Biophysical Chemistry 7 (1978) 269–277


# INTRINSIC AND EXTRINSIC FLUORESCENCE OF HISTONES H2A AND H2B: A CONFORMATIONAL STUDY

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Received 14 March 1977
Revised manuscript received 28 May 1977

Intrinsic and extrinsic fluorescence measurements suggest that H2A and H2B histones, in a partially secondary structure, self-aggregate into assemblies in which some tyrosine groups are buried in a hydrophobic environment and show enhanced fluorescence. 2-p-toluidinylnaphthalene-6-sulfonate (TNS) indicates heterogeneity among the binding sites whose number depends on the pH values of the solutions. Warfarin, used as hydrophobic probe, shows that during the process of self-association and cross-complexing of the two histones there is the covering of some hydrophobic sites of the proteins.

## 1. Introduction

In a recent paper on fluorescence of histones H1 and H5 [1], we have shown the existence of buried tyrosine groups displaying enhanced fluorescence in contrast with the data observed for several other proteins [2]. Here we report the results obtained on two other histones, H2A (129 residues) and H2B (125 residues), which are classified as moderately lysine-rich [3]. It is known that the increase of ionic strength or pH of H2A and H2B solutions causes a conformational transition of these macromolecules and the formation of large structures owing to a self-aggregation phenomenon [4–9]. On the other hand a strong interaction between H2A-H2B pair was demonstrated and it has been shown that the formation of the cross-complex is competitive with the self-aggregation [9,10–13]. Furthermore studies at different temperatures showed hydrophobic interactions between segments of H2A molecules [14].

The use of a hydrophonic probe as 8-anilinonaphthalene-sulfonate (ANS) to study enzyme aggregation was suggested in the case of glutamate dehydrogenase solution [15,16]. In this paper we report the behaviour of H2A and H2B solutions when titrated with 2-p-toluidinylnaphthalene-6-sulfonate (TNS) which is a dye similar to ANS. We also used as hydrophobic probe, the warfarin molecule [17], an anticoagulant drug, which up to today has only been used in the binding with plasma albumins. The circular dichroism (CD) and intrinsic and extrinsic fluorescence data obtained were related to the secondary structure as well as to the aggregation capacity of the two proteins.

## 2. Materials and methods

Calf thymus samples of histones H2A and H2B and of the segments 1–58 and 63–125 of the histone H2B were kindly furnished by Professor E.M. Bradbury (Portsmouth Polytechnic, Hants, England) who reported elsewhere the extraction and the purification methods [4,5,8]. 2-p-toluidinylnaphthalene-6-sufonate (potassium salt) and R-warfarin (sodium salt) were a SIGMA product No. T-8753 and an ENDO Lab. (N.Y.) product respectively and were used without further purification.

The histone concentration was determined by the absorbance at 276 nm utilizing 1340 $M^{-1}$ $cm^{-1}$ as extinction coefficient of the tyrosine group. The concentrations of TNS and warfarin were determined utilizing $1.89 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 317 nm [18] and $1.39 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 308 nm [19] as extinction coefficients respectively.

Intrinsic fluorescence of the proteins is reported as a quantum yield ratio ($R_{TYR} = Q_{histone}/Q_{L\text{-tyrosine}}$) with respect to a $10^{-5}$ M solution of L-tyrosine in $2 \times 10^{-2}$ M tris buffer, pH 7 and the measurements were carried out with the procedure reported in the preceding paper [1] in 1 cm quartz cells. The measurements of the intrinsic and extrinsic fluorescence were always carried out on histone solutions prepared at the fixed conditions of pH and ionic strength, one day before, to avoid kinetic effects. TNS and warfarin were added to the histone solution directly in the fluorescence cell drawing the proper amount of probe from concentrated stock solutions with Terumo microsyringes.

All fluorescence measurements were carried out at 25°C.

Emission fluorescence spectra of both TNS and warfarin were obtained with an excitation light of 340 nm. Correction was always made for the absorption of the excitation light but no correction was required for the emitted light. Quantum yields of TNS solutions were calculated by the area of the emission spectrum by comparison with the area of the spectrum of a $10^{-6}$ M solution of TNS in sec-butanol assuming 0.5 as quantum yield [18]. Fluorescence intensity measurements of warfarin solutions were carried out at 410 nm.

Absorption spectra were registered with a Hitachi–Perkin Elmer EPS-3T spectrophotometer utilizing 1–0.1 cm quartz cells. pH measurements were carried out with a pHM-52 Radiometer pHmeter. Fluorescence spectra were obtained with a Perkin-Elmer-3L spectrofluorimeter supplied with a Xenon lamp and thermostating attachment.

## 3. Results and discussion

### 3.1. Intrinsic fluorescence

Bradbury et al. [8] on the basis of CD and NMR data suggested that the adding of salt to H2A solutions results α-helical conformational changes involving segments 47–66 and 78–88. At the same time the central part of the molecules (25–113) self-aggregates into large assemblies. Previously D'Anna and Isenberg [7], measuring fluorescence anisotropy and ellipticity, have proposed the segment 85–105 as helical region which does not include the three tyrosine groups located at the positions 39, 50 and 57 [3]. Quantum yield measurements carried out on H1 solutions allowed us to show a rather new behaviour for a tyrosine in a protein sequence i.e. buried and unquenched [1]. Since H2A and H2B do not contain tryptophan groups like H1, we studied these macromolecules by fluorescence quantum yield measurements to check the existence of other tyrosines of such a type. On the other hand the results could be useful to obtain supplementary information on the structure of histones H2A and H2B in aqueous solutions. In fig. 1 there are quantum yield and CD data obtained by increasing ionic strength of H2A solutions at two different pHs (3.5 and 7.5). The addition of phosphate buffer (no NaCl) to the solution of H2A in pure water (fig. 1, squares) causes a conformational transition as pointed out by the CD data. In the solutions which do not hold NaCl, the gain of secondary structure is higher at pH 7.5 than at pH 3.5 but

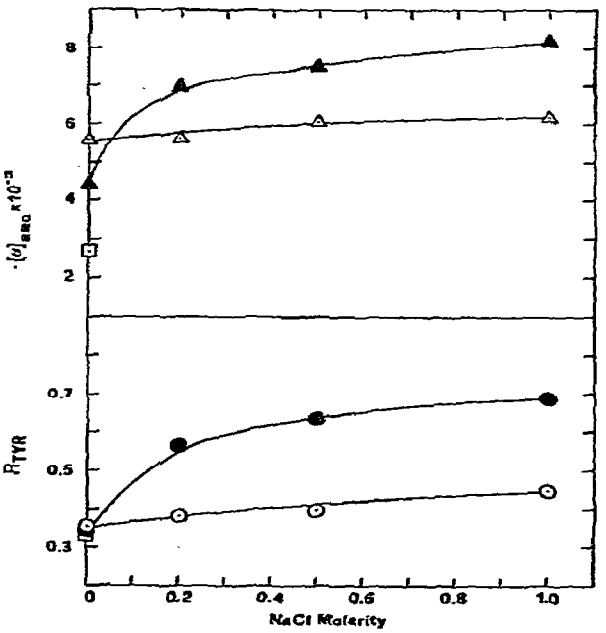


Fig. 1. Ellipticity at 220 nm (upper) and quantum yield ratio (lower) for H2A solutions with changes in NaCl concentration at pH 3.5 (black) and 7.5 (white). The squares refer to histone solution in pure water (no NaCl, neither buffer). Protein concentration, $1 \times 10^{-5}$ M; phosphate buffer 20 mM.

in both cases there is no significant change in the fluorescence of the tyrosine groups. The classification of tyrosyl residues proposed by Cowgill [2] suggests two possible hypotheses for the result at [NaCl] = 0: either all tyrosine groups are not included in the structured segment of the protein or they are included but some of them have enhanced fluorescence and some are quenched so that there is a perfect compensation. Though Bradbury et al. [8] demonstrated that at low ionic strength (10 mM NaCl) tyrosine peaks are already perturbed, we are inclined to not include the tyrosines in the α-helical segment of the molecule owing to the results obtained at pH 3.5 adding salt (fig. 1). In fact, the addition of salt is followed by the increase of ellipticity which parallels a large increase in fluorescence. The graduality of the phenomenon seems distinctive of a process in which the formation of secondary structure accompanies the self-aggregation. A similar curve was found by Edelhoch et al. in the study of self-association of reduced apoA-II [20]. We think that the perturbation of the NMR peaks of the tyrosines in 10 mM NaCl can be the result of the aggregation since the concentration used in those experiments is much higher than that

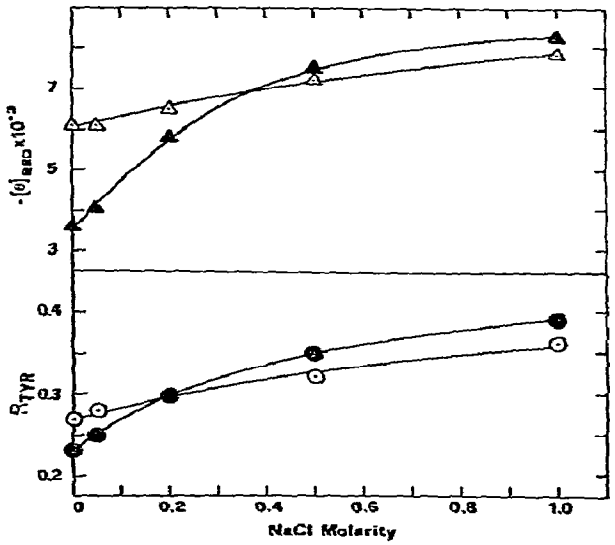


Fig. 2. Ellipticity at 220 nm (upper) and quantum yield ratio (lower) for H2B solutions with changes in NaCl concentration at pH 3.5 (black) and 7.5 (white). Protein concentration, $1 \times 10^{-5}$ M; phosphate buffer 20 mM.

used in our fluorescence measurements. Therefore we conclude that the addition of salt aggregates the protein and increases its α-helical content (CD data of fig. 1). It is in this moment that tyrosines are interested to the process and at least one of them should by of type I [2] (exposed and unquenched) or similar to the tyrosine found in the histone H1 [1] (buried in a hydrophobic environment and unquenched). At pH 7.5, using $10^{-5}$ M protein concentration, the addition of salt does not promote large increases of the helical aggregates and the tyrosine fluorescence does not change in appreciable extent.

The behaviour of the histone H2B whose data (CD and quantum yield) are reported in fig. 2 is partly similar to that of H2A. An α-helix structure of the residues 60–102 with a self-aggregation including the part 31–102 have been suggested by Bradbury et al. [4,5,21], whereas D'Anna and Isenberg [6] proposed 33 residues in α-helical structure at pH 3.5 and only 20 at pH 7.4 involving the segment 65–84. The addition of salt at pH 3.5 (fig. 2) causes a gradual increase of both secondary structure and tyrosine quantum yield. The increase of the pH (no NaCl) results the same phenomenon, i.e. more secondary structure and enhancement of fluorescence. Histone H2B contains five tyrosines in position 37, 40, 42, 83, 121 [3] and NMR spectra [4,5,19] indicated that tyrosine 83 is included in the α-helical segment. Therefore the enhancement of fluorescence could be due to the change of the degree of hydration of the peptide bond (type I, [2]). On the other hand, the aggregation by salt of the region 31–102 can decrease the polarity of the environment around tyrosine 83 as well as around some others (37, 40, 42) resulting in a gradual increase of fluorescence with the degree of aggregation of the structured molecules. It is interesting to compare the data of two corresponding solutions: pH 7.5 without and pH 3.5, 0.22 M NaCl (fig. 2). The two solutions contain molecules with the same degree of secondary structure (same ellipticity, – 6,000) but show different fluorescence. We think that the degree of aggregation is higher at pH 3.5 than at pH 7.5 even if the amount of α-helix is the same. This result can be due to the presence in position 68, 71 and 76 of the carboxyl groups of some aspartic and glutamic residues which are dissociated at pH 7.5 so that the aggregation of this part of molecule is partially hindered. It must be noted that these carboxyl groups

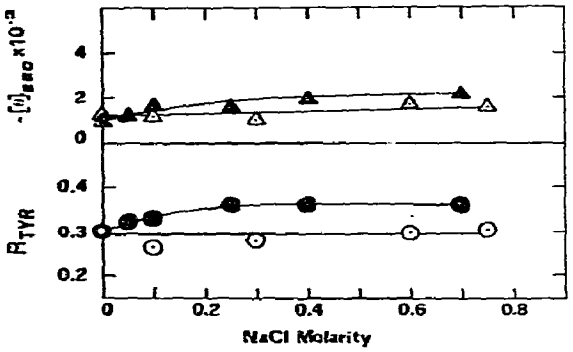


Fig. 3. Ellipticity at 220 nm (upper) and quantum yield ratio (lower) for 1–58 H2B segment solutions with changes in NaCl concentration at pH 3.5 (black) and 7.5 (white) Protein concentration, $1 \times 10^{-5}$ M; phosphate buffer 20 mM.

do not quench tyrosine fluorescence as found in other proteins [2]. Lastly we must suggest at both pH almost the same structure since in 1 M NaCl ellipticity and quantum yield values of the solution at pH 7.5 are only a little bit lower than those at pH 3.5. This result contrasts with the data reported by D'Anna and Isenberg [6]. Data of figs. 3 and 4 were obtained using the segments 1–58 and 63–125 of the histone H2B. The segment 1–58 is very little structured at pH 3.5 and it is unstructured at pH 7.5. These results agree with the optical rotatory dispersion (ORD) and NMR data shown by Bradbury et al. [5]. The aggregation at pH 3.5 shown by NMR spectra is also indicated by the gradual increase of the fluorescence at least of one of the tyrosines 37, 40 and 42. However we found for the segment 63–125 (fig. 4) a behaviour different from that shown by ORD. In fact, data of fig. 4 indicate that at pH 3.5 there is no structure with [NaCl] $\leqslant 0.4$ whereas ORD data show a fast increase on addition of the salt [5]. The large difference in the protein concentration used in the two experiments could give a proper explanation. Furthermore data of fig. 4 indicate that at pH 7.5, using a protein concentration $10^{-5}$ M, the segment 63–125 cannot gain a secondary structure.

### 3.2. Extrinsic fluorescence

The preceding data concerning the intrinsic fluorescence of the histones H2A and H2B suggest, as

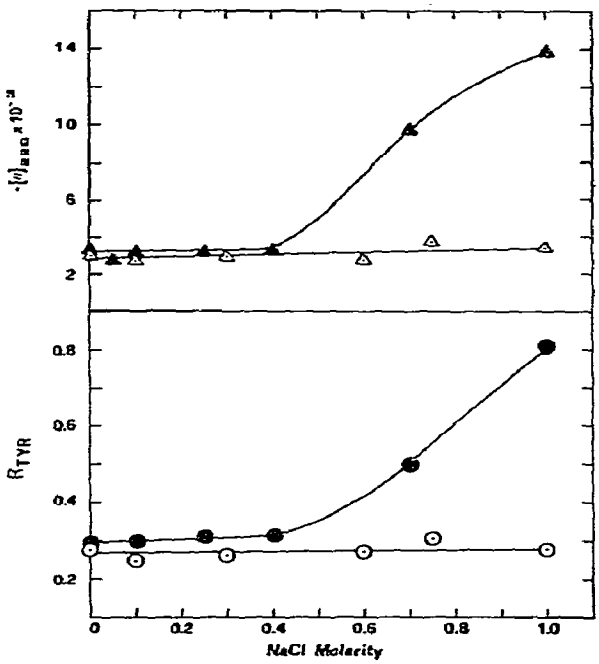


Fig. 4. Ellipticity at 220 nm (upper) and quantum yield ratio (lower) for 63–125 H2B segment solutions with changes in NaCl concentration at pH 3.5 (black) and 7.5 (white). Protein concentration, $1 \times 10^{-5}$ M; phosphate buffer 20 mM.

reported in the section 3.1., an aggregation phenomenon involving the regions of the molecules which include some tyrosine groups. These regions have an apolar character and hydrophobic interactions have been suggested between them [8,14]. The use of some hydrophobic probes which have been widely used in the last years in the studies of protein structures [22,23], prompted us to utilize TNS and warfarin to obtain additional data about the self-aggregation process of the histones.

The blue shift of the maximum of fluorescence and the increase of the quantum yield of TNS on binding to a macromolecule are reported as indicative of hydrophobic sites upon the chain [18,24,25]. In figs. 5 and 6 there are the results obtained in the titration with TNS of solutions of the histone H2B at constant protein concentration. Data of fig. 5 show three evident features:

(a) the values of $\lambda_{max}$ obtained at pH 3.5 are higher

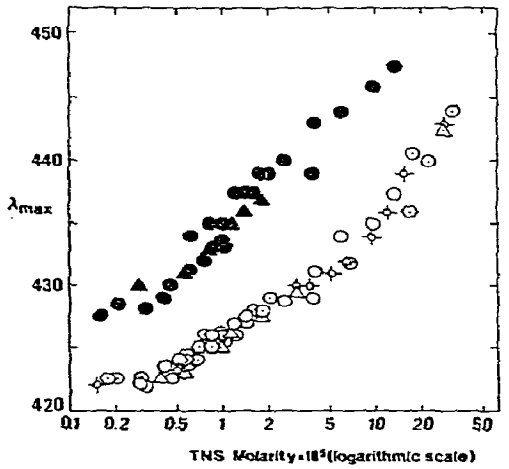


Fig. 5. Maximum of emission fluorescence (nm) of TNS-H2B solutions at pH 3.5 (●, ▲) and 7.5 (○, △, ◈, ◇) in phosphate buffer 20 mM. H2B molarity: ●, ◈, $1 \times 10^{-5}$; ▲, △, $2.5 \times 10^{-5}$; ○, $4.6 \times 10^{-5}$; ◇, $7 \times 10^{-5}$.

than those at pH 7.5;

(b) $\lambda_{max}$ at any pH increases on increasing the probe/protein ratio;

(c) no dependence of $\lambda_{max}$ was observed on protein concentration in the range of H2B concentrations studied.

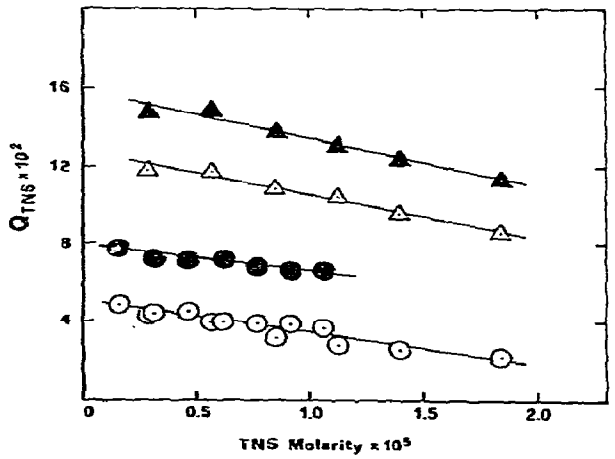


Fig. 6. Quantum yield of TNS-H2B solutions at pH 3.5 (●, ▲) anf 7.5 (○, △) in phosphate buffer 20 mM. H2B molarity: ●, ○, $1 \times 10^{-5}$; ▲, △, $2.5 \times 10^{-5}$.

On the basis of the interpretation of TNS behaviour reported by McClure and Edelman [24], it is possible to conclude that the result (a) indicates that at pH 3.5 the sites on the protein are less hydrophobic than those at pH 7.5. This could be due to the loss of some sites on increasing the pH so that more hydrophobic ones remain on the protein or to the appearing of new and more hydrophobic sites when the partially structured molecules self-aggregate. To make a choice of the two hypotheses it is necessary to consider the quantum yields reported in fig. 6 where it is possible to see that the lower is the pH, the higher is the TNS quantum yields, considering the same protein concentration. The data of figs. 5 and 6 are apparently contrasting since spectra with higher $\lambda_{max}$ give higher quantum yields [18,24,25]. The quantum yields of fig. 6 are calculated on the basis of the total probe concentration which includes bound and free TNS. The amount of bound TNS depends on both the binding constant and number of sites upon the macromolecule [25]. We did not obtain, in agreement with Beyer et al. [26], the value for these parameters. In fact we do not think that it is possible to obtain Scatchard type binding data with te results of figs. 5 and 6 since $\lambda_{max}$ shifts during titration so that fluorescence intensity measurements cannot give the fraction of bound probe. However, if the lower values for $\lambda_{max}$ at pH 7.5 mean more hydrophobic sites, higher binding constants might characterize the binding process. Therefore at pH 3.5 there are higher quantum yields since there are more sites than at pH 7.5 so that more dye is bound to the protein. On changing the pH, the new tertiary structure and/or the dissociation of carboxyl side groups causes the loss of some sites. At pH 7.5 there are less sites but they are on the average more hydrophobic. The second feature (b) of fig. 5 is indicative of an heterogeneity of the binding sites on the protein. In fact TNS is non-fluorescent in water so that the shift of $\lambda_{max}$, increasing the probe/protein ratio, must be the result of band combination of differently bound species. We think that conformational effects of TNS on the histone, as suggested by Holler et al. [27] in the binding of the TNS to the L-isoleucyl-tRNA synthetase, can be ruled out. In fact at pH 3.5 the histone H2B is mainly disordered (CD data of fig. 2) so that the only conformational effect might be the gain of structure. In this case $\lambda_{max}$ should decrease as demonstrated changing the pH. It

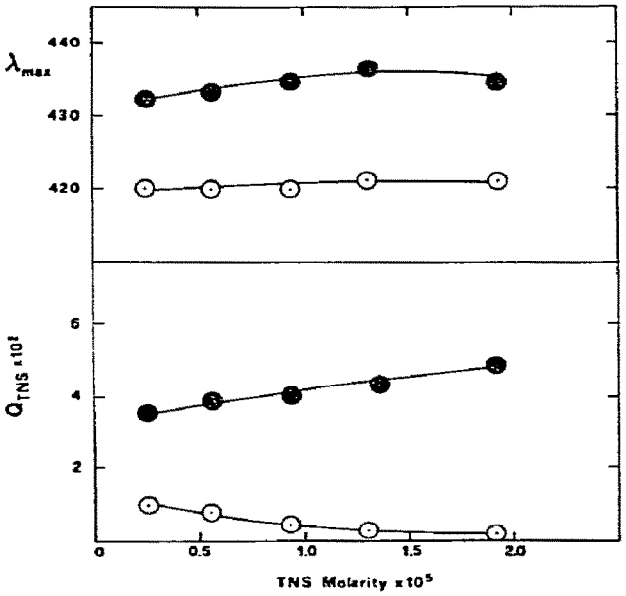


Fig. 7. Maximum of emission fluorescence (upper, nm) and quantum yield (lower) of TNS-H2A solutions at pH 3.5 (●) and 7.5 (⊙) in phosphate buffer 20 mM. H2B molarity: 1 × $10^{-5}$.

seems more likely that first TNS molecules bound to H2B increase the possibility to saturate less hydrophobic sites on increasing dye concentration. Finally, the invariability of $\lambda_{max}$ with protein concentration (point (c); fig. 5) suggests that in the range of concentration used the aggregation of the histone does not affect the quality of the sites.

The fluorescence data obtained with the H2A histone and shown in fig. 7 are similar to those of H2B and confirm for this type of proteins more sites at acid pH but less hydrophobic than at pH 7.5 where the proteins are partially structured and associated. The quantum yield data at pH 3.5 of fig. 7 support the interpretation given for the H2B solutions. The increase of the quantum yield with TNS at constant protein concentration is absurd if we do not suppose that the first TNS molecules promote saturation of new sites. So that there is no decreasing of the fraction of bound dye as suggested by the theory on one class of independent binding sites [28].

The use of the warfarin, an anticoagulant drug, as fluorescence probe is rather new in the sense that this molecule has only been used in the binding with plasma albumins [17]. In this paper we suggest the use of warfarin as hydrophobic probe useful in the conformational studies of macromolecules in water solutions. The addition of H2A or H2B to warfarin solution is followed by an increase of the fluorescence of the dye At any probe/protein ratio it was noted only a very small blue shift of the maximum of the fluorescence spectra so that the extrapolation for a fixed wavelengt to $C_{warf.} \rightarrow 0$ gave the molar intensity fluorescence of the bound warfarin useful to obtain Scatchard type plot for a stated protein concentration with a procedure analogous to that reported by other authors [29, 30]. In the figs. 8 and 9 there are the data obtained at pH 7.5 using different but constant protein concentrations. No data were collected at pH 3.5 because of the warfarin insolubility at this pH. In the range of dye/protein ratios used in the study, the straight lines obtained suggest one class of independent binding sites [28]. A general result characterizes the binding of warfarin to H2A and H2B: on increasing protein concentration, the number of binding sites on the protein decreases. In the range of protein concentration $2.5 \times 10^{-5}$ to $1 \times 10^{-4}$ M the number of sites decreases from 2.5 to 1, in the case of H2A, and from 2

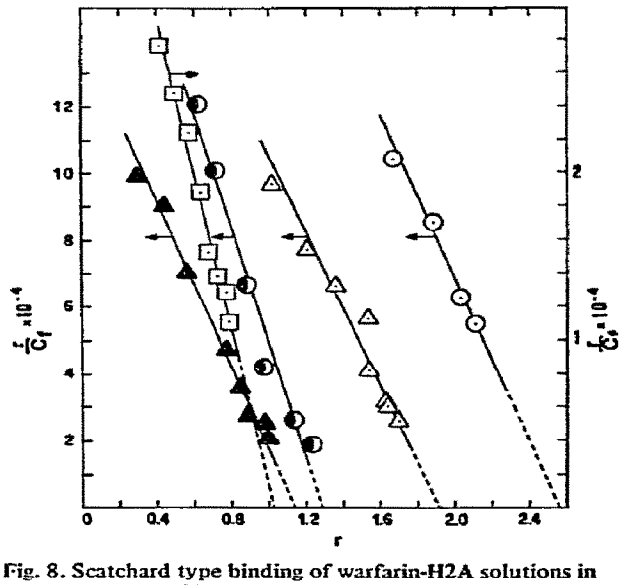


Fig. 8. Scatchard type binding of warfarin-H2A solutions in phosphate buffer 20 mM, pH 7.5. H2A molarity: ⊙, $2.5 \times 10^{-5}$; ▲, $3.5 \times 10^{-5}$; ○, $5.0 \times 10^{-5}$; △, $7.5 \times 10^{-5}$; ■, $1 \times 10^{-4}$.

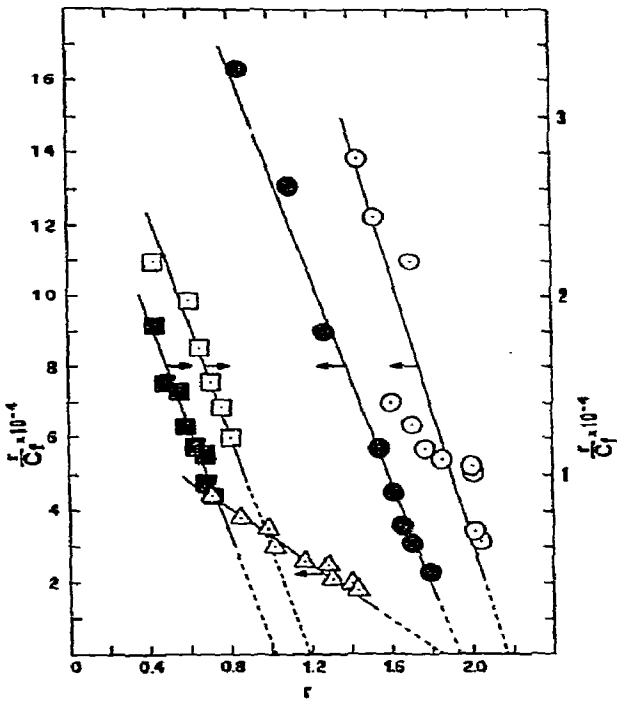


Fig. 9. Scatchard type binding data of warfarin-H2B solutions in phosphate buffer 20 mM, pH 7.5. H2B molarity: ○, 2.5 × $10^{-5}$; ●, 3.5 × $10^{-5}$; ▲, 5.0 × $10^{-5}$; ▣, 7.5 × $10^{-5}$; ■, 1 × $10^{-4}$.

to about 1 in the case of H2B. The number of moles of bound warfarin per mole of protein is rather little and considering that about a fourth of the histone has positive charge, it is possible to conclude that the binding of warfarin with H2A and H2B should involve not electrostatic type interactions, but probably hydrophobic ones. We think that the self-association of H2A and H2B causes the decreasing of the binding sites available to warfarin on the protein molecule. This should mean that warfarin binds at the same region of the macromolecule which is involved in the aggregation. In the range of protein concentrations used there is also a change of the values of the binding constant. In fact, on increasing the histone concentration, the binding constants decrease of an order of magnitude of about 3–4 at a protein concentration 1 × $10^{-4}$ M (fig. 8, H2A system) and 5 × $10^{-5}$ M (fig. 9, H2B system). This behaviour could be due to the loss, upon aggregation of the histone,

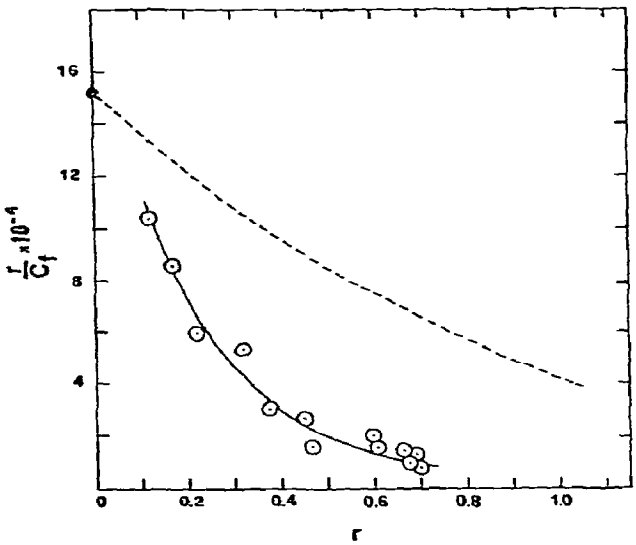


Fig. 10. Scatchard type binding data of warfarin-(H2A + H2B) solutions in phosphate buffer 20 mM, pH 7.5. Dotted line is calculated (see text). H2A and H2B molarity: 5 × $10^{-5}$.

of the sites which display the highest degree of affinity toward the warfarin molecule. This hypothesis contrasts with the straight lines of figs. 8 and 9 which suggest only one class of independent binding sites. However, it is also possible that the technique used by us to obtain the data does not allow to notice any curvature of the Scatchard plots. On the other hand, another hypothesis could suggest that the aggregation of the protein molecules is followed by a conformational change that causes the decreasing of the free energy change of the interaction process, i.e. of the binding constants. We think that more data are necessary in order to suggest a more definite interpretation.

In fig. 10 there are the results obtain when warfarin is added to a 1:1 mixture of H2A and H2B. In the same figure there is the theoretic curve calculated on the assumption that in solution, warfarin is divided among the two proteins, binding independently. The calculation was carried out using binding constants ($K_{H2A}$, $K_{H2B}$) and number of sites ($n_{H2A}$, $n_{H2B}$) obtained from figs. 8 and 9 for a concentration of each protein 5 × $10^{-5}$ M which is the same used in the case of mixture. This procedure is similar to that suggested for a macromolecule with two classes of independent sites of binding [28]. In fact it is possible to

consider the mixture of histones H2A and H2B, each at a concentration $5 \times 10^{-5}$ M, as a solution containing only one type of molecule formed by the junction of one molecule of H2A with one molecule of H2B. On the basis of the data of figs. 8 and 9, we calculated, as suggested by the theory [28], the dotted curve of fig. 10 and the intercept on the ordinate $(n_{H2A}K_{H2A} + n_{H2B}K_{H2B})$. The experimental and calculated curves greatly differ. We think that this result is indicative of the association of the histone H2A with H2B. It is very difficult to interpret the experimental curve which might suggest not independent classes of sites upon the complex formed by the two histones. However it is possible to see a decreasing of the number of sites at disposal of the warfarin molecules on the cross-aggregation. This type of association seems more effective than self-aggregation on deprinving warfarin of suitable sites for the binding.

## 4. Conclusions

Histones H2A and H2B show high quantum yield of tyrosine groups when they have secondary structure and are self-aggregated. It is very likely that some tyrosines have $R_{TYR}$ values $\geq 1$ considering that the data of figs. 1 and 2 have been calculated on the basis of all tyrosines included in the chain. The enhancement of fluorescence of the tyrosines can be due only to two possibilities: either the fluorescence increases because of the change of the degree of hydration of the peptide bond when the tyrosine residues are included in an $\alpha$-helical segment, or the enhancement is the consequence of the decreasing of the polarity of the environment around the tyrosine ring when the macromolecules self-aggregate [1,2]. We think that in the case of histones H2A and H2B the first hypothesis must be ruled out since it is an all or none process which is not indicated by the shape of the curves of figs. 1 and 2. Assuming the mechanism of self-aggregation proposed by Bradbury et al. [8], it is possible to attribute the increase of the fluorescence in the histones H2A and H2B to a process similar to that shown in the study of histone H1. However it is necessary to point out that in the histone H1 the tyrosine is included in a intra-chain compact structure whereas some of the tyrosines of the histone H2A and H2B result included in inter-chain assemblies. These structures are formed by hydrophobic interaction between segments which include some sites suitable for the binding of hydrophobic probes such as TNS and warfarin. The interaction of these molecules with the H2A and H2B histones shows that on the protein both the type and the number of sites depend on tertiary structure of the macromolecules. TNS is more sensitive to the degree of hydrophobicity of the sites than warfarin so that it is possible to show an heterogeneity upon the proteins. At acid pH both histones have a higher number of sites which are on the average less hydrophobic than those at pH 7.5. However it must be pointed out that at pH 7.5, when the proteins are structured and self-aggregated, histones retain the capacity for hydrophobic interactions. On the other hand, warfarin seemed to show a dependence of the number of sites at pH 7.5 on the histone concentration, i.e. on the degree of histone aggregation. Therefore we can conclude that the self-association of the histone and/or the cross-complexing causes the decreasing of the number of hydrophobic sites upon the proteins.

## Acknowledgement

The authors thank Professor E.M. Bradbury for the kind gift of some samples of histones. This work was supported by Consiglio Nazionale delle Ricerche, Roma, Italy, grants No. 7400468.03 – No. 75.00757.03.

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