADP; 0.1 mg enzyme; 10 μg DNA. The incorporation of AMP and ATP was determined as described previously[8]. A. Relative chain length of RNA, derived from the relation of incorporated [8-^{14}C]-AMP to incorporated [γ-^{32}P]ATP (closed symbols). B. Relative number of synthesized RNA chains, in pmoles incorporated [γ-^{32}P]ATP (open symbols). ●, ○, ■, □: γ irradiation, Expts. I and II, dose in krad; ▲, △: ultraviolet irradiation, exposure time in min at a dose rate of $2.76 \cdot 10^6$ ergs $\cdot$ cm^{-3} $\cdot$ min^{-1}.

Fig. 4. Sedimentation coefficient $s^c_{20,w}$ of the RNA polymerase–DNA complex. 1 and 2, nonirradiated DNA, Expts. I and II; 3, irradiated DNA, 10 krad at 200 μg/ml; 4 and 5, irradiated DNA, 12 krad at 200 μg/ml; 6, irradiated DNA, 20 krad at 200 μg/ml. Sedimentation in binding buffer: 0.01 M Tris buffer (pH 7.9), 0.05 M KCl, 5 mM $MgCl_2$, 0.5 mM β-mercaptoethylamine, 0.05 mM EDTA.

number of RNA chains after γ-irradiation is due to the formation of new binding sites for RNA polymerase as shown in the following section.

Binding of RNA polymerase to irradiated DNA

The amount of RNA polymerase bound to DNA is limited by the number of binding sites on the DNA strand. According to RICHARDSON[10] one enzyme molecule is bound per $5 \cdot 10^5$ daltons of T7-DNA (813 nucleotide pairs). The amount of bound enzyme can be measured in the analytical ultracentrifuge, as the enzyme–DNA complex sediments faster than free DNA (Fig. 4). The sedimentation coefficient $s^c_{20,w}$ of unirradiated DNA increases after adding RNA polymerase in a suitable binding buffer. In agreement with results of other authors[10,15], saturation is reached at a weight ratio of 2.5 : 1 (enzyme to DNA). Addition of higher amounts of enzyme does not increase the sedimentation coefficient. The binding capacity of irradiated DNA behaves quite differently. There is only a slight increase of the sedimentation coefficient with increasing amounts of enzyme, and saturation is reached only at a weight ratio of about 8 : 1 (enzyme/DNA).

To determine the number of enzyme molecules bound to DNA, we calculated the molecular weight of the complex from $s^\circ_{20,w}$ and $[\eta]$ according to the method described previously[9] (Table II). Taking the molecular weight of RNA polymerase of $8.8 \cdot 10^5$ (ref. 10) one enzyme molecule is bound per 650 nucleotide pairs of untreated DNA in the saturation range. The saturated complex between enzyme and irradiated DNA contains about 1.6 times more enzyme; in this case one enzyme molecule is bound per 400 nucleotide pairs.

To compare the binding capacity of irradiated DNA with its loss of priming activity it was necessary, however, to obtain more data about the number of binding

TABLE II

MOLECULAR WEIGHT OF THE ENZYME–DNA COMPLEX

$s^\circ_{20,w}$ and $[\eta]$ were obtained by extrapolating the data measured at three different concentrations to zero concentration (*cf.* COQUERELLE *et al.*[9]). Measurements were performed in binding buffer (see legend of Fig. 4) at low ionic strength where the primary component of the enzyme has a molecular weight of $8.8 \cdot 10^5$ daltons[10,29].

Sample	$s^\circ_{20,w} \times 10^{13}$ (*sec*)	$[\eta]$	$M_{s\eta} \times 10^{-6}$	*Enzyme molecules per DNA molecule*	*Nucleotide pairs per one enzyme molecule*
DNA, unirradiated	23.0	65	11.05	—	—
Enzyme, 40 μg/ml DNA, 20 μg/ml	38.2	111	30.9	22.5	800
Enzyme, 60 μg/ml DNA, 20 μg/ml	41.9	105	34.6	26.8	670
Enzyme, 80 μg/ml DNA, 20 μg/ml	37.2	157	35.2	27.4	654
DNA, irradiated (12 krad at 200 μg/ml)	11.0	10.0	1.44	—	—
Enzyme, 180 μg/ml irradiated DNA, 20 μg/ml	20.6	31.0	6.45	5.68	410

sites. The filter technique of JONES AND BERG[11] is suitable for the purpose although it yields only relative values for the binding capacity.

The ability of irradiated unlabeled T4 DNA to prevent the formation of the complex between ^{14}C-labeled T4 DNA with RNA polymerase was used to determine its binding capacity to the enzyme. As in Fig. 5A there is an increase in the radioactivity retained on the filter after incubating [^{14}C]DNA with increasing amounts of enzyme. Saturation is reached at a weight ratio (enzyme/DNA) of 1:2, in agreement with the results of other authors[11,16]. On adding competing unlabeled DNA to the reaction mixture, less [^{14}C]DNA is bound to the enzyme and therefore less radioactivity is retained on the filter. From these data, the relative binding capacity of unlabeled DNA to that of labeled DNA can be calculated (see legend of Fig. 5).

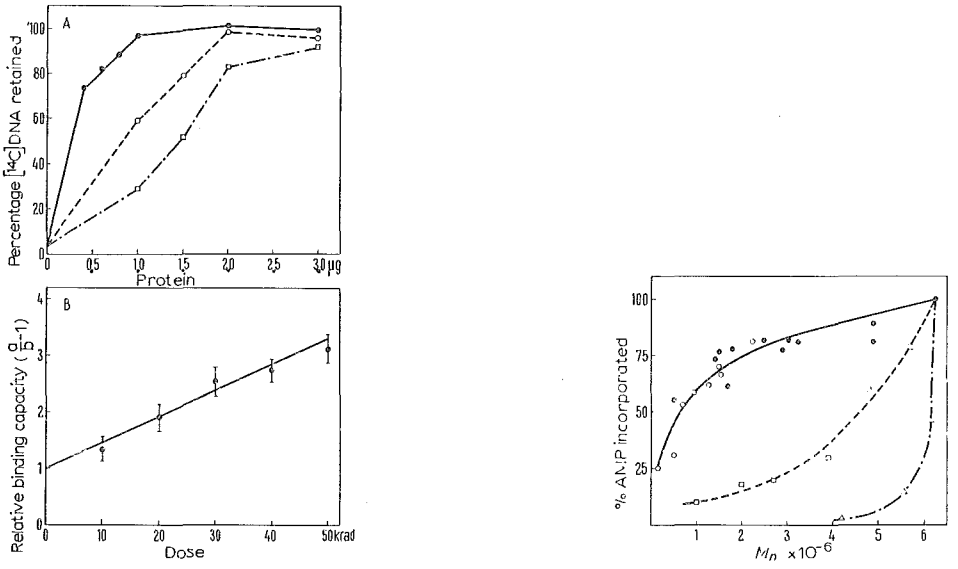


Fig. 5. A. RNA polymerase-dependent retention of ^{14}C-labeled T4 DNA. Reaction mixtures in a final volume of 0.25 ml contained: 2 μg [^{14}C]DNA (approx. 2500 counts/min; 10 μmoles Tris buffer (pH 8.0); 1 μmole $MgCl_2$; 3 μmoles β-mercaptoethylamine and RNA polymerase as indicated on the abscissa. ●–●, reaction mixture with [^{14}C]DNA only; ○– – –○, reaction mixture + 2 μg unlabeled T4 DNA; □–·–□, reaction mixture + 2 μg unlabeled, irradiated T4 DNA (30 krad at 200 μg/ml). The mixture was incubated for 5 min at 37°, the reaction was stopped with 2 ml of ice-cold 0.01 M Tris–0.05 M NaCl solution, immediately filtered through the membrane (Millipore filter, Typ HA, plain, white) and the filter was washed with 40 ml of the same solution. Filters were dried and counted in a liquid scintillation spectrometer (Nuclear Chicago, 40 mg 2,5-diphenyloxazole + 1 mg 1,4-bis-(5-phenyloxazolyl-2)benzene per 10 ml dioxan). B. Relative binding capacity of irradiated T4 DNA to that of nonirradiated DNA *vs.* dose. Provided there is no saturation of enzyme, the relative binding capacity of a given DNA sample can b eexpressed by $((a/b)-1)$, where a is radioactivity on the filter using samples with [^{14}C]DNA only and b is radioactivity on the filter using samples with [^{14}C]DNA + unlabeled DNA. Average values of $((a/b)-1) \pm$S.E. ($n = 24$–30). Irradiation of T4-DNA solution was performed at a concentration of 200 μg/ml in N_2.

Fig. 6. Priming activity and molecular weight of degraded DNA. For determining the priming activity see Fig. 1. AMP incorporation related to the AMP incorporation on untreated DNA. □, samples, degraded by γ irradiation; △, samples, degraded by ultraviolet light; ●, samples, degraded by deoxyribonuclease; ○, samples, degraded by ultrasound.

The relative binding ability of T4 DNA was determined after γ-irradiation in several series of experiments (Fig. 5B). There is a linear increase in the binding capacity with dose. After 12 krad, the binding ability is about 1.6 times higher than the binding capacity of nonirradiated DNA, in agreement with the results obtained by the determination of the molecular weight of the complex. It should be emphasized, that in these binding experiments, the T4 DNA was irradiated at a concentration of 200 μg/ml where the radiosensitivity is 2.5 times higher than in solutions of 500 μg/ml (ref. 6) which concentration had been used in all our other experiments (Figs. 1, 2 and 4).

Nature of the critical event in the DNA strand

Assuming the RNA synthesis to be stopped by a specific lesion in the strand of irradiated DNA, some experiments were performed to describe the nature of this critical event. Various kinds of alterations in the DNA molecule might form this specific lesion: destruction of nucleotide bases, breaks of the single nucleotide strand, breaks of the double helix, crosslinks between the molecules and splitting of hydrogen bonds. To determine the significance of breaks for the loss of priming activity after irradiation, we compared the activity of DNA samples degraded by various agents.

The significance of double breaks as critical event to inhibit the priming ability can be judged from Fig. 6 where the priming activity is plotted *versus* the molecular weight of the various DNA samples. After γ-irradiation priming activity decreases roughly in proportion to molecular weight. Degradation by ultrasound or by deoxyribonuclease, however, exerts a much smaller effect on priming activity; a distinct loss can be seen only at DNA molecules smaller than 10^6 daltons. These observations are in agreement with the result of similar experiments performed by KARKAS AND CHARGAFF[17] and by LLOYD *et al.*[18].

TABLE III

RELATION BETWEEN MOLECULAR WEIGHT AND PRIMING ACTIVITY AT VARIOUS DEGRADED DNA SAMPLES

Priming activity determined as described in the legend to Fig. 1.

Mode of degradation	$M_n \times 10^{-6}$		*Number of single breaks per molecule*	*Priming activity (% of the control)*
	Native	*Denatured*		
Control	6.08	1.50	2.04	100
Deoxyribonuclease	4.91	1.04	2.72	81.1
	4.90	0.57	6.60	89.5
	3.25	0.85	1.83	81.0
	2.90	0.51	3.79	77.6
	2.50	0.54	2.63	81.7
	1.73	0.15	9.5	60.3
Ultrasound	3.04	1.58	0	81.7
	2.18	1.20	0	66.5
	1.58	0.82	0	69.4
	1.27	0.56	0.31	62.8
	0.96	0.46	0.09	58.6
	0.71	0.33	0.15	53.1

The mechanism of degradation by ultrasound is distinctly different from that by nuclease. Whereas ultrasound or shearing break both strands simultaneously[19], the enzyme splits one strand only. Breaks of the double helix after enzymic degradation are produced only when two single breaks occur in opposite positions or at a distance of not more than three nucleotide pairs[6]. For this reason DNA samples degraded by alkaline deoxyribonuclease always carry a number of single breaks in the molecule as shown in Table III. In samples degraded by ultrasound, the molecular weight of denatured DNA is always half that of native material, indicating the absence of single breaks. However, the priming activity of these two kinds of samples does not differ. We conclude, therefore, that single breaks produced by enzymic degradation affect the copying process in RNA synthesis very slightly, if at all. Corresponding conclusions were drawn recently by WARNAAR *et al.*[20].

On the other hand, ultraviolet light is very effectively destroying the priming activity of DNA even before any degradation of the molecule can be measured (Fig. 6).

DISCUSSION

Shape of the inactivation curve of priming activity

An analysis of the inactivation of priming activity can be performed under three different assumptions: (1) The absorbed radiation energy destroys preferentially the binding sites for RNA polymerase. In this case, the dose-effect curve should follow a straight line in a semilogarithmic plot. (2) The inactivation may be due to the fact that γ-irradiation creates new binding sites for the polymerase which, however, are not effective in enzymatic synthesis[5]. Under this assumption the reciprocal relative priming activity of irradiated DNA samples follows the equation[5]

$$A_0/A_z = 1+k_1D/n \qquad (1)$$

where A_0 is the activity of nonirradiated DNA, A_z the activity of irradiated DNA, n the original number of binding sites and k_1 the number of binding sites formed per unit of dose (D in rad). (3) The RNA polymerase synthesizing RNA along the RNA strand is stopped by a specific radiation-induced lesion. To describe the shape of the inactivation curve according to this mechanism, we consider the interval on the DNA strand transcribed by one enzyme molecule. In unirradiated DNA we assign this interval the length L_0. There will be a reduction of this length if a critical lesion is produced by irradiation within this interval of the DNA strand. According to the theory of BRESLER *et al.*[21] concerning the recombination length of DNA for transformation, the interval L_z between the binding site and the critical lesion follows the equation:

$$L_z = L_0\,(1-e^{-z})/z \qquad (2)$$

where z is the number of critical lesions on a DNA strand within the length L_0. Since the amount A of RNA synthesized is proportional to the length L, L_0/L_z equals the reciprocal relative priming ability A_0/A_z. Hence

$$A_0/A_z = z/(1-e^{-z}) \qquad (3)$$

To compare the theoretical inactivation curves resulting from Eqns. 1 and 3 with the experimental data, we plotted the reciprocal relative activity A_0/A_z against the dose (Fig. 7). The experimental points follow approximately a straight line, only in the higher dose range a small deviation can be noticed. A straight line in this kind

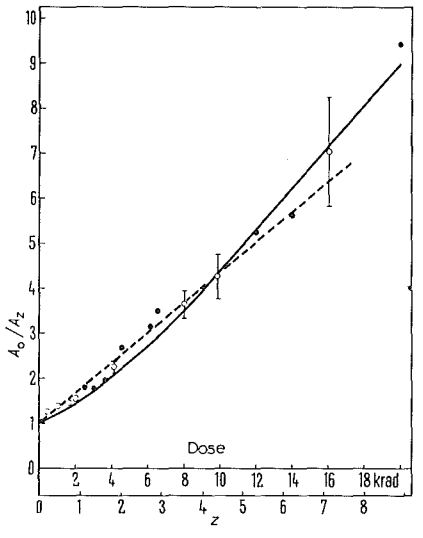


Fig. 7. Plot of reciprocal relative priming activity (A_0/A_z) *vs.* radiation dose. Data of Fig. 1: ●, $\bar{\delta}$. – – –, theoretical curve according to WEISS AND WHEELER[5] (Eqn. 1); ———, theoretical curve according to BRESLER *et al.*[21], z: number of lesions in L (Eqn. 3).

of plot is also obtained according to Eqn. 1, whereas the dose response curve acccording to Eqn. 3 shows a curvilinear shape.

As can be seen in Fig. 7, however, the theoretical curves are very similar and do not permit one to distinguish between the various mechanisms. It is necessary therefore, to consider our experiments concerning the length of RNA chains and the binding capacity of DNA in order to decide which of the mechanisms under discussion is of importance.

Binding capacity and chain length of RNA

In our experiments (Fig. 5 and Table II), the actual number of binding sites formed by irradiation was determined as well as the number and length of RNA chains (Figs. 2 and 3). It was found that the length of RNA chains decreases distinctly after γ-irradiation of the primer DNA, whereas the number of RNA chains is much less affected. From these data the first mechanism of inactivation can be excluded as a sharp decrease in the number of binding sites and in the number of RNA chains would be expected, whereas an increase in the binding sites and only a small decrease in the number of RNA chains was observed in a dose range, in which the priming activity decreases to 30 % of the control (*cf.* Figs. 1, 3 and 5).

According to the second mechanism, new binding sites should be formed by γ-irradiation. Our data allow one to determine the number of binding sites after irradiation. It can be calculated from Fig. 5 that $0.31 \cdot 10^{-7}$ new binding sites are formed per rad and per nucleotide pair when irradiation is performed at 500 μg/ml. On the other hand, this number of new binding sites can also be calculated according to Eqn. 1, provided the proposed mechanism is valid. Since n is known from our binding experiments ($n = 1.25 \cdot 10^{-3}$ binding sites per nucleotide pair), k_1 in Eqn. 1 can be computed from the slope of the dose-effect curve (Fig. 7). We obtain $4.25 \cdot 10^{-7}$

new binding sites per rad and per nucleotide pair ($= k_1$). This calculated value of k_1 is much higher than the actual number ($k_1 = 0.31 \cdot 10^{-7}$). We may conclude, therefore, that the formation of binding sites ineffective for RNA synthesis contributes less than 10 % to the inactivation of priming ability.

The binding capacity of a given DNA sample can also be determined by testing the RNA synthesis with mixtures of untreated and irradiated DNA[22,23]. The addition of irradiated DNA to the reaction mixture containing untreated DNA exerted a strong inhibition of RNA synthesis, since the enzyme is bound to irradiated DNA without being capable to support RNA synthesis. According to the data of ZIMMERMANN *et al.*[22], a degree of competition was observed similar to the one found in our binding experiment with labeled T4 DNA. Apparently, the binding capacity of DNA is enhanced after irradiation also in the presence of nucleotide triphosphates.

The third mechanism remains to be discussed. The pronounced loss of chain length of synthesized RNA suggests that the enzyme is stopped during the process of RNA synthesis by a specific radiation-induced lesion on the DNA strand. The contribution of this mechanism to the total loss of priming activity can be determined comparing the relative reciprocal length of RNA chains L_0/L_z with A_0/A_z (Fig. 4A and Table I). The decrease in chain length of RNA almost equals that in overall priming activity. For instance, at 4 krad, $L_0/L_z = 2.08$ and 2.27 in accordance with the value for $A_0/A_z = 2.27 \pm 0.17$ (S.E.). We may conclude that the loss of priming activity is mainly due to formation of critical lesions along the DNA strand.

Nature of critical lesion

In our experiments concerning the nature of critical lesions, it became obvious that single breaks produced by enzymic degradation do not affect the copying process in RNA synthesis. Critical lesions are abundantly formed, however, by ultraviolet light. It was shown recently by MICHALKE AND BREMER[25] that the pyrimidine dimers formed in ultraviolet-irradiated bacteria stop the transcription *in vivo*. As pyrimidine dimers are not formed after γ-irradiation[26], we have to consider other radiation-induced changes in the DNA strand.

Alterations of the base structure may be not a major factor in the loss of priming activity of DNA. This can be concluded from experiments with DNA samples which have been exposed to hydrogen radicals generated in an electrodeless high-frequency gas discharge[27]. During this exposure, much more bases are damaged in comparison to the formation of single breaks (relation 20 : 1), whereas after γ-irradiation the number of altered bases is slightly higher than the number of breaks (relation 1.6 : 1). The loss of priming activity of a treated DNA sample corresponds to the number of single breaks, respectively, not to that of altered bases[27]. Similar conclusions were drawn by WEISS AND WHEELER[5] from experiments on the base composition of RNA synthesized with irradiated DNA as primer.

We suggest, therefore, that a single break induced by ionizing radiation stops the process of transcription. Whereas by enzymic degradation only a single phosphoester bond is hydrolyzed, several chemical reactions occur in the case of radiation-induced single breaks. As shown recently by SIMON[28] in connexion with the break, either the nucleotide base bound to the broken sugar or the whole nucleoside with oxydized sugar are lost. This deficient base in the strand may actually be responsible for the inhibition of RNA synthesis.

Effect of ultraviolet light on the priming activity

In an earlier paper[4], ultraviolet light was found to be a strong inhibitor of the priming activity of DNA. As shown in Figs. 2 and 3 and in Table I, the chain length of synthesized RNA as well as the number of RNA chains are distinctly reduced after ultraviolet irradiation (Fig. 3B). In contrast to the effect of γ-irradiation, we have to assume that either ultraviolet light preferentially destroys the specific binding sites for RNA polymerase or, alternatively, that new binding sites are formed which are no longer capable of giving rise to RNA chains. The formation of new binding sites by ultraviolet light has already been demonstrated by ZIMMERMANN *et al.*[22] who showed that RNA synthesis on untreated DNA is strongly inhibited by ultraviolet-irradiated DNA. The reduction in chain length of RNA synthesized on ultraviolet-irradiated DNA supports the hypothesis that there are specific lesions in the ultraviolet-irradiated DNA which can stop the process of RNA synthesis. Since ultraviolet irradiation causes only a few breaks in DNA (Fig. 6), we may conclude in agreement with previous discussions[4,25] that the dimers formed between neighbouring pyrimidines have to be considered as such specific lesions.

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REFERENCES

1 H. HARRINGTON, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 59.
2 F. K. ZIMMERMANN, H. KRÖGER, U. HAGEN AND K. KECK, *Biochim. Biophys. Acta*, 87 (1964) 160.
3 J. J. WEISS AND C. M. WHEELER, *Nature*, 203 (1964) 291.
4 U. HAGEN, K. KECK, H. KRÖGER, F. K. ZIMMERMANN AND TH. LÜCKING, *Biochim. Biophys. Acta*, 95 (1965) 418.
5 J. J. WEISS AND C. M. WHEELER, *Biochim. Biophys. Acta*, 145 (1967) 68.
6 U. HAGEN, *Biochim. Biophys. Acta*, 134 (1967) 45.
7 M. CHAMBERLIN AND P. BERG, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 81.
8 E. E. PETERSEN, H. KRÖGER AND U. HAGEN, *Biochim. Biophys. Acta*, 161 (1968) 325.
9 TH. COQUERELLE, L. BOHNE, U. HAGEN AND J. MERKWITZ, *Z. Naturforsch.*, 24b (1969) 885.
10 J. P. RICHARDSON, *J. Mol. Biol.*, 21 (1966) 83.
11 O. W. JONES AND P. BERG, *J. Mol. Biol.*, 22 (1966) 199.
12 G. HOTZ, Habilitationsschrift, Karlsruhe, 1965.
13 C. G. KURLAND, *J. Mol. Biol.*, 2 (1960) 83.
14 U. MAITRA AND J. HURWITZ, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 815.
15 N. W. STEAD AND O. W. JONES, *J. Mol. Biol.*, 26 (1967) 131.
16 N. W. STEAD AND O. W. JONES, *Biochim. Biophys. Acta*, 145 (1967) 679.
17 J. D. KARKAS AND E. CHARGAFF, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 664.
18 R. H. LLOYD, B. H. NICHOLSON AND A. R. PEACOCKE, *Biochem. J.*, 104 (1967) 999.
19 P. F. DAVISON AND D. FREIFELDER, *J. Mol. Biol.*, 16 (1966) 490.
20 S. O. WARNAAR, G. MULDER, I. VAN DEN SIGTENHORST-VAN DER SLUIS, L. W. VAN KESTEREN AND J. A. COHEN, *Biochim. Biophys. Acta*, 174 (1969) 239.
21 J. E. BRESLER, V. L. KALININ AND D. A. PEROMOV, *Biopolymers*, 2 (1964) 135.
22 F. K. ZIMMERMANN, H. KRÖGER AND TH. LÜCKING, *Biochem. Z.*, 342 (1965) 115.
23 H. KRÖGER AND L. SCHUCHMANN, *Biochem. Z.*, 346 (1966) 191.
24 J. P. RICHARDSON, *J. Mol. Biol.*, 21 (1966) 115.
25 H. MICHALKE AND H. BREMER, *J. Mol. Biol.*, 41 (1969) 1.
26 A. WACKER AND E.-R. LOCHMANN, *Z. Naturforsch.*, 17b (1962) 351.
27 H. JUNG, U. HAGEN, M. ULLRICH AND E. E. PETERSEN, *Z. Naturforsch.*, in the press.
28 M. SIMON, *Intern. J. Radiation Biol.*, in the press.
29 J. P. RICHARDSON, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 1616.