Biophysical Chemistry 13 (1981) 89–96
North-Holland Publishing Company

# LOCATION OF THE ETHIDIUM BINDING SITES OF HIGH AFFINITY IN CHROMATIN

D. GENEST, G. SABEUR, Ph. WAHL and G. AUBEL-SADRON
*Centre de Biophysique Moléculiare, C.N.R.S., 45045 Orleans Cedex, France*

Received 4 February 1980
Revised manuscript received 3 August 1980

The influence of H1 and H5 histones proteins upon the accessibility of ethidium bromide into chromatin is studied by steady-state fluorescence anisotropy in the range of *r*-values ([Dye]/[Phosphate]) smaller than 0.01. This corresponds to the very strong binding process. When H1 and H5 are present, the DNA segment which contains the binding sites is 25–30 base pairs long, even if H1 and H5 are digested by trypsin or by natural proteolysis, but presumably still interacting with the DNA chromatin. On the contrary, when H1 or H5 are separated from chromatin by an increase of the ionic strength, ethidium binds to a segment of DNA about 55–60 base pairs long. We may explain the results by assuming that the ethidium sites are located on a continuous segment constituting about one half of the linker, the other half interacting with H1 and H5. When chromatin is depleted from these proteins, the high affinity sites are distributed all along the linker.

## 1. Introduction

It has been shown that ethidium bromide (EB) binds to DNA chromatin by the mean of two kinds of intercalation sites [1–4,22], whereas it only binds to one kind of intercalation sites in deproteinized DNA. By measuring anisotropy decay of EB fluorescence due to excitation energy transfers, we recently showed [5] that the chromatin sites of high affinity were clustered on a DNA segment 28 base pairs long, which was assumed to constitute half of the "linker" DNA.

One generally admits that histones H1 and H5 interact with all or part of the linker. By removing these proteins from chromatin, one expects to modify the number of the high affinity sites, since these sites are assumed to be also situated on the "linker". In the present work, such a modification of the high affinity sites number has been quantitatively studied by static fluorescence anisotropy measurements.

## 2. Materials and methods

### 2.1. Chromatin preparations

Chromatin from Ehrlich ascites tumor cells sensitive (EAS) or resistant (EAR) to the anthracyclin daunorubicin were extracted from nuclei as already described (see the preceding paper and refs. [7] and [8]).

Chicken erythrocytes (CE) nuclei were prepared from freshly collected blood according to Hewish and Burgoyne [9]. CE chromatin digested by micrococcal nuclease was extracted under the same conditions as those used for Ehrlich ascites chromatins.

### 2.2. Depletion and digestion of H1 and H5

*Method 1.* The depletion of H1 from EAS chromatin has been performed according to the method described by Olenbusch et al. [10]. 11 ml of chromatin (OD = 4) were centrifuged in presence of 0.6 M NaCl in a SW50 rotor during 15 hr at 45 000 rmp. The pellet was redissolved in 4 ml of $2 \times 10^{-4}$ M EDTA (ph 7.5) and dyalized against the same medium during 24 hr in the cold room.

*Method 2.* Depletion of H1 from EAR chromatin and of H5 and H1 from CE chromatin has been performed according to the method described by Bolund and Johns [11], in which the lysine rich histones are dissociated by NaCl and extracted by AG50 W2 resin. The resin was precipitated by a 10 mn centrifugation at 3 000 rmp and the supernatant was dialized against

the wished buffer during 24 hr at 4°C.

*Method 3.* H1 was dissociated from EAS chromatin by 0.45 M NaCl, but was not removed from the sample.

*Method 4.* Trypsin (EC 34.21.4 Sigma) digestion of EAS chromatin ($A_{260}$ = 10) had been performed in 10 mM Tris-HCl buffer (pH 8, EDTA 2 × $10^{-4}$ M) at 37°C for 10 mn with 0.2 μg enzyme. The reaction was stopped by addition of 25 fold (w/w) excess of soybean trypsin inhibitor (Sigma).

*Method 5.* We observed that our preparations of CE and specially of EAR chromatin were not totally devoid of contaminating proteolysis activity. We made use of a more or less fast histone degradation in order to study aged samples. In this method, EC chromatin was about 3 weeks old and EAR chromatin 1–2 weeks old.

### *2.3. Electrophoresis control*

Electrophoreses of histones were performed on SDS-polyacrilamide gels according to Weintraub et al. [12]. Proteins were stained with comassie blue (fig. 1).

### *2.4. Circular dichroism measurements*

These measurements of chromatin were performed on a Jobin-Yvon dichrograph III with a cell compartment thermostated at 20°C. The optical densities of samples were always comprised between 0.5 and 1. Results are expressed by the value of the ellipticity ($\theta$).

### *2.5. Steady-state fluorescence anisotropy measurements*

The fluorescence anisotropy $A$ of EB bound to chromatin has been measured with a spectrofluorimeter Jobin-Yvon (type Bearn) equipped with Polaroids placed on the excitation and emission beams. Let $I_V$ and $I_H$ be the vertical and horizontal compo-

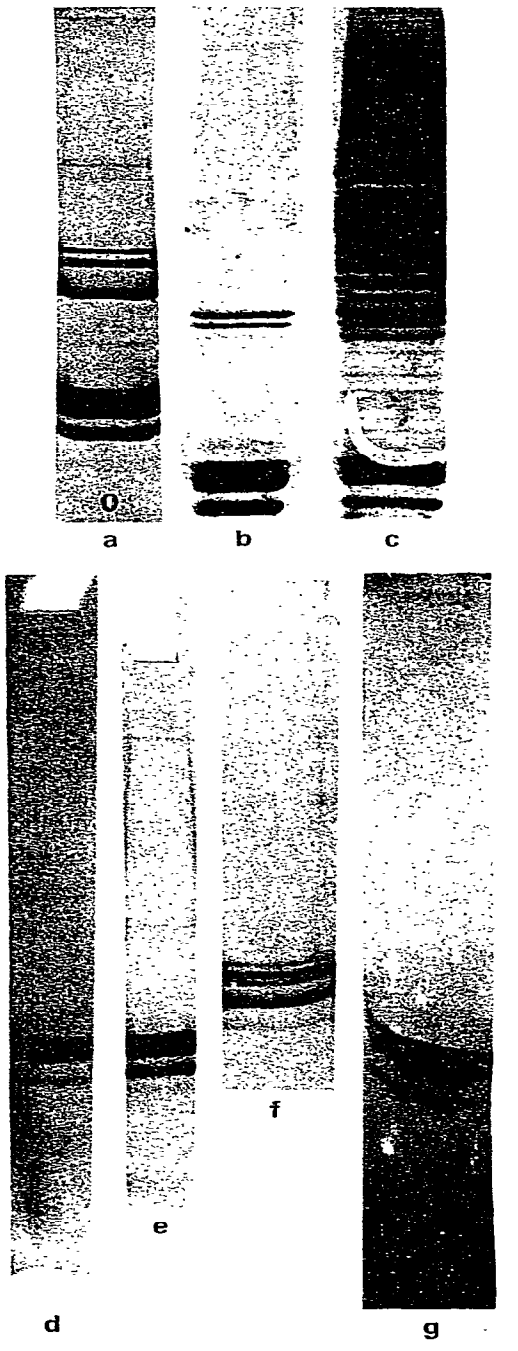


Fig. 1. SDS electrophoretic gels of chromatin proteins. a) CE native, b) EAR native, c) EAS native, d) naturally proteolyzed chromatin (method 5), e) H1-resin extracted EAR chromatin (method 2), f) trypsin digested EAS chromatin (methoc 4) g) 0.6 M NaCl extracted EAS chromatin (method 1).

nents of the fluorescence intensity when the electric field of the exciting light is vertical. Then $A$ is defined by

$$A = (I_V - I_H)/(I_V + 2I_H).$$

We corrected the effect of monochromators polarization [13] and we substracted the light scattering when necessary with a blank made of a chromatin sample in which no ethidium was added.

All measurements were performed at 20°C, the excitation and emission wavelengths being 490 nm and 640 nm respectively. In all cases, the chromatin concentration was greater than $0.75 \times 10^{-3}$ M (in nucleotides) taking the molar absorptivity of chromatin at 260 nm equal to 7 300 litres $mole^{-1}$ $cm^{-1}$. Small aliquots of EB were added to a 2 ml chromatin sample in such a way as the [phosphate]/[dye] (P/D) ratio was comprised between 2 000 and 100. Under these conditions, practically all ethidium molecules were bound to chromatin, and the number of ligands per nucleotide $r$ was practically equal to D/P. The range of $r$ was then comprised between 0.0005 and 0.01. The extrapolation of $A$ to $r = 0$ gives the brownian anisotropy.

### 2.6. Analysis of the experimental results

It has been shown in a previous work that the first EB molecules are clustered on a segment of the nucleosomal DNA [5]. Let $N$ be the number of base pairs of this segment. One can define the average number of EB molecules bound per nucleotide in the binding segment by

$$r' = r \times 200/N, \tag{1}$$

where 200 is the number of base pairs of the nucleosome.

The fluorescence anisotropy $A$ of EB–chromatin complexes depends, on the one hand, upon the brownian motions of the chromophores and, on the other hand, upon the excitation transfers between EB molecules. If $A_B$ is the value of $A$ when there is no energy migration, and $A_T$ the value of $A$, when all motions are suppressed, one can write as previously [5,14] that the time-dependent functions of anisotropy are related by

$$A(t) = A_B(t)\, A_T(t)$$

and that $A_B(t)$ is independent of $r$, whereas $A_T(t)$ is dependent of $r$ and $N$.

The static anisotropy $A$, measured under a continuous excitation, is related to $A(t)$ by the following expression:

$$A = \int_0^\infty A(t) S(t)\, dt \Big/ \int_0^\infty S(t)\, dt, \tag{2}$$

where $S(t)$ is the fluorescence decay of EB bound to chromatin.

Similarly, let us define the static anisotropies:

$$A_B = \int_0^\infty A_B(t) S(t)\, dt \Big/ \int_0^\infty S(t)\, dt, \tag{3}$$

$$A_T = \int_0^\infty A_T(t) S(t)\, dt \Big/ \int_0^\infty S(t)\, dt. \tag{4}$$

One has with a good approximation (see the appendix):

$$A = A_B\, A_T, \tag{5}$$

so that the ratio of the two measurable anisotropies $A$ and $A_B$ may be written:

$$A_B/A = 1/A_T. \tag{6}$$

According to eq. (4), $A_T$ may be obtained from the two functions $S(t)$ and $A_T(t)$ which can be determined as described in our previous work [5]. $S(t)$ was obtained from transient fluorescence measurements and was found equal to

$$S(t) = 0.10\, e^{-t/\tau_1} + 0.90\, e^{-t/\tau_2},$$

with $\tau_1 \simeq 8$ ns and $\tau_2 \simeq 23$ ns, and $A_T(t)$ was determined by a computer simulation of the energy migration along the array of EB molecules bound to a $N$ base-pairs long DNA segment. $A_T(t)$ depends on $r'$ and $N$, or, according to equation (1), on $r$ and $N$. $A_T(t)$ was computed numerically for $N$ values comprised between 10 and 60, and $r$ values ranging from 0.001 to 0.01. $A_T$ was obtained by expression (4) in which the numerator was evaluated by numerical integration. It was found that the curves representing $1/A_T$ as a function of $r$ with $N$ fixed, were straight lines as can be seen in fig. 2. The variation of the slope of these lines with $N$ is represented in fig. 3. According to these calculations and expression (6),

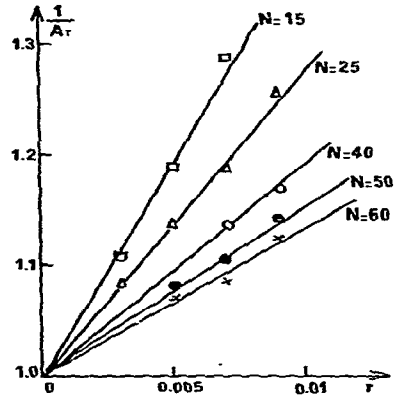


Fig. 2. Variation of $A/A_T$ as a function of $r$ for sifferent $\Lambda$ values.

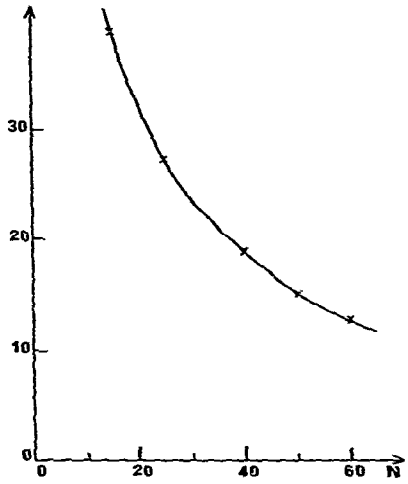


Fig. 3. Variation of the slope of the curves shown on fig. 2 as a function of $N$.

the experimental quantity $A_B/A$ varies linearly with $r$ and its slope depends on $N$, the value of which can be determined with the curve represented on fig. 3.

## 3. Results

### 3.1. Circular dichroism

The ellipticity values of chromatin samples obtained by the different methods described in section 2 are given in table 1. It can be seen that the circular dichroism of chromatin solutions in 0.45 M NaCl remains very similar to the native chromatin one, although under these conditions chromatin is depleted from H1. On the other side the circular dichroism of chromatin prepared by method 2 has a high value. This may be related to the electron microscopy studies of Thoma et al. [23] which showed

Table 1
Circular dichroism and fluorescence anisotropy results of the different chromatin samples used in this work

| Source of | Method of H1 and H5 | $\theta$ (deg. cm$^2$ | $\frac{d}{dr}(\frac{A_B}{A})$ | $N$ |
|---|---|---|---|---|
| EAS | native | 2150 | 24 | 29 |
| | trypsin digestion (method 4) | 2390 | 20 | 36 |
| | 0.6 M NaCl + ultracentrigu-tion (method 1) | – | 13.2 | 56 |
| | 0.45 M NaCl (method 3) | 2600 | 13 | 58 |
| EAR | native | 2300 | 24 | 29 |
| | natural proteolysis (method 5) | 2300 | 23 | 31 |
| | 0.45 M NaCl + Resin AG 50W2 (method 2) | 4200 | 12.9 | 58 |
| CE | native | 2700 | 26 | 26 |
| | natural proteolysis (method 5) | – | 21.4 | 34 |
| | 0.45 M NaCl + Resin AG 50W2 (method 2) | 4900 | 12 | 62 |

that H1-depleted chromatin lost his nucleosomal structure at very low ionic strength.

When H1 or H5 is digested by trypsin or by natural proteolysis (methods 4 and 5), the circular dichroism remains unchanged, and therefore the structure of chromatin does not seem to be destroyed.

### *3.2. Fluorescence anisotropy*

a) Native chromatin. It was experimentally found that for $r$ values lower than 0.01 the $A_B/A$ ratio was a linear function of $r$ (fig. 4), the slope of which was comprised between 23 and 26 (table 1). By reporting these values on the curve represented on fig. 3, according to the method described above, a nucleosomal DNA segment with a length $N$ comprised between 25 and 30 base pairs, was determined. These values agree with our previous estimation based on anisotropy decay measurements [5].

b) H1-depleted chromatin (methods 1, 2 and 3). In this case, $A_B/A$ was also a linear function of $r$ for $r$ smaller than 0.01 (fig. 4). The values of the slope were comprised between 12 and 13 (table 1), corresponding to a DNA segment of 55 to 60 base pairs long. This is about the length of the whole linker.

c) H1-digested chromatin (methods 4 and 5). The variation of $A_B/A$ with $r$ is very similar to the variation obtained with native chromatin (fig. 4). According to table 1 the slopes of the straight lines were comprised between 20 and 23, corresponding to a DNA segment of 30–36 base pairs long. Therefore the digestion of H1 by trypsin or by natural proteolysis does not seem to affect very much the accessibility of EB to chromatin.

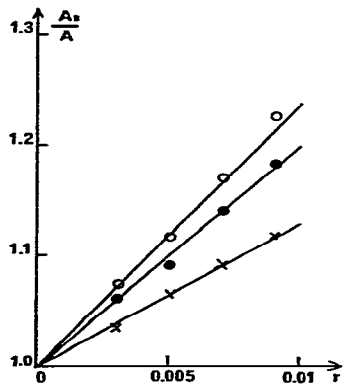


Fig. 4. Some experimental curves representing $A_B/A$ as a function of $r$, obtained with EAS chromatin (ooo native chromatin; ●●● H1-digested chromatin by method 4; ××× H1-depleted chromatin by method 3).

## 4. Discussion

Our value of ellipticity at 283 nm for the native chromatin agrees with the value of de Murcia et al. [5]. As pointed out by these authors, this small value corresponds to the native form of chromatin. In chromatin depleted from H1 (or H5), and at very low ionic strength, one observes a higher ellipticity. which, according to the same authors, corresponds to an inorganized form (without nucleosomal structure). This fact was also shown by Thoma et al. [23] who found that at very low ionic strength H1-depleted chromatin does not present a nucleosomal structure contrary to H1-containing chromatin. At higher ionic strength, Thoma et al. found that the nucleosomal structure remains even when chromatin is depleted from H1. We agree with their finding since the dissociation of H1 by 0.45 M NaCl modified only slightly the ellipticity. On the other side, trypsin or natural proteolysis of H1 and H5 does not affect the circular dichroism, even at very low ionic strength.

In the present work we set up a method based on static polarization measurements allowing the determination of the length $N$ of the nucleosomal DNA which contains the high affinity sites of ethidium bromide. In the case of native chromatin, where H1 and H5 are present, $N$ is 25–30 base pairs, in good agreement with the value previously obtained by us with anisotropy decay measurements [5]. This strengthens the confidence in the method used here. When chromatin is depleted from H1, $N$ increases to 55–60 base pairs and then new EB binding sites of high affinity become available. This result was obtained when H1 and H5 were extracted by resin or ultracentrifugation as well as when they were simply dissociated from chromatin by salt and remained in the surrounding buffer.

Finally the values of the circular dichroism and of the fluorescence polarization measurements lead us to classify our samples into three classes.

1) $\theta \simeq 2200$ deg cm$^2$ dmole$^{-1}$ and $N \simeq 25$–35

base pairs. This was obtained for native chromatin and for samples in which H1 (or H5) were digested but still fixed at their sites.

2) $\theta \simeq 2200$ deg cm$^2$ dmole$^{-1}$ and $N \simeq 55$–$60$ base pairs. This was obtained for chromatin depleted from H1 in 0.45 M NaCl.

3) $\theta \simeq 4500$ deg cm$^2$ dmole$^{-1}$ and $N \simeq 55$–$60$ base pairs. This is the case of chromatin depleted from H1 in 0.2 mM EDTA.

Arguments have been presented by us and others, which favour the view that the high affinity binding sites of ethidium bromide in native chromatin are located on the linker DNA of nucleosomes (4,5). Since the linker is about 60 base pairs long, the binding segment constitutes about half of it. Our present results on native chromatin confirm these previous findings. They may be understood if one admits that, in native chromatin, H1 (or H5) interacts with half of the linker DNA (15) and that ethidium preferentially binds to the remaining part of it. The interaction of H1 and H5 with chromatin does not seem to be altered by proteolysis of these proteins since the length of the binding DNA segment is not affected by this treatment (first class of sample). This means that the interaction of H1 and H5 is strong enough to persist even when the peptide chain of these proteins is broken by enzymatic digestion.

The second class of samples are obtained by removing H1 from chromatin dissolved in high ionic strength solvent. In this case, the nucleosomal structure is preserved as shown by the low value of the circular dichroism at 283 nm and the electron micrography of Thoma et al. [23]. The whole linker (60 base pairs) becomes available for the ethidium binding. This does not exclude the possibility that H1 (or H5) can also interact with the core nucleosome as suggested [16,17].

In the third class of samples, which correspond to H1 (or H5) depleted chromatin at very low ionic strength, the nucleosomal structure is not preserved, but the length of the DNA segment which binds ethidium is still 60 base pairs. This probability means that the histones H2a, H2b, H3 and H4 still interact with the 140 base pairs DNA of the core nuclesome and that the DNA segment containing the high affinity sites is the linker, as it was in the second class of samples.

Let us now recall some of the published studies of chromatin digestion by micrococcal endonuclease. A short digestion leads to the complete mononucleosomes containing 200 base pairs. Further digestion yields two stable fragments of 160–180 base pairs and 140–150 base pairs (the core particle) respectively [15,18–20]. It has been generally found that the histome H1 is still associated with the nucleosome fragment of 160–180 base pairs [18–21], whereas H1 is not found associated with the core particles. This agrees with the fact that part of H1 or H5 is bound to about 30 base pairs of the linker DNA and that micrococcal nuclease first attacks the linker regions which do not interact with H1 and H5.

There is a striking parallelism between these results and our results with ethidium chromatin complexes. The strong binding sites of ethidium, representing 14% of the nucleosomal DNA, appear to be the same as the sites which are first attacked by the nuclease.

*Footnote*: After that the present work has been submitted for publication, Wu et al. (Biochem. 19 (1980) 626) found that high affinity binding sites exist on isolated core particles. This result is in contradiction with the work of Erard et al. (Nucl. Ac. Res. 6 (1979) 3231) and with the analysis presented here. This divergence might come from the preparation of core particles since the circular dichroism at 283 nm of the core particles used by Wu et al. is somewhat higher than the value reported by Erard et al. We are at the present time working about this problem and it will be discussed elsewhere.

## Appendix

According to the present work and previous determinations [5] the expressions of $A_B(t)$ and $S(t)$ are:

$$A_B(t) = A_0(\alpha_1 e^{-t/\theta_1} + \alpha_2 e^{-t/\theta_2}),$$

with $A_0 \simeq 0.30$, $\alpha_1 \simeq 0.125$, $\alpha_2 = 0.875$, $\theta_1 = 6.5$ ns, $\theta_2 = 435$ ns,

$$S(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2},$$

with $a_1 = 0.10$, $a_2 = 0.90$, $\tau_1 = 8$ ns, $\tau_2 = 23$ ns. It follows that $\int_0^\infty S(t)\,dt = 21.5$ ns and $\int_0^\infty A_B(t)S(t)\,dt = A_0 \times 18.5$ ns.

The expression of $A_B(t)S(t)$ is given by:

$$A_B(t)S(t) = A_0 \sum_{i=1}^{4} c_i \, e^{-t/\rho_i}$$

with $c_1 = 0.012, c_2 = 0.113, c_3 = 0.087, c_4 = 0.788$, $\rho_1 = 3.6$ ns, $\rho_2 = 5.1$ ns, $\rho_3 = 7.9$ ns, $\rho_4 = 21.8$ ns.

Taking into account the expressions (2), (3) and (4) of the text, let us compute the expression of $A/A_B - A_T$. We have:

$$\frac{A}{A_B} - A_T = \frac{\int_0^\infty A_B(t)S(t)A_T(t)\,dt}{\int_0^\infty S(t)\,dt} \times \frac{\int_0^\infty S(t)\,dt}{\int_0^\infty A_B(t)S(t)\,dt}$$

$$- \frac{\int_0^\infty A_T(t)S(t)\,dt}{\int_0^\infty S(t)\,dt} = \frac{\int_0^\infty A_B(t)S(t)A_T(t)\,dt}{A_0 \times 18.5}$$

$$- \frac{1}{21.5}\int A_T(t)S(t)\,dt.$$

If one replaces $A_B(t)S(t)$ and $S(t)$ by their values, one obtains:

$$A/A_B - A_T = A_1 + A_2 + A_3 + A_4,$$

with

$$A_1 = \int_0^\infty \frac{0.012}{18.5} e^{-t/3.6} A_T(t)\,dt,$$

$$A_2 = \int_0^\infty \frac{0.113}{18.5} e^{-t/5.1} A_T(t)\,dt,$$

$$A_3 = \int_0^\infty \left[\frac{0.087}{18.5} e^{-t/7.9} - \frac{0.1}{21.5} e^{-t/8}\right] A_T(t)\,dt,$$

$$A_4 = \int_0^\infty \left[\frac{0.788}{18.5} e^{-t/21.8} - \frac{0.9}{21.5} e^{-t/23}\right] A_T(t)\,dt.$$

Under the conditions of our study, $A_T(t)$ was always comprised between 0.6 and 1 when $t$ varied from 0 to 80 ns, so that:

$$0.001 \leqslant A_1 \leqslant 0.002,$$

$$0.019 \leqslant A_2 \leqslant 0.031,$$

$$A_3 \simeq 0,$$

$$-0.034 \leqslant A_4 \leqslant -0.021.$$

Therefore, we can write:

$$-0.015 \leqslant A/A_B - A_T \leqslant 0.012.$$

It can be easily shown that the truncature introduced by limiting the integration to 80 ns does not affect the computed values. Under our experimental conditions, $A/A_B$ was always greater than 0.43, therefore:

$$\frac{|A/A_B - A_T|}{A/A_B} < 0.03.$$

Then, $A/A_B$ differs from $A_T$ by less than 3%, which justifies the use of relation (5).

**Acknowledgements**

The authors thank Dr. J.C. Maurizot for the use of his CD apparatus and Dr. B. Malfoy for very helpful discussions.

**References**

[1] J.J. Lawrence and M. Louis, FEBS Letters 40 (1974) 9.
[2] M. Angerer, S. Georghiou and E.N. Moudrianakis, Biochem. 13 (1974) 1075.
[3] J.J. Lawrence and M. Daune, Biochem. 15 (1976) 3301.
[4] J. Paoletti, B.B. Magee and P.T. Magee, Biochem. 16 (1976) 351.
[5] D. Genest, G. Sabeur, Ph. Wahl and J.-C. Auchet, Biophys. Chem. 13 (1981) 77.
[6] G. De Murcia, G.C. Das, M. Erard and M. Daune, Nucleic Acids Res. 5 (1978) 523.
[7] J. Paoletti, Biochem. Biophys. Res. Comm. 81 (1978) 193.
[8] G. Sabeur, D. Genest and G. Aubel-Sadron, Biochem. Biophys. Res. Comm. 88 (1979) 722.
[9] D.R. Hewish and L.A. Burgoyne, Biophys. Res. Comm. 52 (1973) 504.
[10] H.H. Olenbusch, B. Olivera, D. Tuan and N.J. Davidson, J. Mol. Biol. 25 (1967) 299.
[11] L.A. Bolund and E.W. Johns, Eur. J. Biochem. (1973) 546.
[12] H. Weintraub, F. Palterk and Van Lente, Cell 6 (1975) 85.
[13] T. Azzumi and S.P. MacGlynn, J. Chem. Phys. 37 (1962) 2413.
[14] D. Genest and Ph. Wahl, Biophys. Chem. 7 (1978) 317.
[15] A.J. Varshalvsky, V. Bakayev and G.P. Georgiev, Nucleic Acids Res. 3 (1976) 477.
[16] W.M. Bonner, Nucleic Acids Res. 5 (1978) 71.
[17] S.A. Newman and P. Noon, Nucleic Acids Res. 6 (1979) 609.

[18] M. Noll and R.D. Kornberg, J. Mol. Biol. 109 (1977) 393.
[19] B. Ramsay Shaw, T.M. Herman, R.T. Kovacic, G.S. Beaudreau and K.E. Van Holde, Proc. Nat. Acad. Sci. USA 73 (1976) 505.
[20] J.P. Whitlock and R.T. Simpson, Biochem. 15 (1976) 3307.
[21] R.T. Simpson, Biochem. 17 (1978) 5524.
[22] M. Erard, G.C. Das, G. de Murcia, A. Mazen, J. Pouyet, M. Champagne and M. Daune, Nucleic Acids Res. 6 (1979) 3231.
[23] F. Thoma, Th. Koller and A. Klug, J. Cell Biol. 83 (1979) 403.