Interactions of Calf-Thymus Histone Fractions in Aqueous Solution with 8-Anilidonaphthalene-1-sulphonic Acid

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1. The interactions of histone fractions with 8-anilidonaphthalene-1-sulphonic acid were investigated by fluorimetry and spectrofluorimetry and the results were interpreted with the aid of equilibrium-dialysis techniques. 2. Characteristic differences were found between the various histone fractions, and with fractions F3 and F2a the binding was found to be salt-dependent. 3. Evidence was obtained indicating a slow change of the physical state of fractions F3 and F2a in the presence of salt, and the binding by these two fractions in the presence of salt was greater by an order of magnitude than by fractions F1 and F2b. 4. Conditions favouring binding were also those favouring histone aggregation; SO_4^{2-} ions activated binding at a lower concentration than Cl^- ions; urea, guanidinium ions and high concentrations of I^- ions were inhibitory to binding. 5. After histones had been kept in the presence of salt for a long time the reversal of interaction on decreasing the salt concentration was incomplete. 6. The inhibition of binding by fraction F2a in the presence of urea or fraction F2b depended on the time sequence of addition of the reagents. 7. Artificial nucleoproteins made by precipitating DNA with the histone fractions in neutral 0.14M-sodium chloride showed the same order of interaction as was found for the fractions in solution. 8. Comparison of the binding by fraction F2a with that by bovine plasma albumin showed that in both cases there were a large number of weakly binding sites but that fraction F2a lacked the small number of strongly binding sites found in albumin. No slow change of binding in the presence of salt was found for albumin. 9. Binding by fraction F2b increased the affinity of the protein for further molecules of the adsorbate. 10. The results are discussed in relation to the close relationship between binding and aggregation and the possible role of non-polar interactions as determined by the balance between polar and non-polar amino acids in the histone fractions.

There have been a number of investigations of interactions of histones in tissue sections with anionic dye molecules (Alfert & Geschwind, 1953; Swift, 1964), but less attention has been given to the interactions in histone solutions. Loeb (1964) studied the interaction of eosin with whole histone and also with some fractions differing in lysine and arginine content, and in each case a strong interaction was found. Shepherd & Noland (1965) have used the interaction between whole histone and ANS* to determine the protein quantitatively.

In the present paper the interaction between certain histone fractions and the anion of ANS is described. It was shown by some preliminary experiments that histone fractions differed greatly in the extent of interaction with ANS and that

* Abbreviation: ANS, 8-anilidonaphthalene-1-sulphonic acid.

there was also considerable variation for a given fraction under different conditions. The work now reported resulted from an attempt to investigate the binding phenomenon in more detail.

It was found that histone dissolved in dilute buffers in the pH range 1.5–7 interacted rather weakly with ANS but that a stronger interaction could be induced by increasing the salt concentration. This was interpreted as indicating that a change of physical state of the histone molecules had occurred as a result of the increase in salt concentration. The binding of ANS proved a ready means of characterizing this change.

The compound ANS was used in this work because of its convenient property of being almost non-fluorescent in aqueous solution (quantum yield 0.004) but brightly fluorescent when adsorbed by proteins (e.g. with bovine plasma albumin the

quantum yield is 0.75; Weber & Laurence, 1954; Weber & Young, 1964). Mayhew & Roe (1964) have described the use of the corresponding 8-*p*-toluidinonaphthalene-1-sulphonic acid as a fluorochrome for ascites-tumour cells.

EXPERIMENTAL

Histone fractions. The histone fractions used were those previously identified and characterized in this Laboratory (Butler, 1964; Johns, 1964; Phillips, 1962), namely fractions F1, F2a, F2b and F3. Some results were also obtained with subfractions of fractions F2a (Phillips & Johns, 1965). The histones were dissolved in water as hydrochlorides and stored frozen at -8° for up to 2 weeks. Amino acid analyses are given in Table 1.

Bovine plasma albumin. Crystalline bovine plasma albumin (batch no. 23351) was from Armour Laboratories, Eastbourne, Sussex.

ANS. This was purified from Eastman-Kodak (Rochester, N.Y., U.S.A.) material by crystallization as the ammonium salt after precipitation with $(\text{NH}_4)_2\text{SO}_4$ from slightly alkaline solution.

Degraded DNA. This was obtained by ultrasonic irradiation in a N_2 atmosphere at 20° of a sample obtained from calf thymus by the method of Kay, Simmons & Dounce (1952). The viscosity of the DNA was 0.8 dl./g. at a shear rate of 400 sec^{-1} . The DNA was degraded to make it possible for histone fractions to precipitate different fragments of the DNA molecule. In the experiments reported, however, the precipitation of DNA appeared to be non-specific.

Fluorescence intensity. This was measured at room temperature (i.e. $21-23^{\circ}$) in a Locarte fluorimeter with excitation at $365 \text{ m}\mu$ and a pale-yellow (LF6) filter between the

fluorescent solution and the photo-cell. 5-Dimethylaminonaphthalene-1-sulphonic acid was used as fluorescent standard. The fluorescent intensities, I , were expressed in units equivalent to the fluorescence of a solution of 5-dimethylaminonaphthalene-1-sulphonic acid of 1 mg./l. in dilute (less than 3 mm) borate buffer, pH 9.

Fluorescence spectra. These were measured in the Aminco-Bowman spectrophotofluorimeter with excitation at $375 \text{ m}\mu$ by using slit arrangement no. 3. The spectra were not corrected for variations in the sensitivity of the photo-cell with wavelength etc., but the spectra of ANS in ethanol and methanol were determined as a control.

The wavelength scale was set from the direction of longer wavelength to avoid errors due to back-lash in the drive mechanism. To determine the position of the maximum graphically, the top of the curve was cut by the line produced from the bisectors of the constant intensity lines drawn between opposite sides of the fluorescence-intensity curve.

Equilibrium dialysis. The buffered protein solution was contained in Visking 18/32 in. tubing tied at both ends and immersed in the same volume of external solution of the same salt concentrations as the protein solution. The initial distribution of ANS was shown to be without effect on the final equilibrium. The ANS was added equally to the internal and external solutions in the experiments reported. After 3 hr. at room temperature to ensure that the configurational change of the histone was near completion, the solutions were placed on a rotating turntable in a cold room at 2° for 40 hr. to complete the equilibrium. The ANS concentration was determined inside and outside the sac after equilibrium was obtained. Adsorption of ANS by the dialysis sac and loss of protein to the external solution were small (less than 5%) and the Donnan effect was negligible at the salt concentrations used.

Table 1. *Amino acid analysis of the calf-thymus histone fractions used in this work*

Amino acid values are given as moles/100 moles of all amino acids found. These values were kindly determined by Miss P. Simson and Mrs D. Purkiss.

Amino acids	Amino acid composition						Type of grouping
	Fraction F2a	Fraction F2a1	Fraction F2a2	Fraction F3	Fraction F2b	Fraction F1	
Total diacidic*	10.7	10.3	16.0	14.7	15.4	8.0	
Total dibasic	26.0	24.5	23.5	24.6	25.8	27.2	
Arginine	13.3	12.5	9.9	12.4	8.0	3.1	Basic
Lysine	9.7	9.9	10.8	9.6	15.5	23.6	Basic
Phenylalanine	1.8	2.2	1.3	2.6	1.3	0.7	Neutral non-polar
Leucine + isoleucine	16.5	14.0	15.9	12.9	9.1	6.7	Neutral non-polar
Valine	7.4	8.4	6.4	6.8	8.9	5.8	Neutral non-polar
Proline	3.4	1.6	4.1	5.3	5.2	9.3	Neutral non-polar
Alanine	8.9	7.9	12.5	13.8	12.0	23.0	Neutral non-polar
Glycine	13.8	14.8	10.3	6.3	7.0	6.4	Neutral non-polar
Serine	2.9	2.2	3.1	3.7	7.5	5.8	Neutral hydroxyl
Threonine	5.4	6.1	4.0	6.4	5.3	5.6	Neutral hydroxyl
Tyrosine	2.8	3.7	2.5	2.5	2.3	1.4	Neutral hydroxyl
Histidine	3.0	2.1	2.8	2.6	2.3	0.5	Basic
Cysteine (+ cystine)	0.2	None	Trace	0.6	0.3	0.1	Thiol

* About 40% of the carboxyl groups of the proteins are in the form of the amide (D. M. P. Phillips, unpublished work).

Protein concentrations. These were measured by Kjeldahl determinations of nitrogen. The following nitrogen contents of the isoelectric proteins would be expected on the basis of amino acid composition, including amide nitrogen: fraction F1, 18.8%; fraction F2a, 19.8%; fraction F3, 19.4%; bovine plasma albumin, 16.1%. The values for nitrogen contents of histones were kindly provided by Dr D. M. P. Phillips.

ANS concentration. This was measured at 350m μ , the extinction at this wavelength being little affected (less than 3%) by adsorption by protein. The molar extinction coefficient was $5.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 350m μ . At low extinctions ANS concentration was measured fluorimetrically in the presence of excess (0.25%) of bovine plasma albumin.

Interpretation of binding data. The equation of Scatchard (1949) was used to evaluate the data:

$$\bar{\nu}/F = k(n - \bar{\nu}) \quad (1)$$

where $\bar{\nu}$ is the ratio of concentrations (M) of bound ANS (B) to protein (P), F is the concentration (M) of unbound ANS, and k and n are respectively the association constant (M^{-1}) and the number of sites on the protein molecule available for adsorption. This equation is valuable in revealing the reliability of the extrapolation required to obtain n . In certain cases the state of the histone molecules was found to depend on their previous history and there is consequently an approximation in treating all states of the histone molecule as equilibrium states.

For any actual state of the protein molecule, however, interactions with ANS and salt appeared to be rapid, and from the more limited approach of characterizing time-dependent or metastable states of the histone molecule the system may be treated as an equilibrium according to eqn. (1).

For fluorimetric estimations, when $\bar{\nu}$ is less than n , eqn. (1) may be rewritten:

$$i/(i_{\infty} - i) = knP \quad (2)$$

where i is the fluorescent intensity of the ANS-histone complex and i_{∞} is the intensity of the same total ANS concentration at high protein concentration when all the available ANS is bound. In formulating this equation, i is taken proportional to B and $(i_{\infty} - i)$ proportional to F .

Measurements of pH. These were made with a Pye Dynacap pH-meter at room temperature without dilution of the samples. A buffer solution made from Soloid buffer solution tablets (Burroughs Wellcome and Co., Beckenham, Kent) (pH 4.0 at 25°) was used to standardize the instrument.

RESULTS

Effect of sodium chloride concentration and time. Preliminary experiments showed that the fluorescence of ANS-histone solutions was generally greater in 17mM-acetate buffer, pH 4.7, containing sodium chloride (0.3M) than in the acetate buffer alone. The fluorescence also depended on the time elapsing after mixing the histone and the sodium chloride solution, but not on the time of addition of ANS. The results were interpreted as indicating a slow change of the state of the histone molecules because of the presence of the salt, the configuration

of the histone in sodium chloride solution interacting better with ANS than that in the absence of sodium chloride.

In Fig. 1, certain aspects of these changes are shown as a function of time after the addition of salt. All results refer to an ANS-fraction F2a mixture in dilute acetate buffer, pH 4.7, and except for curve D incubation was at room temperature throughout. In the absence of sodium chloride the fluorescence intensity was constant at 0.9 unit over 24hr. Curves A and B result when the sodium chloride concentration was raised to 0.16M at zero time. In curve A the protein concentration was 0.26g./l. during incubation, whereas in curve B the concentration was 8g./l. but samples were diluted to 0.26g./l. at the time of measurement. The reaction was somewhat more rapid in the more concentrated protein solution. Curve C represents the time-course of a reaction similar to that for curve A but in 0.3M-sodium chloride. In this case the intensity at 1.7hr. was 87% of that at 17hr. In contrast, curve D gives the result of an experiment similar to that for curve C but in which incubation was carried out in a cold room at 2°.

Time after mixing histone and salt solutions (hr.)

Fig. 1. Time-course of the fluorescence reaction between fraction F2a and ANS. At the time of measurement, the concentration of fraction F2a was 0.26g./l., that of ANS was 2mg./l., and that of acetate buffer, pH 4.7, was 17mM. Curve A ($\bullet$), 0.16M-NaCl; concn. of fraction F2a throughout, 0.26g./l. Curve B ($\blacktriangle$), 0.16M-NaCl; concn. of fraction F2a during incubation, 8g./l. Curve C ($\triangle$), 0.3M-NaCl; concn. of fraction F2a as for curve A . Curve D ($\circ$), conditions as for curve C but incubation was at 2° instead of at room temperature; loops from curve D are changes in fluorescence intensity when samples were brought to room temperature at various times. Curve E ($\square$), decrease in fluorescence intensity when NaCl concentration was decreased to 10mM after incubation for 18hr. at 0.3M-NaCl; the loop at 17hr. is the result of adding salt to adjust the concentration to 0.3M.

Samples were taken to the fluorimeter at room temperature for immediate measurement. As the solution warmed up to room temperature the fluorescence intensity decreased but then increased again. The initial fall was attributed to a shift with temperature in the rapid interaction equilibrium between ANS and histone and the subsequent rise to a change in histone configuration at the higher temperature. If the minima in the loops are taken as estimates of the state of the histone before warming and consequent configurational change, it appears that the effect of salt is slower by about 50-fold at 2° as compared with 22°. In curve *E* the reverse reaction was studied, i.e. a solution containing 8g. of fraction F2a/l. in 0.3M-sodium chloride was preincubated for 24hr. at room temperature and then diluted 30-fold with acetate buffer without sodium chloride. The fluorescence intensity fell rapidly and then more slowly but did not attain the low value (approx. 0.9) typical of fraction F2a in 10mM-sodium chloride. In further experiments it was found that after shorter (1hr.) preincubation in salt the change on dilution was completely reversible. Subsequent fluorimetric values presented are all the result of incubation for 17–19hr. at room temperature, the change in intensity with time being then less than 1%/hr.

With ANS-bovine plasma albumin mixtures there was no slow change with time over the same period. Increase in the sodium chloride concentration caused an immediate fall in fluorescence

intensity but this was too rapid for the rate to be observed. A similar fall is recorded at the end of curve *E* but with this fraction (F2a) there was a subsequent slower rise, in contrast with that with bovine plasma albumin, which remains constant in intensity after the initial rapid decrease.

Fig. 2 illustrates the effect of sodium chloride for all the histone fractions used. The F2a–F3 group showed a sharp transition in the region 0–0.25M-sodium chloride. Time has little effect on the fluorescence of ANS–fraction F2b mixtures and none on ANS–fraction F1 mixtures.

Effect of other salts. Fig. 3 shows that bivalent anions were active at a concentration 30-fold less than that of chloride. Sodium iodide and guanidinium chloride were less effective at the higher concentrations.

Effect of pH. In phosphate buffer, pH 7, and in hydrochloric acid, pH 1.5–2.5, the salt activation of fraction F2a was similar to that observed in the acetate buffer. There were slight differences in fluorescence intensity but the shape of the activation curves was qualitatively the same throughout the pH range 1.5–7.

Equilibrium dialysis. Table 2 gives the amount of ANS bound by various histone fractions. Comparison between the results in Table 2 and Fig. 2 shows that differences in intensity as between various fractions and at various salt concentrations (for fraction F2a) are largely the result of changes in the amount of ANS bound, rather than to differences in the fluorescence of bound ANS. The error in *B* (Table 2) is about $\pm 3 \mu\text{M}$ and so the effect of salt on fraction F1 is not significant. In view of the experimental error, no more extensive dialysis experiments were done in the absence of sodium chloride. It was considered that the results with

Fig. 2. Effect of NaCl on the fluorescence intensity of ANS-histone mixtures. Concentrations of ANS, protein and acetate buffer and pH were as given in Fig. 1. Results were read after the mixtures had stood overnight at room temperature. $\blacktriangle$, Fraction F2a1; $\square$, fraction F2a2; $\bullet$, fraction F2a3; $\circ$, fraction F3; $\triangle$, fraction F2b; $\blacksquare$, fraction F1.

Fig. 3. Effects of various salts on the fluorescence of ANS in fraction F2a solution. Concentrations of ANS, fraction F2a and acetate buffer and pH were as given in Fig. 1, except that acetate buffer was omitted when sodium phosphate was used. $\bullet$, Na_2SO_4 ; $\blacktriangle$, sodium phosphate, pH 7; $\square$, NaI; $\circ$, NaCl; $\triangle$, guanidinium chloride.

Table 2. *Interaction of ANS with histone fractions*

All values are of B (eqns. 1 and 2), the concentration of ANS bound. The protein concentration (P) was 0.65 g./l. and the average ANS concentration inside and outside the dialysis sac was $67\mu\text{M}$ (in 17 mm-acetate buffer, pH 4.7, at 2°). N.D., Not determined.

Concn. of NaCl (M)	Concn. of ANS bound (μM)			
	Fraction F1	Fraction F2a	Fraction F2b	Fraction F3
0	10	9	N.D.	N.D.
0.3	5	64	15	45

Fig. 4. Scatchard plot of equilibrium-dialysis data for fraction F2a (Δ) and bovine plasma albumin ($\circ$) in 17 mm-acetate buffer, pH 4.6, containing NaCl (0.3 M) at 2°. The value $\bar{v}$ indicates the number of strongly interacting sites of bovine plasma albumin. The value $(\bar{v}/F)$ is a measure of the strength of binding of fraction F2a. The molecular weights of bovine plasma albumin and fraction F2a were taken as 69000 and 20000 respectively.

fraction F2a, at pH 4.7 in 0.3 M-sodium chloride, could be taken as typical of the F2a–F3 group and this system was compared with bovine plasma albumin under the same conditions. The results are shown plotted according to eqn. (1) in Fig. 4. The curve for fraction F2a shows that binding is on a large number (more than 40) of weakly binding sites. The value of nk for fraction F2a was $1.4 \times 10^5 \text{ M}^{-1}$. Bovine plasma albumin had about eight strongly binding sites and a large number of more weakly binding sites. In spite of the difference in detail between fraction F2a and bovine plasma albumin, the amount of ANS bound/g. of protein was of the same order of magnitude for the two

Table 3. *Partition of ANS between histone solutions and protein-free medium*

The histone concentration was 0.65 g./l. in 17 mm-acetate buffer, pH 4.7, containing NaCl (0.3 M). The values given are the ratios of total concentration of ANS inside and outside the dialysis sac. The protein solution was placed inside the sac.

9 × Average concn. of ANS (mM)	ANS concentration ratio			
	Fraction F1	Fraction F2a	Fraction F2b	Fraction F3
1	1.4	3.6	1.6	2.7
2	1.3	3.6	1.5	2.8
10	1.2	3.3	1.8	2.2
20	1.2	3.1	2.0	2.2

Table 4. *Position of maximum in the fluorescence spectra of histone–ANS complexes*

Concentrations and pH were as given in Table 2. The values for ANS in methanol and ethanol were 486 and 480 m μ respectively.

Concn. of NaCl (M)	$\lambda_{\text{max.}}$ (m μ)			
	Fraction F1	Fraction F2a	Fraction F2b	Fraction F3
0	481	478	482	477
0.3	480	475	477	473

proteins at the concentration used in the experiments.

A line parallel to the abscissa in the Scatchard plot represents the situation where the partition of ANS between the protein solution in the dialysis sac and the external protein-free salt solution is constant at a given protein concentration. The partition coefficient between internal and external solution varied only slowly with ANS concentration with fraction F2a. The corresponding values for the four main fractions are given in Table 3. With fraction F2b there was an increase in partition in favour of the protein solution as the ANS concentration was increased, which shows that binding of ANS increases the affinity of fraction F2b for further molecules of ANS.

Fluorescence spectra and quantum yields. Table 4 lists the positions of the maxima in the fluorescence spectra of ANS with various histone fractions in the presence and absence of salt and the values for ANS in ethanol and methanol. Results were obtained after overnight incubation of the solutions at room temperature. The maxima for methanol and ethanol differed from those given by Stryer (1965) by 10 and 12 m μ respectively. These differences are not unexpected because of differences in photo-cell response, for example.

It was possible to measure i_{∞} (eqn. 2) for fraction F2a, fraction F3 and bovine plasma albumin at protein concentrations in the range 3–10 g./l. The values in 0.3M-sodium chloride, pH 7, were in the proportions 1:0.9:1, and those in 0.3M-sodium chloride, pH 4.7, were in the proportions 1:0.9:0.8 respectively. The quantum yield for ANS with bovine plasma albumin at pH 7 was given by Weber & Young (1964) to be 0.75. Thus the quantum yields for the complexes with fractions F2a and F3 would be 0.75 and 0.67 respectively. Reaction of ANS with fraction F2b or F1 did not result in almost complete binding of the ANS under the same conditions. The highest intensities observed were 0.6 and 0.35 respectively of the values for fraction F2a.

Interactions measured fluorimetrically. From Stryer's (1965) observation that a shift in the fluorescence spectrum of ANS to longer wavelength is accompanied by a decrease in quantum yield, it may be concluded that i_{∞} for fractions F2b and F1 would not be greater than the value for fraction

F2a. Taking the value for fraction F2a as unity, the values 0.8 (fraction F2b) and 0.6 (fraction F1) were considered to be within 20% of the correct values and these values were used in calculating values for binding constants by using eqn. (2) (Table 5). The value of nk for fraction F2a ($5.1 \times 10^4 \text{ M}^{-1}$) and the value from equilibrium dialysis ($14 \times 10^4 \text{ M}^{-1}$) support the interpretation of Fig. 1 (curve D) where the initial fall in intensity was attributed to a thermal dissociation.

Effect of urea. At constant concentrations of ANS, fraction F2a and salt and constant pH (conditions as for Fig. 1) the effect of urea concentrations up to 4M was investigated. With 0.1M-sodium sulphate as activator, 2M-urea decreased the fluorescence intensity by half when added either before sulphate addition or after preincubation for 17 hr. (22°) in sulphate. A similar urea concentration was necessary to 50% inhibit the 0.1M-sodium chloride-activated complex only if addition of urea was delayed until after 17 hr. incubation in chloride. Urea added before the chloride was more effective, 50% inhibition being obtained at 0.4M-urea.

Inhibition of fraction F2a interaction by fraction F2b. The order and time of additions of reagents was also shown to be important in mixtures of fraction F2a with fraction F2b. If 0.13 g. of fraction F2b/l. was present with the same concentration of fraction F2a before the addition of 0.3M-sodium chloride, pH 4.7, the fluorescence was only half that observed with fraction F2a alone. The addition of fraction F2b after the incubation of fraction F2a with salt for 18 hr. decreased the fluorescence intensity by only 3% (ANS concentration $6.5 \mu\text{M}$).

Interaction of ANS with insoluble histone-DNA complexes. Solutions were made containing constant amounts of histone, phosphate buffer, pH 7, sodium chloride and ANS. Various amounts of degraded DNA were added, the precipitated histone-DNA complex was separated by low-speed

Table 5. *Fluorometric determination of the extent of interaction of histones and bovine plasma albumin with ANS*

All values are of nk (eqn. 2). The molecular weight of the histone fractions was taken as 20000 and that of bovine plasma albumin as 69000. Determinations were carried out in 17mm-acetate buffer, pH 4.7, and 2.2mm-phosphate buffer, pH 7.0, both containing NaCl (0.3M).

pH	10 ⁻³ nk (M ⁻¹)				Bovine plasma albumin
	Fraction F1	Fraction F2a	Fraction F2b	Fraction F3	
4.7	3	51	6	40	390
7.0	3	46	10	46	630

Table 6. *Interaction of ANS with histone-DNA co-precipitates*

A 0.5mg. sample of histone fraction was used in each case. The total volume of each solution was 3.55 ml. and it contained NaCl (0.14M), phosphate buffer, pH 7 (2.8mm), and ANS (2.5mg./l.). The values recorded are fractions of the total protein and fractions of total ANS in the precipitate.

Amount of DNA added (mg.)	Fractions of total protein and total ANS in precipitate							
	Fraction F1		Fraction F2b		Fraction F3		Fraction F2a	
	Protein	ANS	Protein	ANS	Protein	ANS	Protein	ANS
0.1	0.22	0	0.37	0.05	0.64	0.11	0.62	0.17
0.2	0.43	0	0.76	0.03	0.94	0.09	0.95	0.12
0.3	0.65	-0.01	0.80	0.05	0.92	0.07	0.91	0.09
0.4	0.81	0	0.80	0.03	0.94	0.07	0.82	0.06
0.5	0.94	0	0.80	0.05	0.93	0.06	0.80	0.05
1.0	0.86	0.01	0.51	0	0.16	0	0.44	0.03

centrifugation and the amounts of histone and ANS in the precipitate were determined by difference from the amount remaining in solution by the method of Lowry, Rosebrough, Farr & Randall (1951) (protein) and fluorimetry (ANS). Table 6 shows that the proportion of ANS bound by the precipitate parallels the relative affinities of the histone, as determined in solution.

DISCUSSION

The results show that ANS binding by histone depends both on the type of histone and also on the physical condition of the histone molecules in solution. Conditions found to favour high fluorescence intensity in solutions containing histone and ANS are also those favouring histone aggregation.

Fractions F3 and F2a1 correspond to the β -histone described by Mauritzen & Stedman (1959) in amino acid composition. These authors found that β -histone was aggregated in solution and the binding of ANS by fractions F3 and F2a1 is marked (Fig. 2), especially in salt, which favours aggregation. In contrast, fraction F1 binds ANS weakly, and Davidson & Shooter (1956) were unable to observe aggregation of this fraction. H. J. Cruft has carried out extensive studies of histone aggregation and has observed (personal communication) that the temperature coefficient of the process is high, as is the coefficient of the salt-induced change in fraction F2a (Fig. 1).

The high activity of sulphate (and phosphate) (Fig. 3) in promoting ANS binding recalls the observation of Ui (1956), who reported that sulphate is more active in causing aggregation than is chloride. The inhibition of the fluorescence reaction by urea or high concentrations of guanidinium ions (Fig. 3) parallels the observations of Ui (1957) that these agents reverse aggregation. Ui (1957) also found that aggregation could be reversed by decreasing the ionic strength of the solution, and the fluorescence reaction is also reversed partially or completely by this procedure.

Mauritzen & Stedman (1959) found that other histone fractions inhibited the aggregation of β -histones. In the present work fraction F2b was effective in inhibiting development of salt-induced changes in fractions F2a and F3. The close correlation between ANS binding and aggregation suggests that the dye-binding method would be a useful aid in defining conditions of aggregation in more detail. The observation in the present work that dye binding depends more on the ionic strength than on the pH is of special interest in this connexion.

The relationship between aggregation and dye binding may not be one of cause and effect. Bradbury, Crane-Robinson, Phillips, Johns & Murray (1965) have shown that there is a relatively

large increase in α -helical content of some histone fractions when the solvent is changed from water to 0.25M-sodium chloride solution. This they attribute to a polyelectrolyte effect, i.e. the salt decreases electrostatic repulsion by screening the charged groups of the protein, thereby favouring a more organized structure of the peptide chains. Aggregation of histones would also be favoured by a decrease in repulsive forces, but ANS binding may be increased by the increase in numbers of neighbouring side chains in a protein conformation not extended into the water by repulsive forces. It was not stated by Bradbury *et al.* (1965) how the changes in α -helical content depended on time and temperature.

The fluorescence spectra and quantum yields confirm that the dye was in a medium of polarity less than or equal to that of ethanol (Laurence, 1952; Stryer, 1965), but there were large differences in the apparent strength of binding of ANS to serum albumin, fraction F2a and fraction F2b in salt in spite of the similarities in fluorescence spectra. The polarity of the protein adsorption sites decreased on the addition of salt, as judged by shifts in the fluorescence spectra. Fractions F2a and F3 are richer in larger non-polar amino acids than are fractions F2b and F1, which contain more serine and threonine. According to D. M. P. Phillips (personal communication) the non-polar amino acids of fractions F2a and F3 are more concentrated in the part of the peptide chain that resists trypsin action and that has a relatively small imbalance in the proportions of acidic and basic amino acids. The existence of a concentration of non-polar amino acids would favour interaction with the non-polar part of the ANS molecules. As ANS binding increases with dye concentration with fraction F2b it seems that with this fraction, and possibly also with the others, the ANS molecules already bound may provide a local environment in the protein favourable to further binding.

There is a correlation between ANS binding and the preparative methods of Johns (1964). Fractions F2a and F3 are extracted at higher concentrations of ethanol in Johns's (1964) procedures. Of fractions F2b and F1, the latter is precipitated by a lower acetone concentration than is fraction F2b. In general, fraction F1 seems to be the most 'hydrophilic' or 'lipophobic' histone and fraction F2a the most 'lipophilic'. The implications of this classification of histones in relation to amino acid composition, aggregation in water and ANS binding are of potential significance. These differences between histones may also have important consequences *in vivo*.

The results with the artificial histone-DNA complexes show that under the conditions used in this work the histone fractions retain some of their

characteristic ability to adsorb ANS when bound to DNA. These results are at variance with the observations of Mayhew & Roe (1964) on staining of whole ascites cells. The nucleochromatin of interphase nuclei appeared to have no interaction *in vivo* with the fluorochrome 8-*p*-toluidino-naphthalene-1-sulphonic acid. As shown with the inhibition of the ANS-fraction F2a reaction by urea and fraction F2b, persistent differences in histone solutions may result from small differences in operational procedures and it is likely that there are many possible differences in artificial histone-DNA complexes depending on the experimental conditions. The likelihood of differences between native and reconstituted nucleoproteins has been emphasized by Murray (1965).

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