internally hydrogen-bonded configuration; and that in bradykinin it spends more time in that configuration. In other words, the secondary structure of these peptides as revealed by CD is a time average of two rapidly interconnecting structures—a disorder structure and a partially ordered one in which a proline residue is internally hydrogen bonded. Given this hypothesis, the CD spectrum of bradykinin was simulated assuming that one of its proline residues spends a quarter of its time internally hydrogen bonded over and above the time spent in that configuration in the analog. Simulation was accomplished by adding to the CD spectrum of the analog the normalized spectrum of two moles of acetylphenylalaninamide in its nonhydrogen-bonded configuration and one-fourth of the normalized difference spectrum of acetylprolinemethylamide produced by dioxane referred to water as calculated from the curves presented in Figure 4A. The simulated spectrum (curve c of Figure 5) is in striking agreement with the observed one (curve a) with respect to both the location of the bands and crossovers and the ratio of amplitudes of the bands ($[\theta]_{234}/[\theta]_{221} = 0.7 \pm 0.1$ for simulated and 0.89 ± 0.03 for observed spectrum), even though Gly replaces Ser in the analog.

In order to test these ideas experimentally and to define more precisely the secondary structure of bradykinin in solution, the author in collaboration with Dr. John M. Stewart has initiated an investigation of the CD behavior of bradykinin, its analogs, its peptide fragments, and model compounds. Results obtained to date are in accord with the views expressed above.

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Physical Studies on Deoxyribonucleic Acid after Covalent Binding of a Carcinogen†

Robert Fuchs and Michel Daune*

ABSTRACT: DNA from various sources was reacted with *N*-acetoxy-*N*-2-acetylaminofluorene and the physical properties of modified DNA were studied. A simple and rapid spectrophotometric method for the determination of fixed carcinogen on native DNA is proposed. Circular dichroism and melting curve analysis showed that modified bases are shifted outside the double helix, while the fixed carcinogen is inserted. Viscosity and light-scattering studies indicated that the fixation of *N*-acetoxy-*N*-2-acetylaminofluorene induces hinge points in the DNA molecule. The existence of cross-links in DNA reacted with carcinogen was demonstrated by the helix–coil transition and light scattering. The stability of these cross-links was studied as a function of pH and temperature. It was found that cross-links were destroyed at high pH values and elevated temperature. The importance of varying amounts of guanine in different DNA samples on cross-link formation is also demonstrated.

It is well established that several hepatic carcinogens bind *in vivo* to liver nucleic acids, proteins and carbohydrates (Miller and Miller, 1967; Farber *et al.*, 1967). AAF[1] is a potent liver carcinogen which, after metabolic activation, binds to liver tRNA, rRNA, DNA, and proteins when administered *in vivo* (Miller *et al.*, 1961; Marroquin and Farber, 1962; Henshaw and Hiatt, 1963; Marroquin and Farber,

† From the Centre de Recherches sur les Macromolecules, 67 Strasbourg, France. *Received October 4, 1971.* Supported by a grant of Ligue Nationale Française contre le Cancer.

[1] Abbreviations used are: AAF, *N*-2-acetylaminofluorene; *N*-OH-AAF: *N*-hydroxy-*N*-2-acetylaminofluorene; *N*-AcO-AAF: *N*-acetoxy-*N*-2-acetylaminofluorene; T_m^{260} and T_m^{305}, melting temperature at 260 and 305 nm, respectively; $(H_{DNA}^{260})_c$, DNA hyperchromicity of variously reacted DNA at 260 nm; $(H_{DNA}^{260})_c$, is a function of the percentage of modified bases; H_{DNA+c}^{260}, total hyperchromicity at 260 nm; A_c^{260}, A_c^{305}, fixed carcinogen absorbances at 260 and 305 nm; A_{DNA+c}^{260}, A_{DNA+c}^{305}, total absorbances of carcinogen reacted DNA at 260 and 305 nm; $(A_{DNA}^{260})_c$, contribution of the DNA in absorbance at 260 nm, when carcinogen is bound to DNA; ΔA_{DNA+c}^{260}, total absorbance increase at 260 nm for reacted DNA samples due to thermal helix–coil transition; $(\Delta A_{DNA}^{260})_c$, contribution of the DNA to the thermal-induced increase of absorbance at 260 nm, when the carcinogen AAF is bound to DNA; index $c = 0$ is relative to nonreacted DNA.

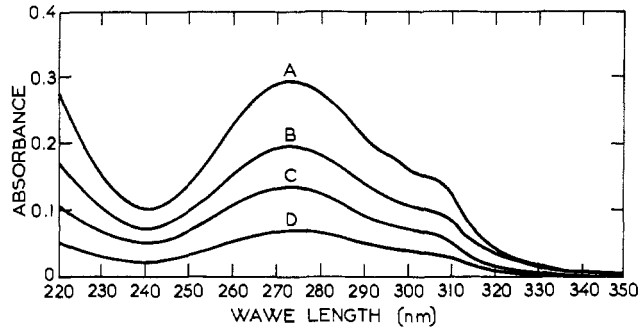

FIGURE 1: Differential spectrum between native calf thymus DNA and samples reacted with various amounts of carcinogen for constant DNA concentration. The respective modified base percentages for A, B, C, and D were 13,0, 8.4, 6.0, and 3.6. 2×10^{-3} M sodium citrate buffer (pH 7) was used.

1965; Irving *et al.*, 1967; Kriek, 1968; Agarwal and Weinstein, 1970; De Baun *et al.*, 1970).

The carcinogenic effect of aromatic amines has been shown (Miller, 1970) to be related to their strong electrophilic metabolites. In the case of AAF the active derivative has been identified (Cramer *et al.*, 1960; Miller and Miller, 1967, 1969) as an ester of *N*-OH-AAF.

The reaction site of DNA treated with *N*-AcO-AAF was demonstrated (Kriek *et al.*, 1967) to be the C_8 of guanine. Recent work (Miller and Miller, 1969; Kapuler and Michelson, 1971; Kriek and Reitsema, 1971; I. B. Weinstein, personal communication) suggests that adenine residues also react to some extent with *N*-AcO-AAF. The results obtained by Kriek and Reitsema (1971) indicate that the reaction between adenine and *N*-AcO-AAF appears to depend largely on the conformation of adenine in the polynucleotides. On the other hand, in a study on the mutagenic effect of *N*-AcO-AAF on transforming DNA, Maher *et al.* (1968), using density gradient sedimentation techniques, observed the formation of a small amount of DNA which appears to be cross-linked.

In the present investigation we studied local changes of conformation in carcinogen-reacted DNA from various sources by helix–coil transition induced by temperature or pH, and circular dichroism. Base pair opening induced by carcinogen binding was observed and the degree of cross-linking was shown to be related to the base composition.

On the other hand, morphological studies (viscosity, light scattering) enabled us to demonstrate the existence of hinge points induced by carcinogen in DNA. A spectrophotometric method for the determination of bonded carcinogen is presented.

Material and Methods

All chemicals were reagent grade (Merck). Native DNA was prepared from calf thymus (Kay *et al.*, 1952), *Hemophilus influenzae* and *Micrococcus lysodekticus* (Bach *et al.*, 1966) and had the following characteristics: hyperchromicity at 260 nm, 40, 43, and 33%; $s_{20,w}$, 21, 22, and 19 S; ϵ_P^{260} 6420, 6380, and 6960, respectively. Protein content was lower than 0.8% in weight in all samples. Native DNA samples were sonicated in 0.1 M NaCl (pH 7) during 4 min at 20 kHz (Litzler, 1967) to yield molecule fragments of about 500,000.

N-AcO-AAF has been synthetized as previously described (Lotlikar *et al.*, 1966). Reaction of DNA with *N*-AcO-AAF was performed according to Miller *et al.* (1966) with minor modifications (Fuchs and Daune, 1971).

The melting curves of native and carcinogen-reacted DNA were measured at 260 and 305 nm with a recorder indicating absorbance *vs.* temperature (Wilhelm *et al.*, 1970). Sodium citrate buffer (2×10^{-3} M, pH 7) was used, except when indicated. Melting curves for neutralized alkaline denatured DNA (Studier, 1965) were also registered.

Light-scattering measurements were performed with a photogoniodiffusiometer FICA (Wippler and Scheibling, 1954) at 5460 Å using benzene as reference. Refractive index increments (dn/dc) were measured with a Rayleigh interferometer (Zeiss). To avoid aggregate formation (R. Fuchs, unpublished data), DNA was denatured by heating the sample in sodium borate buffer (3×10^{-3} M, pH 9.0), containing 1% HCHO (previously treated, Freifelder and Davison, 1963) for 20 min at 5° over T_m^{260}.

Circular dichroism spectra were recorded with a Jouan II dichrograph. Viscosities were determined in a Couette viscometer of Zimm-Crothers type, equipped with an automatic recording device (Prunell and Neimark, 1971). Polarization of fluorescence was measured by means of a polarimeter similar to that described by Weber (1956).

Determination of the Percentage of Modified Bases in Carcinogen-Reacted DNA. Reacted DNA spectrum presents a characteristic shoulder near 305 nm which increases with the amount of fixed fluorene residues (Miller *et al.*, 1966). In recent work (Fuchs and Daune, 1971) we observed a cooperative melting curve at 305 nm in reacted DNA, which implies an interaction of the fluorene residues with the internal field of the double helix. This phenomenon enables us to define an hyperchromicity value at 305 nm (H_c^{305}). On the other hand, Kriek *et al.* (1967) have determined the specific extinction coefficient of 8-(*N*-2-fluorenylacetamido)guanosine (reaction product of guanosine and *N*-AcO-AAF). They found a value of 18,000 at 305 nm. 8-(*N*-2-Fluorenylacetamido)-deoxyguanosine has the same specific extinction coefficient at this wavelength (Kapuler and Michelson, 1971). It is very probable that in denatured DNA the fixed fluorene residues, freed from the double-helix field, have the same optical properties as in guanosine derivatives. We will therefore adopt the value of 18,000 at 305 nm since it has been proved that no loss of *N*-acetyl group is observed *in vitro* (Kriek, 1968, 1969) during the processes of reaction and extraction. We can determine the concentration of bound carcinogen by

$$(C) = \frac{A_c^{305}(H_c^{305} + 1)}{18{,}000} \qquad (1)$$

To determine the DNA concentration we used the differential spectrum between native DNA and variously reacted samples at the same DNA concentration (Figure 1); from the limiting value of 0.65 ± 0.02 found when (C) → O for the A_c^{305}/A_c^{260} ratio of the bound carcinogen one can determine the fluorene residue contribution at 260 nm, $A_c^{260} = A_c^{305}/0.65$; the total base concentration is therefore given by

$$(B) = \frac{A_{DNA+c}^{260}(H_{DNA+c}^{260} + 1) - A_c^{260}(H_c^{260} + 1)}{\epsilon_P^{260}((H_{DNA}^{260})_{c=0} + 1)} \qquad (2)$$

where $(H_{DNA}^{260})_{c=0}$ is the hyperchromicity of the native (noncarcinogen reacted) DNA. This equation is only valid if we assume that $H_c^{260} = H_c^{305}$. This assumption is justified on the following grounds.

In the uv spectrum of 8 (*N*-2-fluorenylacetamido)deoxyguanosine (Kapuler and Michelson, 1971) the two transitions observed at 305 and 275 nm are likely to be the same as those measured in the case of *N*-AcO-AAF. Nonresonant coupling of these transitions with all the other transitions of the bases is responsible for dye hypochromicity. Fluorescence polarization, p, of *N*-AcO-AAF was measured at 25° in 1,2-propanediol, by changing the wavelength λ_{ex} of excitation and keeping constant the emission wavelength of 342 nm. At λ_{ex} = 303, 290, 275, and 260 nm we obtained the following respective values of p 0.33, 0.31, 0.31, and 0.28. It is thus clear that the angle between respective excitation and emission dipoles is almost constant. It may therefore be considered that transition dipoles corresponding to the wavelength 305 and 260 nm are parallel. The corresponding hypochromicities can be assumed equal when the carcinogen molecule is inserted in the internal field of the double helix.

On the other hand, H_c^{260} can be expressed experimentally by

$$H_c^{260} = \lim_{(C)\to 0} \frac{\Delta A_{DNA+c} - (\Delta A_{DNA}^{260})_{c=0}}{A_c^{260}}$$

This value would correspond actually to a single molecule of carcinogen placed in the internal field of a native DNA molecule. For the three binding ratios $A_{DNA+c}^{305}/A_{DNA+c}^{260}$ 0.14, 0.08, and 0.033 respective values of 7, 9, and 16% were found for

$$\frac{\Delta A_{DNA+c}^{260} - (\Delta A_{DNA}^{260})_{c=0}}{A_c^{260}}$$

The experimental error increases as the ratio approaches zero. The last value is thus lying between 12 and 20% and in the limit of experimental accuracy can be considered to be in good agreement with the measured hyperchromicity $H_c^{305} \simeq 20\%$.

Finally, the percentage of modified bases is given by the ratio (C)/(B). The standard curve (Figure 2) for differently reacted samples of DNA permits us direct determination of the percentage of modified bases from the ratio $A_{DNA+c}^{305}/A_{DNA+c}^{260}$.

Determination of Carcinogen-Reacted DNA Helicity. When carcinogen-treated DNA is melted the increase in absorbance at 260 nm (ΔA_{DNA+c}^{260}) may be analyzed as the sum of two contributions. The first is the loss of stacking between base plates (ΔA_{DNA}^{260}) and the second corresponds to the unstacking of a fixed carcinogen with upper and lower corresponding base pairs, (ΔA_c^{260}). A complex relation evidently exists between these two factors. To evaluate the carcinogen contribution we used the equation

$$\Delta A_c^{260} = A_c^{260} \times H_c^{260} \qquad (3)$$

where $H_c^{260} = H_c^{305}$ and $A_c^{260} = A_c^{305}/0.65$. The net contribution of DNA is obtained by

$$(\Delta A_{DNA}^{260})_c = \Delta A_{DNA+c}^{260} - \Delta A_c^{260} \qquad (4)$$

and finally according to the definition of

$$(H_{DNA}^{260})_c = \frac{(\Delta A_{DNA}^{260})_c}{(A_{DNA}^{260})_c}$$

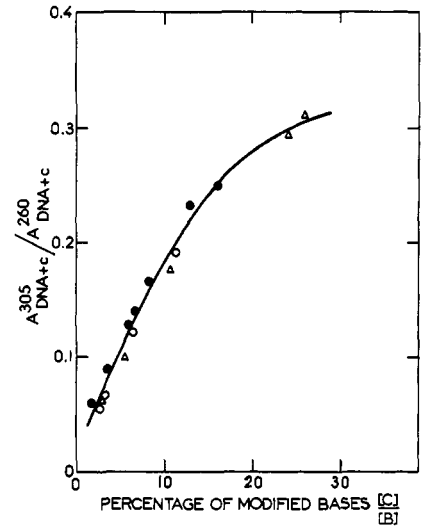

FIGURE 2: Standard curve obtained as described in the text used for the direct determination of percentage of modified bases in DNA. (○) *H. influenzae* DNA; (●) calf thymus DNA; (△) *M. lysodekticus* DNA.

where $(A_{DNA}^{260})_c$ is found by

$$(A_{DNA}^{260})_c = A_{DNA+c}^{260} - A_c^{260} \qquad (5)$$

Combining eq 3, 4, and 5 one obtains

$$(H_{DNA}^{260})_c = \frac{\Delta A_{DNA+c}^{260} - A_c^{305}H_c^{305}/0.65}{A_{DNA+c}^{260} - A_c^{305}/0.65} \qquad (6)$$

The terms on the right-hand side are easily obtained from experiments. In order to compare DNA samples from various sources it is necessary to speak in terms of helicity rather than hyperchromicity. It has been demonstrated for calf thymus DNA that the fraction which remains stacked, as measured by residual temperature-dependent hypochromism, is approximatively equal to the fraction of double helix remaining, as determined by measurements of slowly exchanging hydrogens (Printz and von Hippel, 1965; McConnell and von Hippel, 1970). Percentage of residual helicity can therefore be defined by the equation

$$\varphi = (H_{DNA}^{260})_c/(H_{DNA}^{260})_{c=0} \qquad (7)$$

Results

Melting Curves. Unreacted native DNA melting curves recorded at 260 nm had a very low degree of renaturation, which upon reheating gave no cooperativity (Figure 3). This effect was no longer observed when carcinogen-treated DNA was used. Figure 4 shows a two-step renaturation curve: first, a cooperative renaturation of sigmoidal shape appears, followed by a nonspecific renaturation at lower temperature. The cooperative part increased progressively with the extent of the carcinogen binding. When a second heating cycle was performed the new melting curve coincided with the nonspecific renaturation part followed by a cooperative melting which occurred at a T_m very close to the value observed during the first cycle. Upon cooling, the second renaturation was superimposed on the first one. This second denaturation–renaturation cycle could be repeated many times without

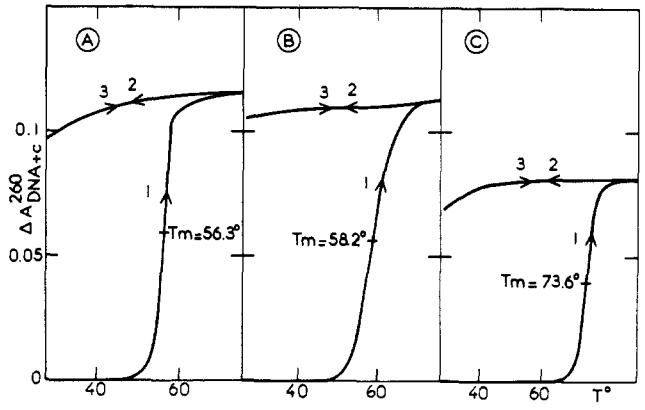

FIGURE 3: Thermal helix–coil transition of native DNA at 260 nm in 2×10^{-3} M sodium citrate buffer (pH 7). (1) First melting; (2) renaturation upon cooling; (3) second melting; (A) *H. influenzae* DNA; (B) calf thymus DNA; (C) *M. lysodekticus* DNA.

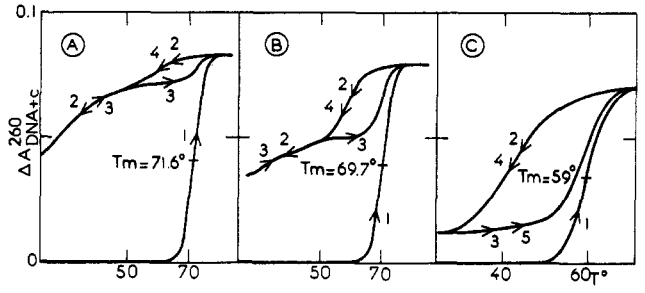

FIGURE 4: Thermal helix–coil transition of native carcinogen-reacted *M. lysodekticus* DNA at 260 nm in 2×10^{-3} M sodium citrate buffer (pH 7). (1, 2) First denaturation–renaturation cycle, respectively; (3, 4) second cycle. The percentage of modified bases was 3.1, 5.6, and 27.3% for A, B, and C, respectively.

variation. The cooperative part of this denaturation–renaturation second cycle shows certain analogies with hysteresis, a well-known physical phenomenon first encountered in ferromagnetism. To verify this analogy, we checked the possibility of obtaining small denaturation–renaturation loops inside the second cycle cooperative part (Figure 5). This criterion has been found to give strong evidence of hysteresis (Everett and Whitton, 1952). Further, the melting behavior is typical or *M. lysodekticus* and *H. influenzae* DNA. In the case of calf thymus DNA the cooperative de-

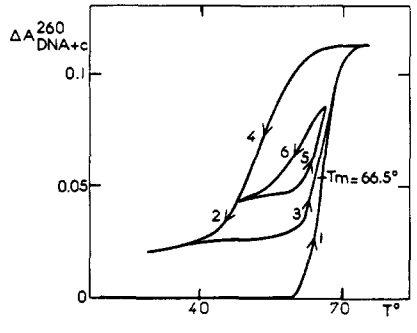

FIGURE 5: Thermal helix–coil transition at 260 nm of *M. lysodekticus* DNA, having 11.1% of modified bases, used to illustrate hysteresis phenomenon. (1, 2) First denaturation–renaturation cycle, respectively; (3, 4) second cycle; (5, 6) small loop inside the second cycle. 2×10^{-3} M sodium buffer (pH 7) was used.

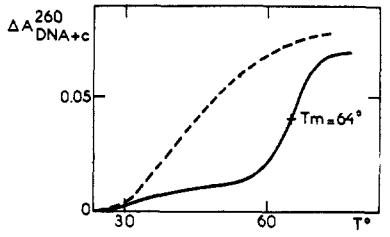

FIGURE 6: Comparative melting curves of reacted (27.3% of modified bases) and unreacted *M. lysodekticus* DNA after alkaline denaturation (Studier, 1965).

naturation–renaturation second cycle was much more attenuated. Larger heterogeneity of calf thymus DNA molecules probably accounts for this observation.

We define the percentage of cooperative renaturation as the ratio of cooperative renaturation part and the first cooperative denaturation part. With sonicated *M. lysodekticus* DNA containing 20% of reacted bases the percentage of cooperative renaturation was 30% instead of 90% for whole DNA molecules reacted to the same extent.

All these observations also applied qualitatively to melting curves measured at 305 nm. On the other hand, after heating to 100° at pH 9, in 5×10^{-2} M sodium borate buffer, cooperative renaturation disappeared. If heating to 100° was carried out in sodium citrate buffer (2×10^{-3} M, pH 7), the loss of cooperative renaturation became less pronounced. The same results were obtained in borate buffer if heating was stopped as soon as full hyperchromicity was obtained. Thus it appears that the combination of both processes of denaturation, alkaline pH and high temperature, is necessary to destroy the cooperative renaturating capacity of carcinogen-treated DNA. These results are summarized in Figure 7.

Light Scattering. NATIVE DNA. From Table I it is seen that after heat denaturation with formaldehyde, native DNA

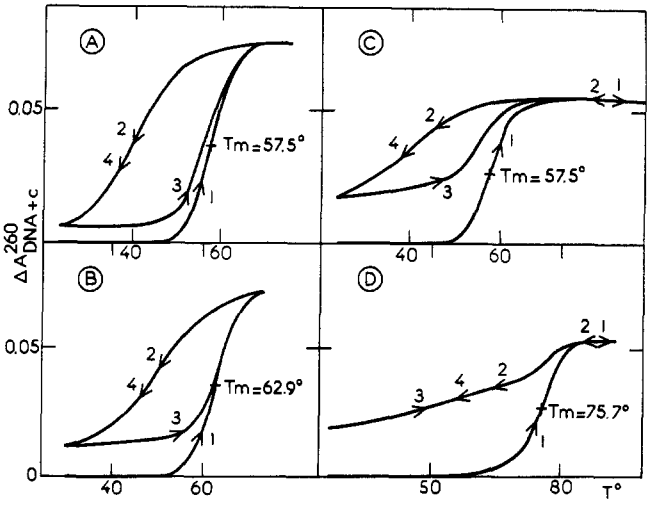

FIGURE 7: Thermal helix–coil transition at 260 nm of *M. lysodekticus* DNA, containing 24.8% of modified bases, used to illustrate cross-link sensitivity to alkaline pH and high-temperature incubation. (1, 2) First denaturation–renaturation cycle, respectively; (3, 4) second cycle. (A) 2×10^{-3} M sodium citrate buffer (pH 7), heated to 75°; (B) 3×10^{-3} M sodium borate buffer (pH 9), heated to 75°; (C) 2×10^{-3} M sodium citrate buffer (pH 7), heated to 95°; (D) 5×10^{-2} M sodium borate buffer (pH 9), heated to 95°.

TABLE I: Light-Scattering Measurements on *H. influenzae* DNA.

Parameters	Native[a]	Denaturated[b]	Native Carcinogen Reacted[c]	Denaturated Carcinogen Reacted[d]
Radius of gyration (Å)	2800	830	2300	2000
Molecular weight	7.1×10^6	2.6×10^6	11.8×10^6	8.3×10^6

[a] DNA concentration was 1.02×10^{-4} g/ml in 0.1 M NaCl (pH 7). [b] DNA concentration was 0.90×10^{-4} g/ml. Denaturation was performed at 5° over T_m for 20 min in sodium borate buffer (4×10^{-3} M, pH 9)–1% HCHO. Light-scattering measurements were carried out in sodium borate buffer (5×10^{-2} M, pH 9). [c] DNA concentration was 0.37×10^{-4} g/ml in 0.1 M NaCl (pH 7). 6.5% of bases was reacted with carcinogen. [d] DNA concentration was 0.31×10^{-4} g/ml. 6.5% of bases was reacted with carcinogen. Denaturation was performed at 5° over Tm for 20 min in sodium buffer (4×10^{-3} M, pH 9)–1% HCHO. Light-scattering measurements were carried out in sodium borate (5×10^{-2} M, pH 9).

has a radius of gyration 3.4 times smaller than native DNA, and a molecular weight which decreases from 7.1×10^6 to 2.6×10^6, *i.e.*, by a factor of 2.8. Thus, there is appreciable evidence for strand separation. Single-strand breaks probably account for the larger loss in molecular weight that would be expected, if duplex molecules dissociated into two complementary subunits.

No such effect was longer observed when a mildly carcinogen-treated DNA was used. The radius of gyration remained the same within experimental errors and the molecular weight decrease was only 1.4-fold. A definite increase of molecular weight was observed after reaction of DNA with the carcinogen. For example, in DNA containing 6.5% of modified bases the molecular weight shifted from 7.1×10^6 to 11.8×10^6. In fact, it is well known that in the case of high molecular weight DNA, light-scattering determination of the molecular weight is always too low (Froelich *et al.*, 1963). For example, if the molecular weight is calculated from a $s_{20,w}$ value according to the Crothers and Zimm formula (1965), one obtains $M_w \simeq 10^7$. When the radius of gyration decreases upon carcinogen binding, the extrapolation of the Zimm plot approaches a more correct value, and this may explain the apparent increase in molecular weight. However, when 11.7% of total bases were modified the molecular weight increased suddenly to 17.6×10^6. This effect will be discussed later.

SONICATED DNA. As seen in Table II and Figure 8a, the radius of gyration of sonicated native DNA following denaturation dropped to one-half its original value, whereas its molecular weight falls from 0.53×10^6 to 0.19×10^6 (2.8-fold decrease). On the other hand, carcinogen-treated DNA did not show any significant difference before and after treatment with formaldehyde (Table II, Figure 8b). Increase in molecular weight after reaction with the carcinogen is also apparent. We must point out that Zimm plots are relative to a single concentration which however is almost the same (1.6×10^{-4} to 1.95×10^{-4} g per ml) in all experiments. At high ionic strength (Tables I and II) the virial coefficient is very small and the molecular weight deviates only slightly from the extrapolated value. In no case is the radius of gyration dependent on concentration. The refractive index increment of carcinogen-treated DNA was checked after dialy-

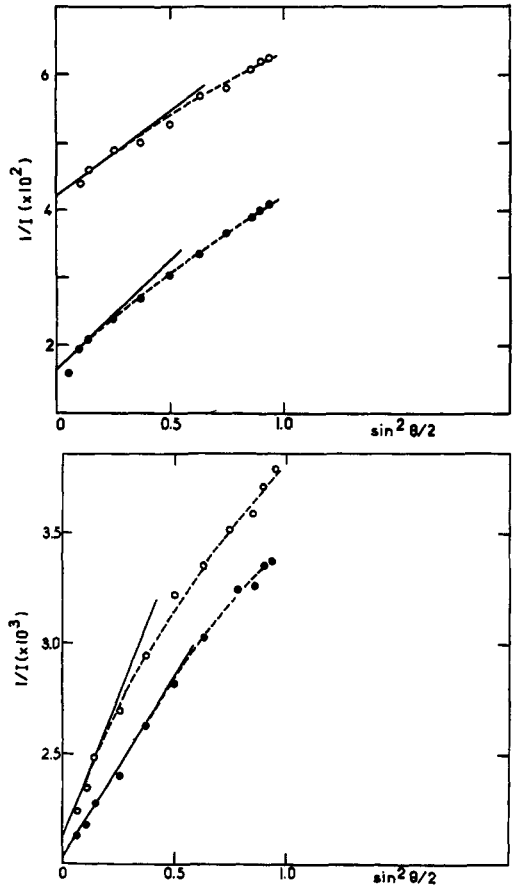

FIGURE 8: Light-scattering measurements on sonicated DNA. (a, top) Native (●) and denaturated (○) calf thymus DNA. The experimental conditions are described in Table II (b, c). (b, bottom) Native (●) and denaturated (○) carcinogen-reacted *M. lysodekticus* DNA. The experimental conditions are described in Table II (d, e).

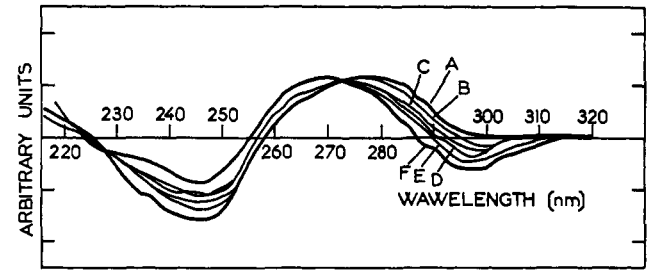

FIGURE 9: Circular dichroism spectra of differently reacted calf thymus DNA in 2×10^{-3} M sodium citrate buffer (pH 7). The DNA concentration was kept constant for all experiments. a, b, c, d, e, and f represent DNA having respectively 0, 1.8, 3.6, 6.0, 8.4, and 13.0%, of modified bases.

TABLE II: Light-Scattering Measurements on Sonicated DNA.[a]

Parameters	Calf Thymus DNA		Carcinogen-reacted *M. lysodekticus* DNA	
	Native[b]	Denaturated[c]	Native[d]	Denaturated[e]
Radius of gyration (Å)	800	400	520	630
Molecular weight	0.53×10^6	0.19×10^6	3.3×10^6	3.5×10^6

[a] All samples were sonicated before carcinogen reaction during 4 min at 20 kHz in 0.1 M NaCl (pH 7). [b] DNA concentration was 1.67×10^{-4} g/ml in 0.1 M NaCl (pH 7). [c] DNA concentration was 1.6×10^{-4} g/ml. Denaturation was performed at 5° over T_m for 20 min in sodium borate buffer (5×10^{-3} M, pH 9)–1% HCHO. Light-scattering measurements were carried out in sodium borate buffer (5×10^{-2} M, pH 9). [d] DNA concentration was 1.95×10^{-4} g/ml in 0.1 M NaCl (pH 7). 20% of bases was reacted with carcinogen. [e] DNA concentration was 1.62×10^{-4} g/ml. 20% of bases was reacted with carcinogen. Denaturation was performed at 5° over T_m for 20 min in sodium borate buffer (4×10^{-3} M, pH 9)–1% HCHO. Light-scattering measurements were carried out in sodium borate buffer (5×10^{-2} M, pH 9).

sis equilibrium (Casassa and Eisenberg, 1964; Cohen and Eisenberg, 1968) and no differences were detected with respect to native DNA. A value of 0.168 ml/g has been found in sodium citrate buffer (2×10^{-3} M, pH 7).

Circular dichroism. With constant amount of differently reacted DNA we observed (Figure 9) a definite decrease of the negative band at 245 nm. On the other hand, as expected, the 300-nm band increased directly with the amount of fixed carcinogen.

Viscosity. Figure 10 shows the effect of carcinogen on DNA reduced viscosity. The sharp decrease observed may be explained on the basis of the bending sites created by the carcinogen.

Discussion

In Figure 2, the standard curve used for the determination of fixed carcinogen on native DNA was obtained as described in Material and Methods. The use of DNA absorbance after melting, as shown in eq 2, was necessary because carcinogen-treated DNA had lower hyperchromicity at 260 nm than native DNA (Figure 11). In order to eliminate this variable we measured absorbance after full melting to determine (C) and (B). For each sample, H^{260}_{DNA+c} and H^{305}_{c} were determined experimentally and used to calculate (C) and (B). The cooperative melting at 305 nm involves a carcinogen interaction with double-helix field (Fuchs and Daune, 1971). Possible carcinogen–carcinogen interaction were eliminated by using moderately carcinogen-treated DNA (<5% of modified bases) which also presents cooperative melting at this wavelength. Modified DNA circular dichroism spectra showed a negative band close to 300 nm. For a given constant amount of fixed carcinogen, the amplitude of this band was independent of the carcinogen/DNA ratio. The carcinogen optical activity is therefore induced by the asymmetrical electric field of the DNA helix on the chromophore, and not by the interaction of two neighboring chromophores.

The linear decrease of DNA helicity with increasing percentage of modified base pairs is shown in Figure 11. The slope of this curve gives the number of base pairs opened by the fixation of one carcinogen molecule. We found a value close to 1 which signifies that the reaction of one carcinogen molecule with DNA involves a loss of hypochromicity corresponding approximatively to the opening of one base plate.

From a dynamic point of view, we may visualize the reaction in two steps. First, *N*-AcO-AAF attacks the C_8 of the guanine made accessible by DNA "breathing" (Kapuler and

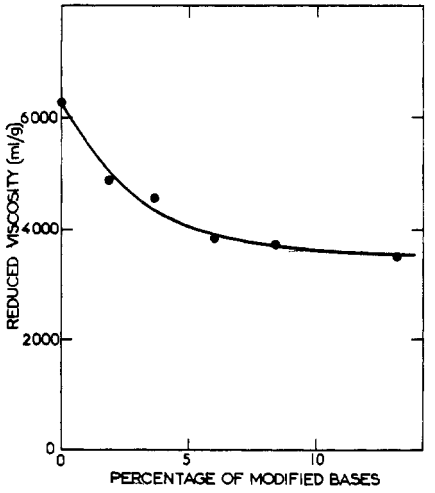

FIGURE 10: Reduced viscosity of variously reacted calf thymus DNA *vs.* percentage of modified bases. All measurements were performed in 0.3 M NaCl (pH 7).

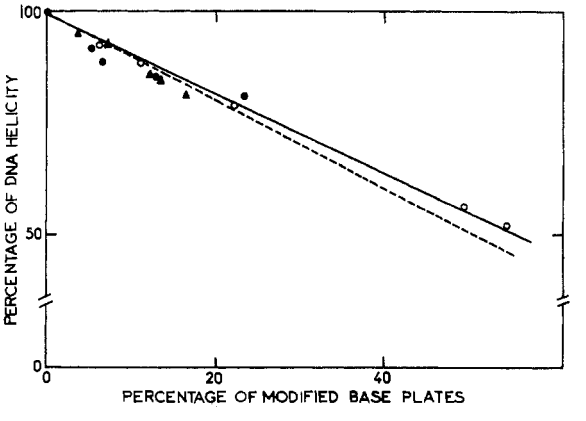

FIGURE 11: Percentage of DNA helicity *vs.* percentage of modified base plates. (▲) Calf thymus DNA, (●) *H. influenzae* DNA, and (○) *M. lysodekticus* DNA. The dotted line represents the relation between helicity and percentage of modified bases if each modified base involves the opening of the corresponding base plate.

Michelson, 1971); then the fixed residue pivots into the double-helix field leaving the modified guanine outside.

In a study of *in vitro* modifications of transfer-RNA by *N*-AcO-AAF (Fink *et al.*, 1970), it was assumed that an anti to syn conformational change of the guanine residue occurred. Recently (Nelson *et al.*, 1971) this result was confirmed by circular dichroism and proton magnetic resonance studies of *N*-AcO-AAF-bound oligoribonucleotides. It may be inferred from these results that the rotation of guanine around the N_9–C_1' bound could cause insertion of the carcinogen molecule into the double helix.

The modification observed in the circular dichroism measurements would thus appear to arise from both the optical activity of the carcinogen and the conformational changes of guanine. The case of adenine is not so well understood. In fact, Miller *et al.* (1966), did not find modified adenine after hydrolysis of reacted DNA. On the other hand, I. B. Weinstein (personal communication) has not yet found direct evidence for *N*-AcO-AAF binding to adenine residues in DNA, but it seems likely that this may also occur. Finally, Kapuler and Michelson (1971), using different hydrolysis techniques, suggest the existence of large amounts of modified adenines in DNA. After careful chromatographic analysis of bases on carcinogen-bound DNA, we have not been able to detect any amount of modified adenine (R. Fuchs, unpublished results).

Morphological changes analyzed by viscosity (Figure 10) and light scattering (Tables I and II) showed a markedly reduced radius of gyration, which is explained by formation of bending sites in DNA by the carcinogen. These are probably located in the reacted base plates.

Initial evidence of cross-linking induced by *N*-AcO-AAF in DNA was obtained by melting curve analysis. The cooperative denaturation–renaturation cycle of carcinogen-treated DNA indicates that strand separation does not occur.

It is also interesting to note that the second denaturation occurs at the same temperature as the first. Therefore the new base pairing is energetically the same as in nonmelted DNA reacted with carcinogen. Thus, cross-links must be present to hold both strands together and permit the reconstitution of the original base pairing. The hysteresis-like phenomenon can be explained if we define the small independent domains (Everett and Whitton, 1952) as DNA sections between two cross-links. In fact, there is no reason why these sections should "switch on and off" at exactly the same temperature, because the sequence of these sections is not likely to be the same. As seen in Figure 6, the persistence of cooperative melting after alkaline denaturation during 1 min at pH 11 (Studier, 1965) is also a criterion supporting the existence of cross-links in carcinogen-reacted DNA. However, cross-links disappeared when DNA was incubated for longer periods in alkaline pH or heated at high temperature (Figure 7).

Confirmation of the existence of cross-links was obtained by light scattering. From Tables I and II it is evident that native and sonicated DNA have a molecular weight and a radius of gyration about three times lower than control samples following alkaline formaldehyde denaturation. In fact, the molecular weight should decrease to half its original value. The higher drop observed can be explained by single-strand breaks. On the other hand, carcinogen-treated DNA shows a reverse effect. If extrapolation could cast some doubt on the validity of the results in the case of native DNA, this is not so with sonicated DNA. Hence, the appreciable increase in molecular weight is probably due to some intermolecular cross-links in addition to intramolecular ones. A slight decrease in the radius of gyration also confirms their existence.

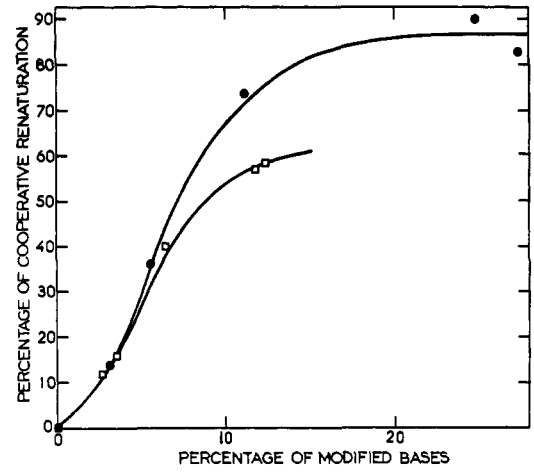

FIGURE 12: Percentage of cooperative renaturation at 260 nm of *M. lysodekticus* (●) and *H. influenzae* (□) DNA *vs.* percentage of modified bases.

The cross-linking reaction must occur to a minor extent because if each bound carcinogen induces a cross-link, a very small amount of fixed carcinogen would result in DNA having a very high percentage of cooperative renaturation. In fact, we observed that important cooperative renaturation occurs only when 5% or more of the bases are modified. As seen in Figure 12, *M. lysodekticus* DNA has a much higher percentage of cooperative renaturation than *H. influenzae* DNA. These results may be interpreted in two ways. (i) Reacted guanine cross-links are produced with the corresponding cytosine in a low yield. If this is so, DNA of different composition would have similar percentage of cooperative renaturation for a given degree of modified bases. Figure 12 shows that this is not the case. (ii) The cross-linking reaction may involve a particular environment. In order to check this hypothesis we attempted to establish a relation between the frequency of occurrence of particular sequences and the percentage of cooperative renaturation. Nearest-neighbor analysis (Josse *et al.*, 1961) of *M. lysodekticus* and *H. influenzae* DNA gave us the relative amounts of different sequences (Table III). The only ones that are more abundant in *M.*

TABLE III: Nearest-Neighbor Frequencies of Bacterial DNAs.[a]

Nearest-Neighbor Sequence	*M. lysodekticus* DNA	*H. influenzae* DNA
ApA, TpT	0.019, 0.017	0.116, 0.116
CpA, TpG	0.052, 0.054	0.067, 0.067
GpA, TpC	0.065, 0.063	0.054, 0.052
CpT, ApG	0.050, 0.049	0.049, 0.050
GpT, ApC	0.056, 0.057	0.048, 0.049
GpG, CpC	0.112, 0.113	0.036, 0.037
TpA	0.011	0.073
ApT	0.022	0.095
CpG	0.139	0.038
GpC	0.121	0.053

[a] From Josse *et al.* (1961).

lysodekticus DNA are GpG, (CpC), and CpG, (GpC). In both cases, the relative amount is about three times higher in *M. lysodekticus* than in *H. influenzae* DNA. It therefore seems that these sequences are involved in some way in the cross-linking reaction. It is clear that guanine plays a major part in the formation of cross-links. Work is in progress to study further the chemical nature and the relative reactivity of adenine and guanine residues with *N*-AcO-AAF using kinetic methods.

Acknowledgments

We are grateful to Dr. Gerald Lemieux for helpful discussions and to Dr. John MacCordick for a critical reviewing of the English text.

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