Ultraviolet Absorption and Optical Rotatory Dispersion Studies of Deoxyribonucleic Acid and Nucleohistone

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This report concerns the ultraviolet absorption and optical rotatory dispersion (ORD) of DNA and of nucleotides down to 185 mJ. An absorption band (y) at about 195 mJ occurs for all these compounds. Similar studies have been made on histones and nucleohistone. These indicate a strong absorption below 200 mJ due to protein. Consequently, if any native DNA is associated with some amount (2—12%) of protein (e.g., histones), the molecular absorption of DNA in the said region becomes masked, and no clear y-maximum due to DNA occurs in the absorption spectrum. The absorption of DNA in the y-band region (ca. 190 mJ) seems to be decreased as a result of DNA-protein interaction. No significant change is observed in the absorption region of DNA at longer wavelengths (250—290 mJ). The y-peak of DNA appears at about 190 mJ on complete removal of proteins. This peak is sharper than the conventional one (x) at 260 mJ and is hyperchromic on denaturation, and involves multiple Cotton effects to any degrees similar to that observed at the second peak (260 mJ).

The absorption (also ORD) features of nucleohistone at the x and y wavelengths do not provide similar information (e.g., denaturation artefacts) regarding the structure of the nucleohistone contained DNA which is apparently distinct from the structures of native and partially denatured (denaturation 10%) DNA. The weaker x—y transition (190 mJ) in the DNA bases is very susceptible to nucleoprotein interaction. This result has been used to explain the influence of nucleoprotein interaction on the configuration of DNA in nucleohistone.

Nucleic acid bases containing many π-electrons have a number of absorption bands near 260 mJ and further in the ultraviolet 1—3. Voet, Gratzer, Cox, and Doty 2 have reported that the higher energy bands near 200 mJ and below this region are present in the spectra of polynucleotides and nucleic acids. For both practical and theoretical reasons the 200 mJ band of DNA and the wavelength of its maximum are of enormous importance 4—6. Voet et al. 2 have reported that no clear maximum is observed near 20 mJ in the spectrum of native DNA. If the native DNA is treated with DNase and then heated at 80° a clear maximum appears at about 190 mJ. Their results further show that the enzymic digest without heating shows a slow turning (inflection) absorption instead of a maximum in the said region. These authors 2 conclude that an absorption feature associated with one or more of the component chromophores undergo a very pronounced redshift when these chromophores are stacked, and thereby mask the DNA maximum near 190 mJ. However, several other investigators 7, 8 including the present author 9, 10, have shown that the spectrum of native DNA which is free from RNA and protein exhibits an absorption band with a maximum in the range 185—200 mJ. This band is more intense than the 260 mJ band by a factor of about 1.9. This spectral change which are brought about by heating the DNA in solution (H2O or D2O) 7, 9, 10, or by drying 7 in solid films are similar 7. In other words, the DNA peak near 200 mJ shows hyperchromicity which depends on DNA structure, in qualitative agreement with the other group of authors 2.

The hypochromism observed at the 200 mJ peak is believed (but not verified) to occur as a result of interference of the lower energy transitions (ca. 200 and 260 mJ) with the higher ones occurring in the vacuum ultraviolet 2, 3. This is probably related to the origin of the hypochromic effect 4, 5. There are several practical benefits of the studies of DNA absorption in the far ultraviolet region. These studies can be used to determine the degree of denaturation 7—9, to estimate the base composition in regions of DNA showing selective denaturation, and to quantify the DNA-protein interaction.

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ABSORPTION AND ORD STUDIES OF DNA

Before one could use the DNA absorption for practical purposes it would be necessary to determine and to rationalize, from fresh experimental data, whether or not DNA has an absorption peak near 200 m\(\mu\). In view of the difference in conclusions by this author, and others, this problem should be approached in directions of understanding the conditions of experiments in the above two cases. The data in the two cases would be different, even if the conditions of the samples studied are different, no matter whether the factors arising from the instrumental limitations (e.g., stray light, photometer calibration) are the same. The sample conditions may be different due to the use of different solvents (salts), dissolved oxygen and carbon dioxide of various amounts, and the presence, in different amounts, of non-DNA materials of characteristic benzoid absorption in the far ultraviolet. For the sake of clarity and comparison, the solvent chosen in the present studies is the same as that used by VOET et al.2.

Absorption studies have been initiated with the DNA nucleotides and the nucleotide mixture in order to check the spectral features of these compounds in the ultraviolet region. DNAs isolated from different bacterial and mammalian sources have been studied in the earlier work of this author. These can be noticed to be contaminated with very little protein, which is reportedly 0.1 – 0.3%, and with negligible amounts of other non-DNA materials. Since proteins also absorb in the far ultraviolet region these were removed as much as possible from the DNAs in those earlier studies. Although it has not been included in the previous reports, it was found during the course of those earlier experiments that proteins contaminating the DNAs could mask the DNA peak near 200 m\(\mu\), and might even change the wavelength of this peak. These features are similar to the effects due to hydroxyl (ca. NaOH) and chloride (ca. NaCl) ions which absorb to a large extent in the said region of DNA absorption. So the protein effect has been considered here in connection with the disappearance of the DNA maximum as reported by VOET et al.; also because it is not clear from the report of these authors what might be the protein content in the DNAs they have used. For the aforesaid purpose, a commercial DNA preparation has been used and the effect of protein on the DNA absorption has been studied by lowering the protein content of the DNA to different extents; viz., 12% – 0.1% relative to DNA. Obviously, the objective of these studies is to test whether the results that are observable without much purification of the DNA are similar to those reported by VOET et al., or whether the disappearance of the DNA y-maximum can be explained to be due to the pronounced red shift of one or more of the component chromophores (e.g., adenines) when these are stacked.

Although the ultraviolet absorption spectra of nucleotides and of DNA up to 185 m\(\mu\) are available in the literature, ORD spectra of the same compounds up to the same wavelength are rare and not sufficient enough to confirm results like the red shift of adenines derived from absorption data. The ORD data of the same compounds in the near ultraviolet region are abundant. Hence, in the hope of developing new ideas which may complement the conclusions from the absorption data, ORD studies of DNA and DNA components in the shorter wavelength have been made.

The second purpose of the present studies of absorption and ORD in the far ultraviolet region is to report on the nucleoprotein interaction and how this interaction determines the DNA configuration in soluble nucleohistone. Both conformational and configurational studies have indicated distinctive alterations of the DNA molecule(s) in nucleohistone. In the present studies attention has been given on the influence of nucleoprotein interaction on the optical transitions (\(\pi - \pi^*\)), at 260 and 190 m\(\mu\), due to the DNA bases, and to explain the structural alterations of DNA which may occur as a consequence of binding of histones to DNA.

Materials and Methods

(a) DNA-nucleotides

The selected deoxy-5'-Nucleotides are obatined from Pabst Laboratory (5'-AMP), Sigma Chemical (5'-CMP, 5'-TMP) and Nutritional Biochem. Corp. (5'-GMP). Samples in water (pH 6.5) showing concordant ratios of their peak to trough absorbances above 230 m\(\mu\) have been studied for absorption in the far ultraviolet.

(b) DNA purification and estimation

Calf thymus DNA has been purchased from Calbiochem. The crude DNA has been found to be contaminated with 12% proteins and 2% RNA relative to DNA. So this DNA is precipitated with isopropanol. 

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several times, air dried and then dissolved in aliquots in freshly boiled deionized double distilled water and 0.015 M NaF. The pH of the two solvents is checked and is found to be in the neutral region. At the end of each precipitation the dissolved DNA is tested for proteins, DNA and RNA; and also for spectral absorption up to 185 mμ. The first three steps of precipitation have been found to lower the protein contamination to 2.8% and the RNA to 0.6%. Further precipitation up to six times reduces the two contaminants to 2% and 0.5% only. Since isopropanol precipitation is found gradually to be less efficient in removing proteins, possibly those bound to DNA, the DNA dissolved in 0.015 M NaF after the sixth precipitation is treated with heat-treated (60°) pronase. The material is then centrifuged at 15,000 rpm for 15 min and dialyzed against the solvent with several changes, so that the released proteins and the added enzyme are removed. The dialyze is next treated with heat-treated (75°) RNase, centrifuged and then dialyzed in similar ways. RNase is used to remove RNA associated with the DNA, also because this helps in dissociating some proteins linked either to DNA or RNA. The dialyzed material is next treated with protease, centrifuged and dialyzed. The treatment of protease is made in order to digest the RNase that might be bound to DNA and the residual protein on DNA. The DNA dialyze after protease treatment contains about 1.5% proteins. It seems that, although the enzymes can remove protein from DNA, only small peptides are removed by dialysis. In order to separate DNA from peptides, DNA is precipitated twice from the suspension by isopropanol and then air dried. The dissolved DNA is finally dialyzed against the solvent until any isopropanol present in the material is removed. This has been verified by checking the absorption of the material in the far ultraviolet. The above procedure reduces the protein contamination to 0.1% and the amount of RNA to any negligible extent. The enzymes are purchased from Calbiochem and each of them has been used at a concentration of 500 μg/ml. Each enzyme treatment is performed at 37° for 3 hrs.

The protein-to-DNA ratio in the commercial DNA and the nucleohistone is estimated from the measurements of optical absorption at 230 and 260 mμ, the shift of the DNA minimum near 230 mμ with the change in histone concentration, and also from the coloration experiments for both DNA and protein. Tests for the amounts of protein, DNA and RNA, have been made by Lowry, diphenylamine (Burton's modification) and orcinol reactions as mentioned in a previous report. For protein estimation, histones (Calbiochem.) have been used as a standard.

(c) Heat denaturation and DNase treatment of DNA

For heat denaturation of DNA, DNA has been heated to 90° for 10 min and then chilled quickly in an ice-water mixture. For enzymatic degradation of DNA, DNA has been treated with DNase (2.5 μg/ml) in a phosphate buffer (0.01 M PO₄, pH 7.0) containing magnesium ions (10⁻³ M). The enzyme treatment has been carried out at 37° for 3 hr. Mg ions and DNase molecules are removed by extensive dialysis against water and then in 0.015 M NaF solvent.

(d) Partially dehistonized nucleohistones

Calf thymus nucleohistone used in the present study has been found to dissolve slowly in water (double-distilled and boiled before use). Magnetic stirring at low speed (4°) is recommended for this purpose. After a few hours (6 hrs) of stirring, a soluble fraction and a gel-like particulate fraction always occurs. After high-speed centrifugation, the sediment and the clear supernatant are retained, and then studied for estimation of DNA and histones, for ultraviolet absorption and for characterization of the configuration of DNA in nucleohistone from linear dichroism (257 mμ) of DNA, polarized fluorescence of intercalated acridine orange (in DNA-dye complex) molecules and from rotational diffusion measurements (for methods of these experiments, see Refs. 32 — 34). The protein-to-DNA ratio and the aforesaid configurational characteristics of the nucleohistone supernatant indicate that nucleohistone which is early dissolved represents mainly DNA (histone-to-ratio 0.05 — 0.12) rather than nucleohistone as a whole. As the particulate fraction is resuspended and stirred again in water for a long time, soluble portions (protein-to-DNA ratio in the range 0.85 — 1.35) characteristic to full nucleohistone (as known from the literatures) are obtained. These partially dehistonized nucleohistones are collected at different intervals and then stored until the particulate fraction is completely dissolved in 3 to 4 days. The present study has been performed on such several collections of the water soluble partial nucleohistones. The histones present in all these nucleohistone solutions are found (by isopropanol precipitation and protein estimation) to be totally bound to DNA.

If the supernatant which is obtained after the first 6 hrs of dissolution is not discarded, the full nucleohistone dissolved in 4 days would indicate a histone-to-DNA ratio of about 1.52.

Partial nucleohistones have also been prepared by dissociating (dialysis) the DNA-bound histones from the full nucleohistone (histone-to-DNA ratio 1.52) in high ionic solvents (1 M — 2.5 M NaCl). The histones that are released in the suspension could not be completely removed by dialysis. In the ORD studies to be reported here, it is essential that the histones made free in ionic suspensions are completely removed. Since this requirement is not met with these preparations, only water soluble, partially dehistonized nucleohistones, as described in the previous paragraph, have been used. In order to study the conformation of DNA in nucleohistone, the latter has been treated at low and high temperatures with neutralized formaldehyde (1%) obtained from the Fischer Scientific Corporation.

(a) Spectrophotometry

Spectra showing steady absorption in the far ultraviolet have been recorded in a Cary model 15 recording
spectrophotometer which is always flushed with nitrogen for hours both before and after insertion of the cuvette containing the sample. The cuvettes are cleaned by aqua regia or ethanol-HCl mixture and the cell baseline is examined before each spectrum is obtained. The stray light level of the spectrophotometer is determined by using pure isopropanol (1 cm) in the sample beam. The proportion of stray light near 190 mμ is found to be much less than 0.001%. With lower pathlengths (0.1, 0.5 cm), the spectra of DNA and nucleotides are checked in order to demonstrate the adherence to the Lambert-Beer law. The absorptions in the spectra reported here are not corrected for the macromolecular scattering because such corrections are found negligible. All measurements of spectra have been carried out at room temperature (25°—27°). The hyperchromicity measured relative to initial absorption at the peak wavelengths, instead of integrated absorptions, has been considered as a measure for denaturation.

(f) Spectropolarimetry

ORD is measured with a Cary 60 recording spectropolarimeter at 27° on 0.006—0.05% solutions in 0.5—0.05 cm cells. Because of the small magnitude of rotations for dilute solutions, the base line below 300 mμ is checked. The signal-to-noise ratio at the peaks and troughs are 2 to 3. The data for nucleotides are shown in terms of molar rotations [m], which equals 10 Θ/(d.M.), where M is the molar concentration of the compound, d the light path (dm) in exclusively NaF or freshly boiled distilled water, and Θ the rotation in degrees. The optical rotations for DNA are shown in terms of [α], given by Θ/d.c., where c is the concentration of DNA in gm per ml.

Results and Explanation

1. Spectra of Nucleotides and of a Mixture of Nucleotides

Fig. 1 shows that the DNA nucleotides (curve a—d) and their physical mixture (curve e) display an absorption band near 200 mμ which is generally more intense than the corresponding band near 260 mμ. The two absorption bands at 200 mμ and 260 mμ will hereafter be referred to as y and x respectively, following the terminology of MASON. The peaks of the y band for dAMP and dTMP occur at about 206 mμ, while those for dGMP and dCMP are near 190 mμ. A similar result has been reported by VOET et al. For dAMP, a double peak has been observed in the y region. The nucleotides show some evidence that the y-band (like the x-band) is composite in nature. Fine structure and perceptible shoulders are present in all spectra and have been shown by arrows. In addition to the spectra of the physical mixture of the DNA nucleotides (curve e), a spectrum of the mixture of nucleotides present in proportions (GC 40%, AT 60%) as in calf thymus DNA has been calculated from the individual spectra of the four nucleotides. This shows no difference in the spectral absorption at the shorter wavelength with respect to the experimentally obtained spectra for the physical mixture. This therefore indicates that the interaction among the nucleotides in solution is little. It will be of interest to see whether the spectral characteristics of the aforesaid compounds in the y-band region are altered when these compounds are hydrogen bonded and stacked, as in native DNA.

2. Spectra of native DNA and denatured DNA

The absorption spectrum of a crude commercial calf thymus DNA (protein 12%, RNA 2%) in 0.015 m NaF is shown in Fig. 2 (curve a). The absorption due to the solvent is shown by the curve at the bottom of the sample spectrum.
The extinction of the contaminating proteins in the y-region (190 m/\(\mu\)) may be about five times more than that of DNA. Due to suppression of the y-absorption of DNA as a consequence of nucleoprotein interaction and also owing to absorption of proteins, one sees the y-absorption of the crude DNA increase in two steps (i.e., discontinuously). The absorption of DNA is predominant at wavelengths up to 192 m/\(\mu\), while below this wavelength (192 – 185 m/\(\mu\)) the protein absorption increases by a factor of 2.5 over the DNA absorption. This has been shown by a dotted line in curve (a), Fig. 2.

The DNA is next heat-denatured and then digested by DNase I. The results are shown in the same figure (2) (curves b and c). The spectra of the heat-denatured and DNase digested DNA (Fig. 2, curves b and c) can be compared with that of the mixture of nucleotides (Fig. 1, curve e) added in proportions as present in calf thymus DNA. From curves (a) and (b) (or c), it can be seen that both the y- and x-bands of the native DNA are hypochromatic. The DNA on heat denaturation tends to show an inflection in the region (190 m/\(\mu\)) of the y-maximum, while there is no such indication in the spectrum of the crude native DNA. Since the y-maximum appears slightly in the spectra of enzymatically degraded DNA, the material has not been heated as others did at elevated temperatures to remove residual hypochromism.

The hyperchromicity upon denaturation at the y-peak (hyperchromicity 34% on heat denaturation; 21% on enzymatic degradation) is higher by about 1 – 8% than that exhibited at the x-peak (hyperchromicity 26% on heat denaturation and 20% on enzymatic degradation) (cf. Fig. 2, curves (a) – (c)). The above results of the masking of the y-maximum in the spectrum of the crude native DNA and the appearance of an inflection instead of a clear maximum in the DNase treated DNA are exactly similar to those reported by Voet et al. Search for the reasons for the observed effect is the subject of the following section.

A close examination of the spectra of the crude native, enzyme (DNase) treated and heat denatured DNA (Fig. 2, curves (a) – (c)) indicates that the y-absorption (at 190 m/\(\mu\)) relative to the x-absorption (at 260 m/\(\mu\)), is different in each sample. The relative absorption (Ey/Ex) for the three samples are 1.68, 1.73 and 2.1, respectively. This variation in the Ey/Ex ratio may be connected to the disappearance of the y-peak of DNA in curve (a). The three samples have the same amount of proteins. The two hyperchromicities observed at the x-peak for the heat-denatured and enzyme treated DNA differ by only 6%. If the x-absorption (Ex) of these treated (i.e., denatured) samples are normalized with respect to their hyperchromicities, the y-absorptions (Ey) will still show an increasing trend. This result and the different y-hyperchromicities (viz. 34% (heat), 21% (DNase treatment)) exhibited by the two denatured samples cannot probably occur due to quite different types of change in the electronic structure of the DNA polymer, which affect the main \(\pi - \pi^*\) transition in the near ultraviolet (ca. 260 m/\(\mu\)). It is therefore proposed that (1) the observed variation in Ey/Ex ration for the native and denatured samples is perhaps mostly due to the various altered structures of the contaminating proteins and to the modified charge distribution in regions of the DNA-helix (or helices) due to denaturation or nucleoprotein inter-
action which may preferentially affect the weaker \( \pi - \pi^* \) component near 190 m\( \mu \). Secondly, (2) the y-peak appears indistinctly (infection) in the spectra of the denatured and enzyme treated DNA because the y-absorption of DNA may be predominant over the protein absorption following denaturation or degradation.

3. Removal of proteins from the DNA in (2) and the protein effect

It follows from the previous results that the y-peak of DNA should partly emerge out of the combined absorption of the two compounds (viz., DNA and protein) if the protein contamination is kept vanishingly small. This can be shown for both native and denatured DNA. Fig. 3 shows the absorption spectra of a native calf thymus DNA at two reduced protein concentrations; namely, 2% (curve (a)) and 0.1% (curve (b)), (for details see Materials and Methods). The y-peak due to DNA appears at 190 m\( \mu \) in the spectrum of DNA if the protein contamination is as low as 0.1%. The peak does not appear if the proteins are in the region 12% - 2%. Considering the relatively small amount of proteins present in the crude DNA, the proteins have not been isolated for determining the protein effect and for the estimation of the protein extinction. Histones of known concentration have been used for these purposes.

In the following analysis the data in Fig. 3 have been used to show the protein effect quantitatively. One can compare the \((\varepsilon_y/\varepsilon_x)\) ratios of the different DNA samples containing proteins of various amounts; viz., 12%, 6%, 2.8%, 2% and 0.1%. The ratios for these samples are 1.68, 2.0, 2.36 and 2.51, at the same wavelengths; namely, 190 and 258 m\( \mu \). The result is that the increase in protein concentration effectually decreases the y-absorption of DNA. The decrease could be explained if the absorption of the two compounds (in native samples) are assumed to be non-additive in the y-region. Presumably this may be characteristic to changes in the two compounds and to any change in the DNA-protein interaction. To test this, the spectra of a contaminated DNA (e.g., the DNA with 2% protein) and of the most purified DNA (protein 0.1%) are compared with each other for absorption. The decreases at the x- and y-peaks, after the final deproteinization (ca. 0.1%), are different. Referring to Fig. 3 (curves (a) and (b)), the decreases at the two peaks are 58% and 34%, respectively. In other words, if the spectra of the contaminated (2% protein) and the purified DNA are normalized at 258 m\( \mu \), the latter (0.1% protein) shows an increase in y-absorption (190 m\( \mu \)) by about 24% relative to the contaminated one (2% protein). The ratios of extinctions of the purified sample (0.1% protein) at 230 and 258 m\( \mu \) or at 230 and 258 m\( \mu \) were, namely, 0.42 and 0.58. These are smaller than the corresponding ratios for the more contaminated (2% protein) sample (viz. \( \varepsilon_{230}/\varepsilon_{258} = 0.46 \) and \( \varepsilon_{280}/\varepsilon_{258} = 0.61 \)). Since proteins absorb more at 230 and 280 m\( \mu \) than at 258 m\( \mu \), the smaller extinction ratios for the purified sample further ensure that the protein contamination in this sample is definitely lower than in the other DNA (viz., with 2% protein). The concentrations of DNA in the two samples are known from the Dische reaction. The DNA extinction coefficients at the x-peak for the two samples is found to be similar. This indicates that any physical effect (e.g., change in charge distribution) that may have caused a relative increase in the y-absorption of the purified sample is not due to small amounts of denaturation or degradation which usually affect the x-absorption of DNA.

The y-absorption of the crude DNA has been found to decrease time dependently if a freshly prepared solution of this DNA is kept for a few hours in cuvette. The spectra of this DNA shown pre-
viously have been recorded as the absorption in the y-region becomes steady. No significant change in the x-absorption has been noticed during the same time. Nor does any change in the y-absorption of the final deproteinized DNA (0.1% protein) occur within the same length of time. This effect seems to imply that the proteins associated with the DNA have a role in altering the DNA configuration. A similar but unsystematic effect (viz., decrease in y-absorption) has been observed by Voet et al. \(^2\). The results may not be very significant to alter the longitudinal transition \((\pi - \pi^*)\) at 258 m\(\mu\) but may perhaps be quite effective in decreasing (24%) the absorption due to the weaker perpendicular transition \((\pi - \pi^*)\) near 190 m\(\mu\). Further verification of the protein effect has been made by studying the absorption of nucleohistone and of histones as given in the following section:

4. Spectra of Nucleohistone and Histone

The spectrum of a partially dehistonized nucleohistone (histone to DNA ratio 0.9 conc. 105 \(\mu\)g/ml) is shown in Fig. 4, curve (a). The y-peak due to

![Ultraviolet absorption curves for nucleohistone and histones](image)

DNA absorption does no appear in the spectrum of native nucleohistone even if the histone-to-DNA ratio is varied from 0.05 \((i.e., 4.8\% \text{ w/w})\) to 0.9 \((i.e., 47\% \text{ w/w})\). The result is obviously due to the predominant absorption of histones. Curve (b) represents the absorption of free histones at a concentration of 10 \(\mu\)g/ml. The absorbance due to total histone \((\text{viz., 49.5} \mu\)g/ml in the above-mentioned nucleohistone would be 0.65 at 190 m\(\mu\), if they are free in solution. So curve (b) represents by weight about 20% of the total histones present in the nucleohistone shown in curve (a). The absorbance of histoen-free DNA of the concentration \((\text{viz., 55} \mu\)g/ml, pathlength 0.1 cm, absorbance calculated from curve (b) of Fig. 3) as present in the nucleohistone is 0.145 at the same wavelength \((\text{viz., 190} \mu\)\). The total sum absorbance of DNA and histones in the nucleohistone would, then, be about 0.8 if there is no interaction between the two polymers. The observed absorbance of nucleohistone is 0.6 and this shows a diminution in the y-absorbance of 0.2 unit. This decrease in absorbance is greater than the contribution in absorbance due to total DNA \((\text{viz., unit 0.145})\) in nucleohistone. This further supports that absorption of both the DNA and histones has been lowered as a result of DNA-protein interaction, and the DNA absorption \((y)\) is totally masked \((\text{cf. absorbance 0.65 of histones; 0.6 of nucleohistone})\). A subtraction of the absorption due to total histone present in the nucleohistone from the nucleohistone absorption gives negative y-absorbance \((\text{viz.,} \ -0.05)\). In other words, the absorption of nucleohistone is not an additive function of the individual absorption of DNA and histones. The histones when free in solution absorb about five times more than DNA does \((\text{cf. curve (b) in Fig. 3 and curve (b) in Fig. 4})\). The Ey/Ex ratio of the nucleohistone at 190 m\(\mu\) is about 5.00 which is clearly larger than that for DNA and is supposedly due to predominant absorption of histones.

Referring back to curve (b) in Fig. 4, the absorption of 12% protein (if these are histone-like) would be equivalent to the absorption of a solution of 60% DNA. If the suppression of the y-absorption of DNA is taken into account, the individual absorption due to DNA and proteins (12%) in the DNA spectrum will be of equal amounts. If this DNA is denatured or degraded \((\text{Fig. 2, curves b and c})\) the y-absorption of DNA becomes greater than the absorption due to proteins. This will therefore cause the appearance of an inflexion in the y-region. Similarly, a 2% protein can have a y-absorption equivalent to 10% of that of full DNA. Apparently a 2% protein should not cause the disappearance of the y-maximum of DNA because the DNA absorption is still predominant. But calcula-
tion from the data of the DNA protein complex indicates that even this small amount of proteins may suppress the y-absorption of DNA by 24% as observed (cf. Fig. 3, curves (a) and (b)).

One can now come to the following conclusions:

(1) The y-absorption of DNA is more sensitive to and is suppressed due to DNA protein interaction than the conventional x-absorption;
(2) The absorption of DNA and proteins in the native complexes are non-additive (i.e., hypochromic in the y-region).

As a result of (1) and (2), the y-maximum of native DNA is masked even though the proteins present may be as low as 2%, and
(3) in the denatured and enzyme digested DNA, the DNA-protein interaction is decreased and the absorption due to denatured DNA appears in the spectrum.

5. ORD Measurements on Nucleotides, Mixtures of Nucleotides and on DNA in Native and Denatured States

Fig. 5 displays the ORD patterns of 5'-deoxymononucleotides and that of a nucleotide mixture. The ORD profiles of the native, enzyme digested and heat-denatured DNA in 0.015 M NaF are shown in Fig. 6. The pertinent parameters of the ORD curves are listed in Table I.

Examination of the above figures reveals the following features:

All mononucleotides and native DNA show about three peaks and two to three troughs, but the magnitude of the peak and trough for the DNA differs significantly from that for the nucleotide mixture, indicating strong base-base interaction in native DNA. As has been observed by others, the Cotton effect in the x-band region (λ > 220 m/μ) is negative for purines but positive for pyrimidines. The Cotton effect is multiple in the y region (λ < 220 m/μ) for both the classes of compounds.

Table I. Cotton effects of mononucleotides and their mixture.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solvent: water</th>
<th>Peaks 1 and 2</th>
<th>Troughs 1 and 2</th>
<th>Peaks 3 and 4</th>
<th>Troughs 3 and 4</th>
<th>Cross over</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ m/μ [10⁻³]</td>
<td>λ m/μ [10⁻³]</td>
<td>λ m/μ [10⁻³]</td>
<td>λ m/μ [10⁻³]</td>
<td>λ m/μ [10⁻³]</td>
<td></td>
</tr>
<tr>
<td>5'-AMP</td>
<td>247</td>
<td>6.24</td>
<td>275</td>
<td>-2.83</td>
<td>212 (inflexion)</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>220</td>
<td>-2.83</td>
<td>204</td>
<td>10.77</td>
<td>192</td>
</tr>
<tr>
<td>5'-GMP</td>
<td>250</td>
<td>0.36</td>
<td>263</td>
<td>-2.37</td>
<td>188</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>290</td>
<td>-2.02</td>
<td>236</td>
<td>1.78</td>
<td>17.0</td>
</tr>
<tr>
<td>5'-CMP</td>
<td>289.5</td>
<td>6.33</td>
<td>230</td>
<td>-11.61</td>
<td>212.5</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246</td>
<td>-7.18</td>
<td>249</td>
<td>201.5</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(slowing)</td>
<td>195</td>
<td>249</td>
<td>201.5</td>
<td>198</td>
</tr>
<tr>
<td>5'-TMP</td>
<td>233</td>
<td>2.63</td>
<td>255</td>
<td>-4.73</td>
<td>201</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td></td>
<td>244</td>
<td>-2.10</td>
<td>261</td>
<td>5.79</td>
<td>263.0</td>
</tr>
<tr>
<td>5'-nucleotide</td>
<td>mixture</td>
<td>220</td>
<td>-0.65</td>
<td>260</td>
<td>1.73</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td></td>
<td>290</td>
<td>-0.65</td>
<td>260</td>
<td>2.57</td>
<td>271.5, 245,</td>
</tr>
</tbody>
</table>
Table II. Cotton effects of DNA in native, denatured and degraded forms.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Condition</th>
<th>Peaks 1 and 2</th>
<th>Trough 1</th>
<th>Peak 3</th>
<th>Trough 3</th>
<th>Crossovers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) native (12% protein)</td>
<td>0.015 M NaF</td>
<td>λ</td>
<td>mμ</td>
<td>x x x 10^-3</td>
<td>λ</td>
<td>mμ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>292</td>
<td>1.60</td>
<td>255</td>
<td>-1.60</td>
<td>199</td>
</tr>
<tr>
<td>(b) native (2% protein)</td>
<td>0.015 M NaF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>293</td>
<td>1.76</td>
<td>256</td>
<td>-2.2</td>
<td>202</td>
</tr>
<tr>
<td>(c) native (0.1% protein)</td>
<td>0.015 M NaF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>292</td>
<td>1.68</td>
<td>257</td>
<td>-3.40</td>
<td>199</td>
</tr>
<tr>
<td>(d) DNA (a) heated at 90°</td>
<td>0.015 M NaF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>292</td>
<td>0.1</td>
<td>263</td>
<td>-1.88</td>
<td>199</td>
</tr>
<tr>
<td>(e) DNA (a) DNase treated, mg-ions removed by dialysis after the treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>290</td>
<td>0.44</td>
<td>265</td>
<td>-0.80</td>
<td>198.5</td>
</tr>
</tbody>
</table>

Fig. 6. Ultraviolet rotatory dispersion of native and denatured calf thymus DNA. Curves (a) native DNA (protein 0.1%), (b) DNA treated with DNase I (2.5 μg/ml) at 37° for 3 hr, Mg ions (10^-3 M) removed by dialysis against isotonic solvent, and (c) DNA heated at 90° for 10 min and rapidly cooled to 0 °C. Pathlength 0.05 cm; concentration of DNA 0.5 mg/ml. Solution: 0.015 M NaF.

The multiple Cotton effects of nucleic acids at 200 and 260 mμ are conformation dependent. As a result of denaturation of DNA, these Cotton effects (peaks, etc.) are greatly reduced. The magnitude of the ORD peak of native DNA near 200 mμ is always greater than that at 290 mμ or 228 mμ, and is highly diminished on denaturation. A close parallelism between the change (i.e., decrease) in Cotton effects near 200 or 260 mμ and the hyperchromicities in absorption in these regions is observed for the heat-denatured and enzyme-treated (i.e., degraded) DNA. This implies that the two \( \pi - \pi^* \) components (200 and 260 mμ) of DNA are equally sensitive to the changes brought about in the secondary structure of DNA.

In order to test the effect of proteins on the optical rotation of DNA, the ORD pattern of the latter has been measured at different contributions of protein (Table II, rows (a) — (c)). The troughs and the peak (3) in the y-region occurred at about similar wavelengths (viz., 217, 199-202; 193-197 mμ). But the maxima and minima are slightly increased in some relation with the removal of proteins. Like extinction ORD in the near ultraviolet is not much affected at higher (12% — 2%) protein content. If the y-maximum in the spectrum of native DNA disappears or goes further away in the shorter wavelengths due to adenine shift, the ORD pattern should change systematically at all wavelengths. The change observed in the y-region is not sufficient to support the prediction of red shift in the stacked DNA bases. The small change in ORD pattern is perhaps mostly related to change in the nucleoprotein interaction and/or the alteration of the configuration of the DNA molecule, with the reduction of proteins. This aspect has been further verified by measuring the optical rotation of nucleohistone at various histone-to-DNA ratio. The results have been shown in the following section and have been used to study the histone-DNA interaction.

The ORD curves of partially dehistonized nucleohistone essentially resolves the 200 mμ band of DNA present in the material irrespective of histone content in the range of 5% — 12%. This is because
histone has relatively less effective (negative) rotational band in the region of 200 m\(\mu\).

6. Configuration of DNA in Nucleohistone and Histone-DNA Interaction

Absorption studies of both the conformation and configuration of the nucleohistone (histone-to-DNA ratio 0.85—1.52) on heat (90\(^\circ\)C, 10 min., rapidly cooled) treatment indicates a hyperchromic rise at 260 m\(\mu\) by about 26%. The hyperchromicity exhibited by a similarly denatured calf thymus DNA (water solvent) is about 36%. This difference in hyperchromic may correspond to a pre-existing denatured (10%) conformation of the DNA in nucleohistone. But the same nucleohistone is found to be quite stable in the presence of 1%—10% neutralized formaldehyde for 3 hrs at 25\(^\circ\)—37\(^\circ\). Neither any red shift nor any rise in absorption at 260 m\(\mu\) is found during the formaldehyde treatment. Under similar conditions a partially denatured DNA always exhibits an additional hyperchromicity and a red-shift by about 1—4 m\(\mu\) (not shown). Obviously, the DNA in nucleohistone is not denatured in a conventional way, and therefore the configuration of this DNA hereafter will be referred to as 'hyperchromic'.

In view of the hyperchromic configuration of nucleohistone, ORD studies of this material have been undertaken in order to find out (1) if there is any correlation between the Cotton effects of nucleohistone and that of DNA denatured in vitro, each studied up to 185 m\(\mu\); and to (2) determine which regions (260 or 200 m\(\mu\)) of the DNA transitions (\(\pi - \pi^*\)) are more susceptible to DNA denaturation in situ.

Histones (Worthington, HL6HA) have been found (also shown by others \(^{22, 26}\)) to have a rotational band at 230 m\(\mu\) and below this wavelength. So any masking effect due to histones on the DNA rotation should be taken into account. The possibility of electronic interactions between proteins and nucleic acid resulting in masked (or perturbed) Cotton effects in the near ultraviolet has been considered by Oriel \(^{36}\). He assumed a simple additivity relation in assessing the contributions of nucleic acid and protein structures to the optical rotation of nucleoprotein. It is questionable whether this assumption would be equally valid in the far ultraviolet region. In other words, it is doubtful that by subtraction (\(i.e.,\) ORD of nucleohistone minus ORD of histones) one would be able to precisely assess the DNA contribution in the region of the \(\pi - \pi^*\) transition near 200 m\(\mu\). This is reasoned because unlike the x-absorption (260 m\(\mu\) peak) the y-absorption (near 200 m\(\mu\)) of DNA has been found previously to be greatly suppressed as a consequence of the DNA-protein interaction. Furthermore, a comparison of the optical rotation of nucleohistone and its components in the same salt solution is not correct because most of the phosphate groups of DNA in the nucleohistone are electrostatically bound to the basic side chains of histones. So a quite different approach has been made here.

The partial nucleohistone(s) (see for preparation the Method section, page 1517) of protein-to-DNA ratios higher than 0.8 have been used for the aforesaid purpose because these are similar in many characteristics to the full nucleohistone (protein/DNA ratio 1.52). The hyperchromism of DNA in the partial (\(i.e.,\) incomplete) nucleohistone, for example, serves as one of the criteria for the DNA-configuration in these materials. The hyperchromicity at 260 m\(\mu\) decreases from a level of 10% if the histone-to-DNA ratio in the partial nucleohistone is lower than 0.8. The value, however, remains constant at 10% if the partial nucleohistone becomes gradually enriched in histones so that the ratio (protein/DNA) is higher than 0.85, until the completely dissolved particulate nucleohistone exhibits a value of 1.52 (\(i.e.,\) 60% histones and 40% DNA).

The results of linear dichroism (\(v.i.z.,\) at 257 m\(\mu\), \(E_{i}/E_{11} = 1.10\)), polarization (R) of AO* fluorescence (\(v.i.z.,\) R = +5—15%) and rotational diffusion (P) (\(v.i.z.,\) P(for polarization) = 0.1) of a partial nucleohistone (protein-to-DNA ratio 0.9) indicate that the DNA-molecule(s) in this material (oriented by flow, fiberizing or streaking) is folded and compact to any degrees similar to that in the full nucleohistone (unpublished). The hyperchromicity also indicates that the histones which are absent in this partial nucleohistone have insignificant role in determining the DNA-configuration. Evidently, the ORD curve of this material is of importance in connection with the problem of DNA-denaturation in nucleohistone, also because the masking effect on ORD due to histones is relatively reduced. The curve (Fig. 7) of this material can be compared with the ORD spectrum of native DNA (Fig. 6, curve a). Any difference in ORD due to different solvents (\(v.i.z.,\) water * AO: Acridine Orange.)
for nucleohistone and 0.015 M NaF for DNA) used for the two compounds should be negligible. Also, the two compounds (nucleohistone and DNA) are stable in their respective solvents.

The results (Fig. 7) indicate that an ORD maximum (195 mμ) and the Cotton effect near 200 mμ are dominant in the spectrum of the partial nucleohistone. These are characteristics of native DNA. The results are different at wavelengths longer than 230 mμ. The Cotton effects and the spectrum (ORD) are diminished (perturbed) in the near ultraviolet region. The reduced Cotton effects in the aforesaid region are comparable to a great extent to those of the heat-denatured DNA (Fig. 2, curve (b)). The trough near 217 mμ occurs in the spectra of the partial nucleohistone and of the native and denatured DNA. This trough may be recognized as the negative link of 220 mμ Cotton effect which may arise from the π−π* transition in the DNA bases. Otherwise, it might correspond to the lower wavelength peptide Cotton effects. The net result in connection with the protein effect is that the nucleohistone DNA behaves native-like in the region of 200 mμ, whereas it acts like denatured (in vitro) DNA in the near ultraviolet (240−290 mμ).

Absorption studies of the partial nucleohistone have previously suggested that the DNA material is hypochromic in absorption near 200 mμ relative to protein-free DNA. The same material, however, exhibits a hyperchromicity of 10% at 260 mμ, as mentioned earlier. The hypochromism near 190 mμ may be partly due to change in the electronic structure of histones when they bind to DNA but the major contribution in hypochromism near 200 mμ is due to DNA. This is reasoned because the hypochromism near 190 mμ is found in all nucleohistone preparations containing different (high or small) amounts of histones. Also, the effect has been found in commercial DNAs containing even small amounts (2%) of protein.

The Cotton effects in the near ultraviolet are attributed mainly to the polarization (longitudinal) behavior of the main π−π* component in the stacked chromophores of DNA. The decreased Cotton effect of nucleohistone in the said region therefore implies that the main π−π* component (260 mμ) has been greatly affected due to denaturation following protein binding. The weaker transition near 200 mμ would have been affected (i.e., it would show hyperchromism as well as highly decreased Cotton effect) if the denaturation in nucleohistone would have had identical (or effect) on the two transitions. In other words, denaturation in situ should have some specific roles on the weaker transition (200 mμ) of DNA which are not observed at 260 mμ.

The hyperchromicities (with respect to native DNA) of nucleohistone and heat-denatured DNA are 10% and 26%, respectively. These different values represent different degrees (amounts) of base-base interaction. But rotations (290 mμ) are not much different in the two cases, and this is probably because the environments and configurations of the DNA-bases in the two materials are different. Most of the negatively charged phosphate groups of DNA in the nucleohistone are electrostatically bound to the basic side chains of the histones, whereas in denatured DNA a large amount of negative charges is unshielded. Shielding of the charges on the DNA phosphates causes the molecule to be highly flexible. Due to binding of histones, the DNA molecule(s) may be folded up and can take a compact configuration, as mentioned beforehand. The tight DNA-packing may cause dehydration which in turn will cause a decrease in base-base interaction. Perhaps due to closeness of the DNA helices and owing to interactions of some specific histones with some DNA-chromophores, the polarization as well as interaction due to the weaker π−π* component (200 mμ) will be improved. Any loss in base-base interaction may be overcome by histone-base interaction. This result presumably will be reflected near
190 m\(\mu\) of the spectrum of DNA so that the ORD band near 200 m\(\mu\) also remains still optically active. Under similar conditions the major \(\pi - \pi^*\) component near 260 m\(\mu\) is highly affected due to loss of base-base interaction. Histone-DNA interaction does not involve this region of the spectrum. The aforesaid explanation is no doubt tentative but the observed results imply that denaturation in vitro and denaturation in situ are not identical as long as the electronic structures of DNA are concerned.

**Discussion**

The absence of a clear maximum at 190 - 195 m\(\mu\) in the spectrum of native DNA has also been observed by Voet et al.\(^2\). These authors have further found that adenine (6-aminopurine) in neutral solution shows a redshift of 19 m\(\mu\) in relation to purine itself. According to Mason\(^35\), a substituent in the 6-position of a purine will cause a charge displacement in directions perpendicular to the molecular axis. This will lower the energy of \(\pi - \pi^*\) transition in the y-region as this transition is polarized transversally to the molecular axis. The major \(\pi - \pi^*\) transition would remain unaffected because the direction of polarization of this transition is along the molecular axis. The masking of the y-maximum of DNA has been attributed by Voet et al.\(^2\) to the pronounced redshift due to substituted adenines and similar bases stacked in DNA.

The present results obtained with commercial DNA do not contradict the experimental data of Voet et al.\(^2\). However, their argument should be tested because there may be other reasons which may cause the disappearance of the y-maximum of DNA. To test the explanation given by Voet et al.\(^2\), we shall first of all consider the features in the y-absorption of the DNA bases and their mixture. The physical mixture of the four DNA nucleotides show a clear y-maximum at 195 m\(\mu\). Also, if one adds up the integrated absorptions of the three of the four DNA bases; namely, guanine, cytosine and thymine, in their monomeric configuration or the absorptions of the four bases in their hypochromic (hydrogen banded as in dA:dT, dG:dC) (unpublished results) configuration, one finds the final peak at 195 - 190. This indicates that the absorption feature of adenine has no relation to the red shift of the DNA y-maximum\(^2\).

The copolymer poly r(A – U) and poly A indicate a maximum at wavelengths 200 – 205 m\(\mu\) and 190 m\(\mu\), respectively\(^2\). These wavelengths are smaller than the wavelengths of the y-maximum due to adenine (cf. 207 m\(\mu\)) and to uracil (cf. 203 m\(\mu\), Voet et al.\(^2\)). Falk\(^7\) has observed that the destacking of the DNA bases by drying or heating causes the y-maximum (185 m\(\mu\)) of DNA to be shifted to longer wavelengths by only 5 m\(\mu\). No disappearance of the inflection due to the y-maximum is observed following these changes. The present results also show that the y-maximum of pure native DNA occurs at a wavelength (viz., 190 m\(\mu\)) slightly shorter than that of the nucleotide mixture and of the enzymatically degraded DNA (cf. 195 m\(\mu\)). In other words, the y-maximum of DNA would suffer a blue shift of 5 m\(\mu\) (no red shift) due to perhaps stacking or hydrogen bonding of these substituents or other chromophores. This may also be true for both poly r(A – U) and poly A because they have been found to have a small amount of stacking and imperfect secondary structure. It is therefore questionable whether (or not) adenine (and similar substituted bases) would behave like a purine rather than an amino purine itself\(^2\). However, the net (averaged over the four DNA bases) blue shift that may be observed\(^7\) for DNA is of the order of 5 m\(\mu\). Such a small shift cannot cause the complete disappearance of the y-maximum.

Voet et al.\(^2\) found that deoxyribose and deoxyribosephosphate begin to show absorption below 190 m\(\mu\). However, these compounds absorb by a factor of 0.01 and 0.05 relative to native DNA\(^2\). So the fact that the absorption band at 200 m\(\mu\) shows a hyperchromic effect on denaturation, of a magnitude somewhat similar to that in the 260 m\(\mu\) region leads one to believe that the 190 m\(\mu\) peak is also sensitive toward the change in the secondary structure of nucleic acids.

The present absorption results together with those previously reported\(^9, 10\), indicate that in addition to the DNA absorption band at 260 m\(\mu\) the peak near 190 m\(\mu\) may be helpful in studying the configuration of DNA in solution or in thin film and their modification in different DNA containing structures. It is, however, essential that the DNA sample be kept absolutely free from proteins and other compounds showing benzoid absorption in the far ultraviolet. Perhaps this is one of the reasons why Voet et al.\(^2\) failed to observe the y-band maximum.
It may be said in passing that contaminating proteins in commercial DNA may not be mainly histone-like. Non-histone nuclear (acidic) proteins may also be present in the contaminating proteins. The nature of the contaminating proteins has not been determined in this study, however, the results of partially delistonized nucleohistone (protein 0.5% to 12%) are found to be qualitatively similar to those of contaminated calf thymus DNA.

ORD spectra of the contaminated DNA and nucleohistone have shown that the minor $\pi - \pi^*$ component near 190 m$\mu$ is more susceptible to DNA denaturation and to DNA-protein interaction than the principal transition near 260 m$\mu$. The Cotton effects and hypochromism are prominent in the y-region, although the DNA in nucleohistone may be slightly denatured for tight packing or dehydration, etc.

Evidences have been found that phage (T2, T4) DNA exhibits denaturation in situ (for various references and configurational studies of phage DNA, see reference 32). If the DNA (phage) molecule is released into the medium, the denaturation effects can be removed perhaps because the internal proteins and polyamines are dissociated from the macromolecule (DNA). Similarly if histones are dissociated from the nucleohistone material, the properties of the remaining material are as if due to DNA. It is therefore evident that nucleoprotein interaction does not produce any gross and permanent change in the DNA structure.

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