Effect of Cross-Linking on the Secondary Structure of DNA I. Cross-Linking by Photodimerization†

Rainer Herbeck, Tain-Jen Yu, and Warner L. Peticolas*

ABSTRACT: An investigation has been made into the effect produced by photo-induced pyrimidine cross-links upon the secondary structure of DNA. We have studied the effect of uv irradiation upon the B ⇄ A transition in DNA brought about by a change of solvent from 70 to 80% ethanol. Circular dichroism (CD) was used to monitor the conformational changes. However, we first showed by means of laser Raman spectroscopy that CD is a reliable monitor of the conformational change, even though the DNA is aggregated in 80% alcohol solutions. It is suggested that this aggregation stabilizes the A form through lateral interaction between the helices. The uv irradiation experiments show that pyrimidine-dimer cross-links induced into the B-form DNA will lock it irreversibly into that conformation and prevent it from going to the A form in 80% EtOH solution. The A-form DNA can tolerate a few cross-links but converts cooperatively to the B form if a larger number of cross-links is introduced. Irradiation-induced pyrimidine cross-links create locally denatured regions in B-form DNA. Upon continued irradiation, the entire DNA molecule becomes denatured.

The question of the secondary and tertiary structure of DNA in eukaryotic cells is one which is difficult to answer. Virtually all of the work on the secondary structure of DNA has been done by x-ray crystallographers using diffraction techniques on oriented fibers or in solution. Although a variety of forms of nucleic acids and polynucleotides has been reported (Arnott, 1970, 1971; Bram, 1971a,b), it is not possible to always be certain exactly which form is present in the cell. In this paper we will restrict ourselves to a simple classification of the various helical forms of DNA which has been suggested by Ivanov et al. (1973, 1974). This classification consists of two families, the A family and the B family. The latter includes those nucleic acids whose ring pucker is of the C(2)-endo or the closely related C(3)-exo type.

Recently Raman spectroscopy has been shown to distinguish between the two conformations of DNA, and Raman spectroscopy may now be regarded as a reliable method for the determination of the helical secondary (A or B) structure of DNA (Erfurth et al., 1972; Erfurth and Peticolas, 1975).

Almost all DNA samples are isolated from DNA–histone complexes in the cell by methods which probably change the tertiary structure and which might well change the secondary structure of the DNA. Consequently any measurements of the secondary structure on such preparations would not necessarily be indicative of the in vivo structure in the cell. In this work we have considered the possibility of stabilizing the secondary and tertiary structures of DNA directly in the cell by means of chemically or photochemically produced cross-links. Hammer et al. (1957) have previously shown that both the secondary and tertiary (sherulitic) structure of synthetic polymers can be stabilized against thermal or solvent disordering by radiation-induced chemical cross-links. Thus we have set about to determine the effect of cross-linking on the stability of its secondary structure of DNA.

There are two well-known methods of producing cross-links in DNA. The one which we have used in this study is ultraviolet irradiation which creates pyrimidine dimers in DNA, of which the largest proportion are thymine dimers (Wacker, 1963; Setlow, 1964a,b; Smith, 1964; Setlow and Carrier, 1963, 1966; Johns et al., 1964). These dimers are cross-links between adjacent thymine (pyrimidine) residues on the same chain and lead to a decrease in the ultraviolet absorbance maximum at 260 nm as well as a decrease in the melting transition temperature, T_m.

Until recently, it has only been possible to study the B ⇌ A transition in oriented fibers by changing the relative humidity of the environment. However, studies of the circular dichroism of solutions of DNA in ethanol–H_2O mixtures have led several investigators to suggest that the secondary structure of DNA goes from the B family to the A family as the fraction of ethanol goes from about 70 to 80% (Brahms and Mommaerts, 1964; Ivanov et al., 1973, 1974; Girod et al., 1973). Unfortunately, while the interpretation based on CD[1] spectra are strongly suggestive, it is not rigorously convincing because of the possibility of aggregation leading to an artifact in the CD spectra. This point has been emphasized by Girod et al., 1973. Consequently, we have set about to determine by means of ultracentrifugation if, in 80% alcohol solutions, aggregation of DNA does in fact occur and, if so, to use Raman spectroscopy as a means of confirming or rejecting the A-type conformation in these solutions and to examine the validity of the CD measurements in solutions of DNA aggregates. Then we have attempted to stabilize the two forms of DNA against further changes by means of photodimerization and have investigated changes in conformation induced by photodimerization.

† From the Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received August 15, 1975.* This work was supported in part by grants from the U.S. Public Health Service (GM 15547) and the National Science Foundation (GB-29709). Dr. Herbeck holds a postdoctoral fellowship No. 430/402/518/5 from the Deutscher Akademischer Austauschdienst, West Germany.

[1] Abbreviations used: uv, ultraviolet; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Sedimentation Constants $s_{w,20}$ of Calf Thymus DNA before and after Irradiation in 70 and 80% EtOH Solutions.

Irradiation Time (min)	Irradiation Solvent (% EtOH)	Sedimentation Solvent (% EtOH)	Sedimentation Constant $s_{w,20}$
0	0	0	15
0	70	70	15
0	80	80	59
2	70	70	14
2	80	80	55
2	70	80	236
2	80	70	46

Experimental Procedure

Highly polymerized calf thymus DNA, obtained from Worthington Biochemical Corporation, was used throughout these experiments. The DNA was washed at 2 °C with 70% ethanol–H_2O solutions containing 10^{-5} M EDTA to remove cations and salt. The DNA was then dried. All the experiments except the Raman experiments were performed on dilute solutions of DNA. These solutions were prepared by dissolving the DNA in 10^{-3} M NaCl solution to give an OD of 5.0. The samples were then diluted with alcohol and water to the proper alcohol concentrations with a final OD of between 0.8 and 1.0, and a final salt concentration of 2×10^{-4} M.

The irradiation experiments were performed using a low-pressure resonance mercury lamp made by Ultra Violet Products. It had an output at 253.7 nm of 2×10^5 ergs mm^{-2} min^{-1} or 2×10^4 J m^{-2} min^{-1}. Thus although all of our data are presented as a function of the time of irradiation, this factor can be used to convert from time to energy per mm^2.

To obtain a rough estimate of the number of cross-links per 100 residues, we have ascribed all of the loss in optical absorbance of the DNA upon irradiation to the formation of pyrimidine dimers. We have neglected the absorbance of the dimers since measurements on purified thymine dimers show that the residual absorbance of the dimer at 260 nm is no more than 1–2% of the unirradiated material (Johns et al., 1964). Calf thymus DNA is 50% purine (30% G, 20% A) and 50% pyrimidine (30% C, 20% T). Consequently, the molar absorbance of the DNA (ϵ_{DNA}) is the average of the molar absorbances of the purines (ϵ_p) and the pyrimidines (ϵ_{py}); i.e., $\epsilon_{DNA} = 0.5\epsilon_p + 0.5\epsilon_{py}$. Since the molar absorbance of the purines is 1.4 times that of the pyrimidines, we may estimate ϵ_{py} from the equation, $\epsilon_{DNA} = 6.6 \times 10^3 = (0.5)(1.4)\epsilon_{py} + 0.5\epsilon_{py}$, to obtain $\epsilon_{py} = 5.5 \times 10^3$.

The number of moles of pyrimidine molecules lost upon irradiation is therefore $\Delta OD/(5.5 \times 10^3)$. The moles of total nucleotide initially present in the DNA is $OD/(6.6 \times 10^3)$. Consequently the number of cross-links per 100 nucleotide residues is very approximately

$$n_x = \frac{OD \times 6.6 \times 10^3 \times 100}{2 \times 5.5 \times 10^3} = \frac{60(\Delta OD)}{OD} \qquad (1)$$

Using this formula we have obtained a rough upper limit of the number of cross-links per 100 nucleotide units.

Absorbance measurements were made in a Cary 14 CM spectrophotometer using 1-cm pathlength cells.

The ultracentrifugation experiments were performed in a Beckman ultracentrifuge, Model E. The sedimentation velocity was computed from the formula $s_{w,20} = (1/r\omega^2)(dr/dt)$ where r is the radial distance of the sample and ω is the circular frequency.

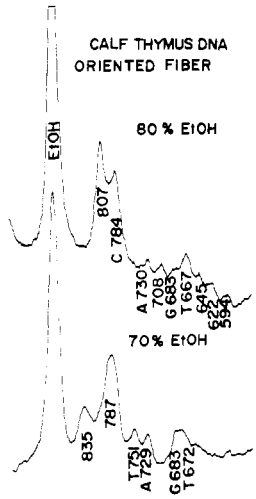

FIGURE 1: Raman spectra of an oriented fiber of calf thymus DNA in 80 and 70% EtOH. The region of the spectrum from 672 to 835 cm^{-1} is sufficient to clearly identify the oriented fiber as being in the A form in 80% EtOH and in the B form in 70% EtOH.

Circular dichroism measurements were made with a 6003 CD attachment to a Cary 60 spectropolarimeter equipped with an Ushio uxL-451 lamp. Samples were measured in 1-cm pathlength cells. Only solutions which showed no turbidity by eye were used. Baselines of the appropriate alcohol–water–salt solution were run for each measurement, and no adjustment of the pen position was made between baseline and sample runs.

Results

The results of the ultracentrifugation experiments are shown in Table I. As suggested by Girod et al. (1973), there appears to be considerable aggregation of the DNA when the concentration of the ethanol is brought to 80%. The s value for our sample of CT DNA in aqueous salt solutions (10^{-4} M NaCl) is about 15 ± 1. In 70% EtOH, the s value we obtained is also 15, showing little if any change in aggregation due to added ethanol. However, at 80% alcohol concentration, the s value increases to about 60. This increase by a factor of 4 in the sedimentation constant clearly indicates the presence of aggregates in dilute solution of DNA in 80% EtOH. This is just what is to be expected from the work of Erfurth and Peticolas (1975) and Bram and Baudy (1974) who show that the A form is stabilized by lateral interactions between the DNA chains. Indeed it seems to be apparent that DNA cannot even exist in the A form as a single isolated chain in solution. Also included in Table I are the S values after uv irradiation. These results will be discussed later.

Since the existence of aggregates of DNA in 80% EtOH solutions might lead to artifacts in the interpretation of CD data, it is desirable to have an independent investigation of the secondary conformation of DNA in 80% EtOH solution. Consequently we have taken the Raman spectra of DNA fibers in 70 and 80% EtOH solutions. Since orientation plays a role in the amount of A form in a sample, we have prepared both oriented and unoriented fibers according to the method described by Erfurth and Peticolas (1975) and submerged these fibers in the corresponding 70 and 80% EtOH solutions. The Raman spectra of these fibers are shown in Figures 1 and 2. Plainly both the oriented and unoriented

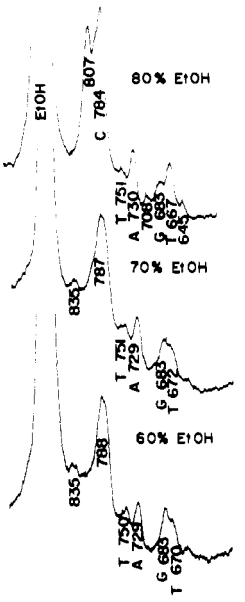

FIGURE 2: Raman spectra of an unoriented fiber of CT DNA in 60, 70, and 80% alcohol. The DNA is in the B family of conformations at 60 and 70% EtOH, but it is in the A family at 80% EtOH.

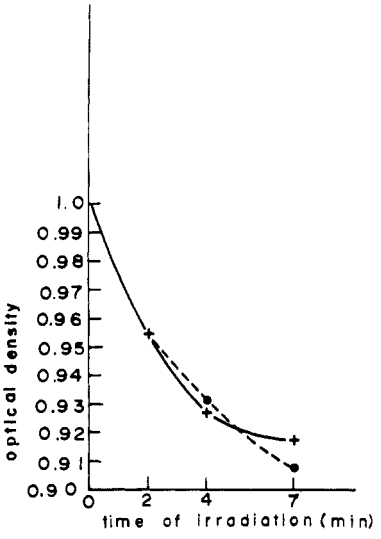

FIGURE 3: Decrease in the OD_{max} at 260 nm for CT DNA in 70 and 80% EtOH vs. time of uv irradiation at 253.7 nm. Solid curve: 70% EtOH. Dashed curve: 80% EtOH.

samples show a band at 807 cm^{-1} which is due to the presence of the A-form DNA in 80% alcohol. It is of interest that, in the oriented fiber, the 807-cm^{-1} band is stronger than the 784-cm^{-1} band due to a ring vibration of the cytosine base, while in the unoriented fibers the 807-cm^{-1} band is weaker. We interpret this result to mean that, in the oriented fiber, there is a higher degree of order of the A-form structure than in the unoriented fibrous material.

The Raman spectrum of DNA in 60% EtOH is shown as the third spectrum of Figure 2. Girod et al. (1973) have suggested that, in 60% EtOH, the DNA is in the C form. From our Raman measurements we cannot tell the difference between the B and C forms. Both belong to the B family insofar as they have B-type C(3)-exo or C(2)-endo ring pucker. However, since the reported CD spectrum at 60% EtOH is plainly different from that at 70%, it may be that the C form is present. In Figure 2, the broadening of the

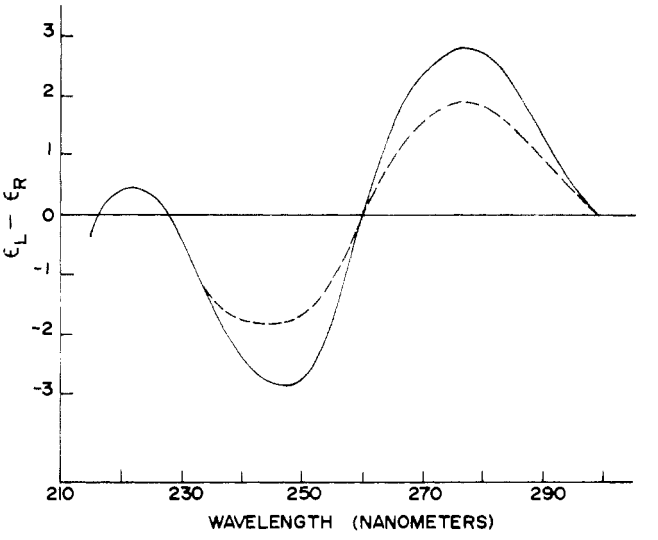

FIGURE 4: CD spectra of CT DNA in 70% EtOH. The solid curve is before uv irradiation, while the dashed curve is after uv irradiation for 4 min.

787-cm^{-1} band in 70% EtOH over that in 60% EtOH may be due to a weak shoulder at 807 cm^{-1}. Consequently, it seems possible that the CD of 70% EtOH may be due to a mixture of the A and C forms which gives rise to CD spectrum which resembles the B form. Thus, we may conclude with reasonable certainty that DNA does go from the B family (including the C form) to the A family in going from 70 to 80% alcohol solutions.

Figure 3 shows a plot of the decrease in the optical density maximum at 260 nm in 70 and 80% EtOH solution as a function of uv irradiation time. A measurement of the optical density change at 270 nm gave essentially similar results. The number of cross-links per 100 nucleotide residues for a specific time of irradiation may be calculated from eq 1. There is very little difference in the amount of OD change of the A and B forms upon irradiation. However, the changes in the CD spectra of A form and B form DNA after uv irradiation are completely different. The CD spectrum (i.e., the maximum at 275 nm) of DNA in 70% EtOH solution decreased in intensity linearly with irradiation. Figure 4 shows the CD spectrum of CT DNA in 70% EtOH before and after irradiation for 4 min. In each case the calculated CD spectrum is based on the total concentration of bases as determined by the uv absorption spectrum (see Experimental Section). Consequently this decrease in CD must represent some slight conformational change in the B form brought about by pyrimidine cross-links. After a very long time of irradiation (10 min), the CD spectrum of the DNA resembles the CD spectrum of denatured DNA (Johnson and Girdon, 1974).

The behavior of the A-form DNA in 80% EtOH is much more complex. Irradiation for short times (less than 2 min with our lamp) results in actually no change in the CD spectrum, although the decrease in the optical density is comparable to that for the B form. For example after uv irradiation for 2 min the CD maximum at 270 nm had decreased by less than 1%, while the optical density decreased by almost 5% (see Figure 3). However, in the next 2 min, the positive long wavelength maximum of the CD spectrum decreased rapidly to that of the corresponding irradiated B-form DNA and the band maximum shifted from 270 to 275 nm, the usual wavelength for B-form DNA. The large

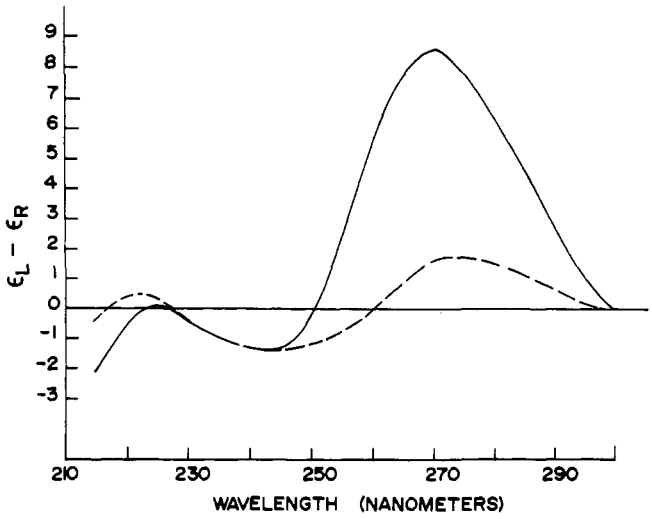

FIGURE 5: The CD spectra of CT DNA in 80% EtOH. (Solid curve) Before irradiation and after irradiation for 2 min; (dashed curve) after uv irradiation for 4 min.

changes after irradiation for 4 min can be seen in Figure 5, where the CD spectra of DNA in 80% EtOH before and after 2- and after 4-min uv irradiation are shown.

Figure 6 shows a plot of the $(\epsilon_L - \epsilon_R)_{max}$ of the long wavelength positive band of both the A-form and B-form DNA vs. time of irradiation in minutes. The slow steady decrease of the maximum of the B form is clearly apparent as is the sharp cooperative change in the maximum of the A form after about 3 min.

Discussion

Our interpretation of these results is as follows. It is important to note that the decrease in the long wavelength CD of B-type DNA is linear with irradiation dosage and, therefore, the conformation changes continuously from B form to fully denatured form. It appears that this decrease in CD is due to the introduction of pyrimidine cross-links. This explanation is in agreement with the interpretation of Shafranovskaya et al. (1973). As the density of the locally denatured regions gets higher and higher with longer irradiation times, the CD spectra resembles more and more that of fully denatured DNA.

On the other hand, there is no measurable change in the CD of A-type DNA during the first 2 min of irradiation when about 2 cross-links per 100 nucleotide residues are introduced. However, as the density of cross-links is increased above about 2 cross-links per 100 residues, the A form can no longer maintain its rigid conformation and it is converted cooperatively to the B form, with locally denatured regions. At this point the conformation of the DNA in 70 and 80% EtOH is identical. Furthermore the DNA is locked into the B form and cannot again be put back into the A form.

To show that the introduction of even a few cross-links by means of photodimerization in 70% alcohol locks the conformation irreversibly into the B form, we took a CT DNA solution in 70% EtOH after irradiation for 2 min and increased the alcohol concentration to 80%. The dashed curve in Figure 7 is the resulting CD spectrum. Clearly after 2-min irradiation in 70% EtOH, the DNA does not go into the A form in 80% EtOH solution. For comparison, the CD spectrum of DNA in 80% EtOH after 2-min irradiation in the same solution is also given in Figure 7 (solid line). Thus

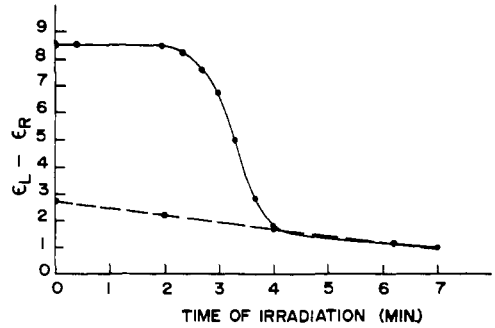

FIGURE 6: A plot of the $\Delta\epsilon_{max}$ for the long wavelength (270 nm) positive band in the CD spectra of A-form (top curve) and B-form DNA (bottom line) as a function of the time of irradiation.

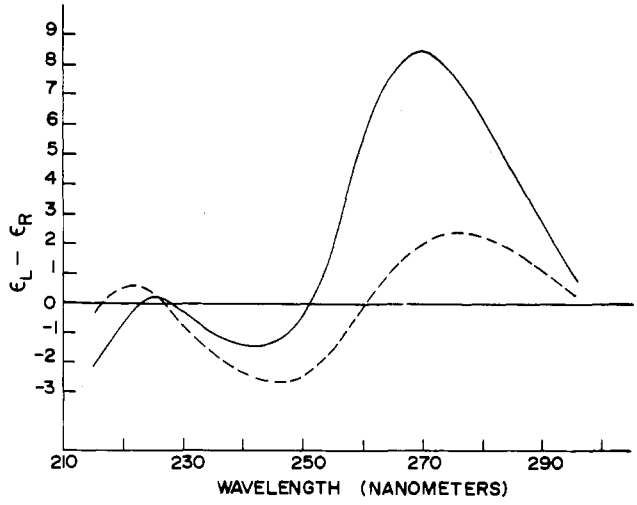

FIGURE 7: CD spectra of CT DNA in 80% EtOH solution after uv irradiation for 2 min. Solid curve is after irradiation in 80% EtOH; dashed curve is after irradiation at 70% EtOH and the concentration of EtOH increased to 80%.

we see that, at low levels of uv irradiation, the A form persists in DNA originally at 80% EtOH solution, but that the B form irradiated in 70% EtOH solutions is locked in irreversibly.

We have seen in Figures 5 and 6 how irradiation of the A form in 80% EtOH for 2 min or less leaves the DNA in the A form. In view of Figure 6, it is to be expected that this A-form DNA would revert to the B form if the alcohol concentration is adjusted to 70%, and we have indeed found this to be true. The CD spectrum of DNA irradiated in 80% EtOH for 2 min and changed to 70% EtOH is completely identical, within the precision of our measurements, with DNA irradiated in 70% EtOH for 2 min.

Finally we may discuss the possibility of artifacts due to aggregation of the DNA. Certain lots of DNA give solutions in 80% EtOH which are slightly turbid to the eye. Such samples, as discussed in the Experimental Section, were discarded. Other lots of DNA gave clear solutions in alcohol–H_2O mixtures. The sedimentation constants measured in Table I were made on clear solutions but, as was discussed above, still showed measureable aggregation. However, such aggregation does not appear to affect the CD spectra of the solution. For example, in Table I we see that irradiation does not seem to affect the state of aggregation so long as the solvent is not changed. Thus the S value in 80% alcohol after irradiation for 2 min changes from 59 to 55, while that in 70% alcohol changes from 15 to 14. On

the other hand DNA irradiated at 70% EtOH and changed to 80% EtOH gives an S value of 236 which is quite large. However, even such a large aggregate still shows the B-type spectrum and not the A-type. Consequently whether one observes the A- or B-type CD spectrum appears to depend only upon the secondary structure of the DNA and is independent of the state of aggregation so long as obviously turbid samples are discarded.

Conclusion

In conclusion we have shown that pyrimidine cross-links induced by photodimerization irreversibly lock the DNA into the B form. If the DNA is in the A form initially, it will be forced into the B form. No aggregation of DNA appears to occur in 70% EtOH but does occur in 80% alcohol. The aggregation probably stabilizes the A-form DNA. In spite of the aggregation, the CD spectra give a correct indication of the A or B form as has been confirmed by laser Raman measurements. Pyrimidine cross-links induced by uv irradiation create locally denatured regions in B-form DNA.

References

Arnott, S. (1970), *Prog. Biophys. Mol. Biol. 21,* 265.

Arnott, S. (1971), *Prog. Biophys. Mol. Biol. 22,* 179–213.

Beabealashvily, R. Sh., Ivanov, V. T., Michenkova, L. E., and Savotchkina, L. P. (1972), *Biochim. Biophys. Acta 259,* 35.

Brahms, J., and Mommaerts, W. F. H. M. (1964), *J. Mol. Biol. 10,* 73.

Bram, S. (1971a), *J. Mol. Biol. 58,* 277.

Bram, S. (1971b), *Nature (London), New Biol. 233,* 161.

Bram, S., and Baudy, P. (1974), *Nature (London) 250,* 414.

Erfurth, S. C., Kiser, E. J., and Peticolas, W. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 938.

Erfurth, S. C., and Peticolas, W. L. (1975), *Biopolymers 14,* 247.

Girod, J. C., Johnson, W. C., Jr., Huntington, S. K., and Maestre, M. F. (1973), *Biochemistry 12,* 5092.

Hammer, C. F., Brandt, W. W., and Peticolas, W. L. (1957), *J. Polymer Sci. 24,* 291.

Ivanov, V. I., Minchenkova, L. E., Minyat, E. E., Frank-Kamenetskii, M. D., and Schyolkina, A. K. (1974), *J. Mol. Biol. 87,* 817.

Ivanov, V. I., Minchenkova, L. E., Schyolkina, A. K., and Poletayev, A. I. (1973), *Biopolymers 12,* 89.

Johns, H. E., Pearsons, M. L., LeBlane, J. C., and Helleiner, C. W. (1964), *J. Mol. Biol. 9,* 503–524.

Johnson, W. C., Jr., and Girdon, J. C. (1974), *Biochim. Biophys. Acta 353,* 193.

Mokulsky, M. A., Kapitonova, K. A., and Mokulskaya, T. D. (1972), *Mol. Biol. (Moscow) 6,* 883.

Setlow, R. B. (1964a), in Mammalian Cytogenetics and Related Problems in Radiobiology, Pavan, C., Chagas, C., Frota-Pesspa, D., and Caldas, L. R., Ed., Oxford, Pergamon Press, p 291.

Setlow, R. B. (1964b), *J. Cell. Physiol., Suppl. 1 64,* 51.

Setlow, R. B., and Carrier, W. L. (1963), *Photochem. Photobiol. 2,* 49.

Setlow, R. B., and Carrier, W. L. (1966), *J. Mol. Biol. 17,* 237.

Shafranovskaya, N. N., Trifonov, E. N., Lazurkin, Yu. S., and Frank-Kamenetskii, M. D. *Nature (London), New Biol. 241,* 58.

Smith, K. C. (1964), *Photophysiology 2,* 329.

Wacker, A. (1963), *Prog. Nucleic Acid Res. 1,* 369.

Wang, J. C., and Saucier, J.-M. (1972), *Nature (London) New Biol. 239,* 167.