Interaction of Clupeine with Deoxyribonucleic Acid. I. Thermal Melting and Sedimentation Studies*

Sadako Inoue and Toshio Ando

ABSTRACT: Thermal melting and sedimentation properties of deoxyribonucleic acid–clupeine complexes prepared in solution have been studied. Soluble complexes markedly stabilized against thermal denaturation were formed by direct mixing of sonicated deoxyribonucleic acid and clupeine at low ionic strength. These complexes exhibited biphasic melting transitions when the arginine:deoxyribonucleic acid–phosphorus ratios were less than unity. The first transition had a melting temperature similar to that of free deoxyribonucleic acid and was ascribable to the melting of uncomplexed portion of deoxyribonucleic acid. The melting temperature for the second transition was characteristic of the complexes. The melting profiles of deoxyribonucleic acid–$(Arg)_{20}$, deoxyribonucleic acid–$(Lys)_{20}$, and deoxyribonucleic acid–$(Orn)_{19}$ also showed biphasic transitions. The stability of the complexes decreased in the order of deoxyribonucleic acid–$(Orn)_{19}$ > deoxyribonucleic acid–$(Lys)_{20}$ > deoxyribonucleic acid–$(Arg)_{20}$ > deoxyribonucleic acid–clupeine. In contrast to these complexes, deoxyribonucleic acid–$(Arg)_4$ complexes showed monophasic melting profiles for varying levels of arginine:phosphorus ratio. Thus clupeine was found to behave more like $(Arg)_{20}$ than $(Arg)_4$ in its interaction with DNA. Deoxyribonucleic acid–clupeine complexes formed by gradient dialysis, on the other hand, were less soluble and exhibited larger turbidity as compared with the complexes formed by the mixing method. The former complexes were precipitated by centrifugation at 25,000g. Chemical analysis of the amounts and composition of the precipitates revealed that the binding of clupeine to deoxyribonucleic acid occurred cooperatively, and DNA molecules could be fractionated into an essentially free species and those fully complexed, when arginine:phosphorus input ratios were less than unity. On the basis of these studies, it has been concluded that the binding of clupeine to deoxyribonucleic acid is irreversible and stoichiometric in solutions of low ionic strength. The present studies failed to demonstrate significant difference between each of the three components of clupeine in the interaction with deoxyribonucleic acid, although their natural occurrence in nearly equal proportion suggests some biological implications.

A number of studies on the structure and properties of chromosomal nucleoproteins have appeared in the literature since the importance of nuclear basic proteins, histones, and protamines, in the expression and regulation of genetic activities of DNA was suggested (Stedman and Stedman, 1950). One of the approaches to the understanding may be achieved by use of simple model systems consisting of cationic homopolypeptides and natural or synthetic polynucleotides (Raukas, 1965; Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Olins *et al.*, 1967, 1968). However, the recent progress in the structural elucidation of histones shows that these synthetic polypeptides are not appropriate model substances for histones. Clupeine, a typical protamine occurring in the sperm nuclei of Pacific herring, is considered to be one of the most suitable model substances at present. It has been fractionated into three molecular species (YI, YII, and Z) and their amino acid sequences have been completely elucidated (Ando *et al.*, 1962; Ando and Suzuki, 1966, 1967) as follows: clupeine YI, H-Ala-Arg_4-Ser-Ser-Ser-Arg-Pro-Ile-Arg_4-Pro-Arg_3-Thr-Thr-Arg_4-Ala-Gly-Arg_4-OH; clupeine YII, H-Pro-Arg_3-Thr-Arg_2-Ala-Ser-Arg-Pro-Val-Arg_4-Pro-Arg_2-Val-Ser-Arg_4-Ala-Arg_4-OH; and clupeine Z, H-Ala-Arg_4-Ser-Arg_2-Ala-Ser-Arg-Pro-Val-Arg_4-Pro-Arg_2-Val-Ser-Arg_4-Ala-Arg_4-OH. Each component of clupeine consists of 31 (30 in YII) amino acid residues of which 20 (21 in Z) are arginine. In addition, arginine constitutes clusters of at most four consecutive residues. This paper reports the results obtained mainly from thermal melting and sedimentation studies of DNA–clupeine complexes.

Experimental Procedures

Materials. DNA was extracted with 2 M NaCl from the frozen testis of Pacific herring and precipitated with ethanol (Watanabe *et al.*, 1953). It was purified by sodium dodecyl sulfate treatment (Kay *et al.*, 1952). The DNA specimen had ϵ_p (260 mμ) of 6300 and its protein content was found to be less than 0.2% by an amino acid analysis of the hydrolyzed sample. The value of intrinsic viscosity $[\eta]$ in 0.1 M NaCl, extrapolated to zero gradient, was 57 in units of 100 ml/g. The molecular weight was calculated to be 6.0×10^6 by use of the equation $[\eta] = 1.45 \times 10^{-6}\ M^{1.12}$ (Doty *et al.*, 1958). Sonicated DNA was obtained by subjecting the DNA solution (1.5 mg/ml) in 0.1 M NaCl to 10 Kc-sonic oscillation for 20 min at 2–4° in a nitrogen atmosphere. A molecular weight of 3.7×10^5 was obtained for the sonicated DNA on the basis of viscosity measurements. This was consistent with the value obtained from the average length of 133 molecules directly measured in an electron micrograph. The solution of the sonicated DNA gave a thermal melting curve essen-

* From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan. *Received April 29, 1969.* This work is supported in part by a grant from the Ministry of Education of Japan. A preliminary account has been reported (Inoue and Ando, 1966).

tially identical with that of the unsonicated DNA under the identical solvent conditions.

Clupeine was prepared from the acetone-dried sperm heads of Pacific herring (Ando *et al.*, 1957). The fractionation of clupeine into three molecular species (YI, YII, and Z) was carried out through a one-step column chromatographic method recently developed in this laboratory (Ando and Watanabe, 1969).

Poly-L-arginine-HCl (degree of polymerization = 90) and poly-L-ornithine-HCl (degree of polymerization = 90) were chemically synthesized and kindly given by Mr. S. Kawashima. Poly-L-lysine-HBr (degree of polymerization = 100) was provided by Ajinomoto Co., Inc. These polyamino acids were partially hydrolyzed with 6 N HCl for 73 hr at room temperature and fractionated into oligomers with definite chain length by column chromatography on CM-cellulose essentially according to the method of Stewart and Stahmann (1962). The degree of polymerization of the oligomers was determined by a colorimetric analysis of the trinitrophenylated products (Satake *et al.*, 1960). For the peptides with a degree of polymerization as large as 20, the fractionation became incomplete. Therefore the degree of polymerization as indicated by the suffix to the parenthesized abbreviation of an amino acid residue is the average for a class.

CM-cellulose was a product of Serva, Heidelberg, Germany. Glass-distilled deionized water was used throughout.

Preparation of DNA–Clupeine and Other Complexes. Soluble complexes were prepared by either of the following two methods.

DIRECT MIXING. DNA–clupeine and other DNA–peptide complexes were prepared by titrating, under constant stirring, the solution of sonicated DNA in 0.01 M NaCl–0.001 M Tris-Cl (pH 7.0) with an equal volume solution of the peptide in the same buffer. The final concentration of DNA was about 5×10^{-5} mole of P/l. and the ratio of the peptide cation: DNA-phosphorus was below unity.

GRADIENT DIALYSIS. The solution of DNA (1×10^{-4} M) and that of the peptide (less than 0.8×10^{-4} M) in 2 M NaCl were mixed under stirring. The ionic strength of the mixture was then gradually reduced by dialysis against 0.6 M NaCl for 2 hr, 0.4 M NaCl for 2 hr, 0.2 M NaCl for 1 hr, and finally against 0.03 M NaCl–0.003 M sodium citrate (pH 7.0) for 16 hr or more at 4°. The dialysis tubing had been boiled in 0.005 M EDTA, washed thoroughly with deionized water and dried at 90° for 24 hr before use. The tubing shrank by the heat treatment and the rate of dialysis decreased. Clupeine and the polyamino acids with a degree of polymerization of about 20 were not lost from the tubing under the above conditions when DNA was present in the solutions. However, when these peptides were dialyzed in the absence of DNA, about half of the initial amount was lost in 24 hr.

Analytical Procedures. Melting profiles of the complexes were obtained in an Ito Model 3QA spectrophotometer equipped with thermally controlled compartment. The samples were heated at a rate of 1.5°/min and the absorbance at 260 mμ was recorded. The temperature was measured by a Cu-constantan thermocouple inserted in a cuvet placed in the compartment. The relative absorbance was absorbance at temperature T/absorbance at room temperature. No correction for thermal expansion or light scattering was made in routine experiments. Melting profiles obtained after correcting for thermal expansion and for light scattering by the method of Leach and Scheraga (1960) showed general patterns similar to those uncorrected. Data obtained from the corrected melting profiles did not change any conclusion described in the present paper.

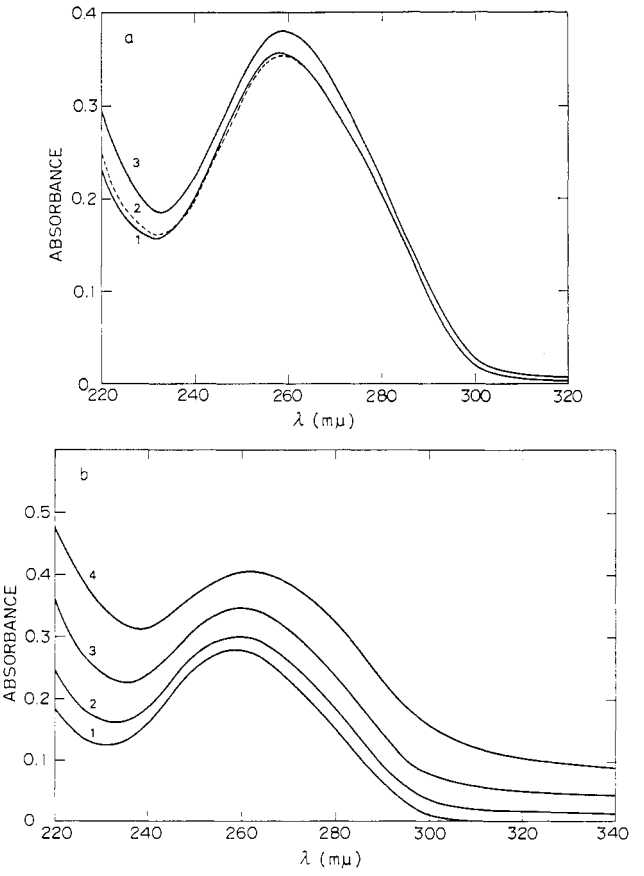

FIGURE 1: (a) Absorption spectra of sonicated DNA and sonicated DNA–clupeine YI complexes formed by direct mixing; solvent: 0.01 M NaCl–0.001 M Tris-Cl (pH 7.0); concentration of DNA: 5.1×10^{-5} M; Arg:P ratios: curve 1, DNA alone; curve 2, 0.20; curve 3, 0.60. (b) Absorption spectra of unsonicated DNA and unsonicated DNA–clupeine Z complexes formed by gradient dialysis. Similar spectra were obtained when sonicated DNA was used; solvent: 0.03 M NaCl–0.003 M sodium citrate (pH 7.0); concentration of DNA: 4.5×10^{-5} M; Arg:P ratios: curve 1, DNA alone; curve 2, 0.20; curve 3, 0.40; curve 4, 0.60.

DNA concentrations were expressed as moles of P/l. Phosphorus was determined by the method of Chen *et al.* (1956).

Peptide concentrations were expressed in terms of mole residue of basic amino acid (Arg or Lys) per liter, which were determined by amino acid analyses (Hitachi KLA-3B automatic analyzer) of the samples hydrolyzed in 6 N HCl for 24 hr at 110° in sealed evacuated tubes. The melting temperature, T_m, was defined as the temperature at which 50% of the hyperchromicity of the transition was attained. The width of the transition was expressed by σ which was defined as the temperature interval required to bring the transition from 25 to 75% of completion.

Results

Absorption Spectra of Complexes. Figure 1a,b shows typical absorption spectra of DNA–clupeine complexes

TABLE I: Melting Transitions of Sonicated DNA and Sonicated DNA–Clupeine Complexes Formed by Direct Mixing.

	Arg:P	First Transition		Second Transition		Hyperchromicity Shown by the Second Transition (%)
		T_m	σ	T_m	σ	
DNA	0	66.5	4.4			
DNA–clupeine YI	0.2	66.5	5.7	80.3	4.0	25.5
	0.4	67.0	5.0	81.3	3.2	51.4
	0.6	68.5	7.9	81.3	1.6	65.0
	0.8	66.0		81.6	1.6	92.6
DNA–clupeine YII	0.2	66.5	6.2	80.0	2.0	24.6
	0.4	67.3	6.7	81.2	2.0	44.0
	0.6	69.0	7.5	81.5	2.0	70.8
	0.8	66.0		81.7	2.3	96.3
DNA–clupeine Z	0.2	66.5	5.5	79.7	2.3	21.6
	0.4	66.5	5.0	80.0	2.8	51.4
	0.6	67.0	5.2	80.5	2.3	76.5
	0.8			81.6	2.4	95.6
DNA–clupeine Mixture[a]	0.2	66.5	4.2	79.3	3.2	30.6
	0.4	66.5	5.0	80.1	2.6	50.7
	0.6	67.7	5.5	81.2	1.8	70.6
	0.8			81.5	2.0	97.0

[a] An equimolar mixture of clupeine YI, YII, and Z.

formed by direct mixing and gradient dialysis, respectively. Similar absorption spectra were obtained for the complexes formed with the other components of clupeine not shown in Figure 1. The complexes formed by gradient dialysis showed greater turbidity than those formed by direct mixing. By gradient dialysis, both the unsonicated and sonicated DNA formed the complexes which showed similar turbidity. However, by direct mixing, soluble complexes could not be formed with unsonicated DNA because of the precipitation of fibrous aggregates.

Thermal Melting Profiles. The complexes formed by direct mixing were subjected to thermal denaturation studies. Typical melting profiles of the sonicated DNA–clupeine YI complexes are reproduced in Figure 2. Biphasic transitions are seen for the complexes at Arg:P ratios below unity. Data relevant to the melting transitions of DNA–clupeine

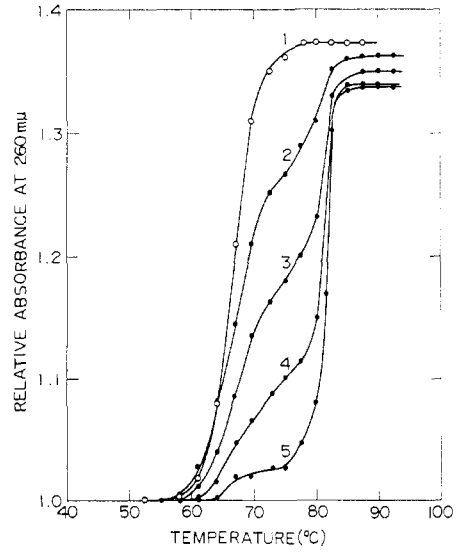

FIGURE 2: Melting profiles of sonicated DNA–clupeine YI complexes formed by direct mixing; Arg:P ratios: curve 1, DNA alone; curve 2, 0.20; curve 3, 0.40; curve 4, 0.60; curve 5, 0.80.

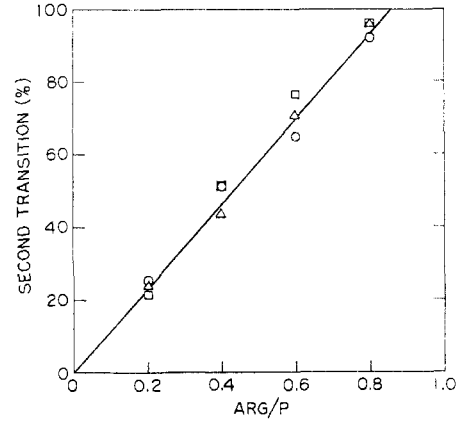

FIGURE 3: Relationship between Arg:P input ratios and relative amounts of hyperchromicity attained by the second transition; (○) clupeine YI; (△) clupeine YII; (□) clupeine Z.

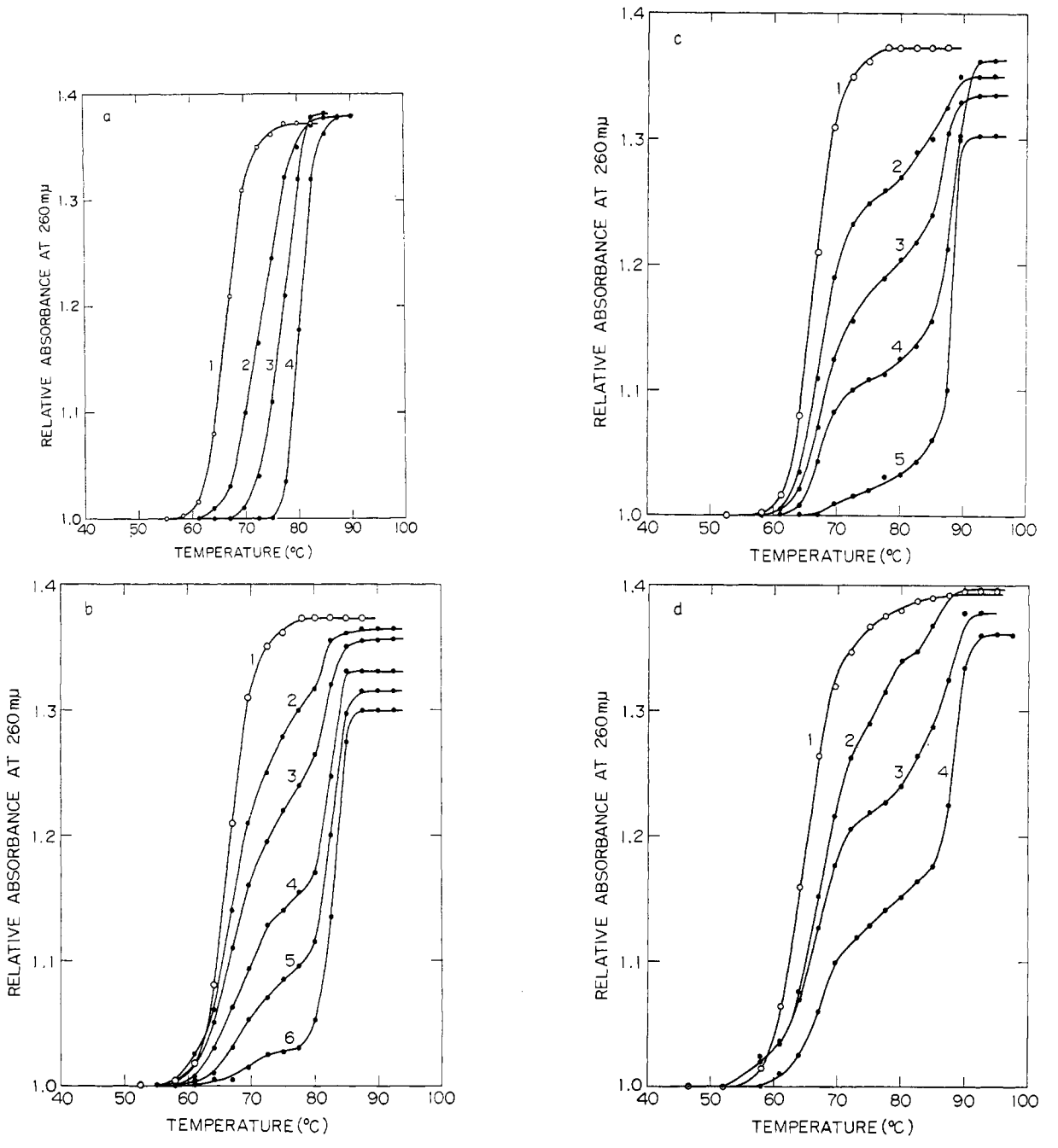

FIGURE 4: Melting profiles of sonicated DNA–peptide complexes formed by direct mixing. (a) DNA–$(Arg)_4$; Arg:P ratios: curve 1, DNA alone; curve 2, 0.20; curve 3, 0.39; curve 4, 0.78. (b) DNA–$(Arg)_{20}$; Arg:P ratios: curve 1, DNA alone; curve 2, 0.16; curve 3, 0.32; curve 4, 0.48; curve 5, 0.64; curve 6, 0.80. (c) DNA–$(Lys)_{20}$; Lys:P ratios: curve 1, DNA alone; curve 2, 0.25; curve 3, 0.50; curve 4, 0.80; curve 5, 1.0. (d) DNA–$(Orn)_{19}$; Orn:P ratios: curve 1, DNA alone; curve 2, 0.20; curve 3, 0.40; curve 4, 0.60.

complexes are listed in Table I. T_m values of the first transitions are similar to the value of DNA alone, while the second transition shows T_m values about 15° higher than this. These biphasic melting profiles suggest the presence of two distinguishable states of DNA (or segments of DNA) in the solutions as suggested by Tsuboi *et al.* (1966) for poly-L-lysine-DNA and poly-L-lysine-poly (I + C) complexes and by Olins *et al.* (1967) for basic homopolypeptide–DNA complexes. Thus the first transition is attributed to the melting of free DNA molecules (or segments of DNA) and the second transition represents the melting of DNA molecules (or segments of DNA) complexed with the peptide. A linear relationship is observed between the Arg:P input ratios and relative amounts of hyperchromicity exhibited by the second transition (Figure 3). Almost all the DNA molecules melt at higher transition when the Arg:P ratio attains 0.8. These results indicate that the binding of clupeine to DNA occurs irreversibly and stoichiometrically under these conditions.

TABLE II: Melting Transitions of Sonicated DNA–Peptide Complexes Formed by Direct Mixing.

	Cation:P	First Transition		Second Transition		Hyperchromicity Shown by the Second Transition (%)
		T_m	σ	T_m	σ	
DNA	0	66.5	4.4			
DNA–$(Arg)_4$	0.20	73.0	6.5			
	0.39	76.9	4.8			
	0.78	80.2	3.5			
DNA–$(Arg)_{20}$	0.16	67.5	6.0	81.5	2.3	17.2
	0.32	67.5	6.5	81.7	2.5	32.2
	0.48	67.5	6.5	82.0	2.6	54.5
	0.64	69.0	7.0	82.5	3.0	69.8
	0.80	69.5	5.0	83.0	2.5	90.0
DNA–$(Lys)_{20}$	0.25	67.5	5.0	86.0	5.5	25.7
	0.50	68.0	5.3	85.8	3.0	43.2
	0.80	68.0	3.6	87.5	3.2	69.0
	1.0	72.5	6.0	88.5	1.5	90.2
DNA–$(Orn)_{19}$	0.20	66.5	7.4	85.5	3.2	13.7
	0.40	65.0	5.8	87.0	6.0	41.7
	0.60	67.0	5.5	88.5	2.5	61.0

Data obtained from the melting profiles of DNA–clupeine YII and DNA–clupeine Z were essentially similar to the data for DNA–clupeine YI. When the unfractionated clupeine or an equimolar mixture of three components was used in place of any one component, no significant difference was observed in the melting profiles (Table I).

These melting profiles were compared with those of the complexes formed between DNA and basic oligo- and polyamino acids. The previous work has shown that the nature of binding of oligo-L-ornithine to DNA is dependent on the degree of polymerization of the peptide (Kawashima *et al.*, 1969). Figure 4a–d shows the melting profiles of DNA–$(Arg)_4$, DNA–$(Arg)_{20}$, DNA–$(Lys)_{20}$, and DNA–$(Orn)_{19}$ complexes formed by the mixing method in 0.01 M NaCl–0.001 M Tris-Cl (pH 7.0). The DNA–$(Arg)_{20}$, DNA–$(Lys)_{20}$, and DNA–$(Orn)_{19}$ complexes show biphasic transitions at cation:P ratios less than unity. The DNA–$(Arg)_4$ complexes exhibit monophasic transitions and their T_m values increase with increasing Arg:P ratio. The finding indicates that the binding of $(Arg)_4$ to DNA is reversible. Although, in the amino acid sequence of clupeine, at most four consecutive arginine residues are separated by the insertion of a few consecutive neutral amino acid residues, the binding of the whole molecule of clupeine to DNA is irreversible as it is the case for $(Arg)_{20}$. In Table II, T_m and σ values are listed for DNA–$(Arg)_4$, DNA–$(Arg)_{20}$, DNA–$(Lys)_{20}$, and DNA–$(Orn)_{19}$. The stability of the irreversible complexes was found to decrease in the order of DNA–$(Orn)_{19}$ > DNA–$(Lys)_{20}$ > DNA–$(Arg)_{20}$ > DNA–clupeine from the data based on the second T_m values. The fact that DNA–$(Arg)_{20}$ is more stable than DNA–clupeine while both peptides have the same number of arginine residues should be due to an effect of neutral amino acid residues inserted between clusters of arginine residues in clupeine molecules. It is also noted that the second transition of DNA–clupeine complexes is sharper than that of DNA–$(Arg)_{20}$ complexes. The second transition for DNA–$(Lys)_{20}$ and DNA–$(Orn)_{19}$ is even broader than for DNA–$(Arg)_{20}$.

Solutions of the complexes formed by gradient dialysis also showed hyperchromicity upon heating. In this case, however, a quantitative expression was difficult because of a large contribution from light scattering.

Sedimentation. Centrifugation at about 25,000*g* of solutions of DNA–clupeine complexes (Arg/P < 1) prepared by gradient dialysis resulted in partial precipitation of DNA from the solutions. The amounts of DNA precipitated and the Arg:P ratios found in the precipitates are given in Table III. The table includes the results for both the sonicated and the unsonicated DNA. In the case of the sonicated DNA, the ratio of the precipitated DNA relative to the total DNA is approximately equal to the Arg:P ratio in the original solution. Furthermore, the Arg:P ratios found in the precipitates are close to unity regardless of the Arg:P input ratios, indicating that the binding occurs at an Arg:P ratio of unity. In other words, DNA molecules are segregated into free species and completely complexed species in which all the phosphates are neutralized by the peptide cations. In the case of unsonicated DNA, the ratios of the precipitated DNA relative to the total DNA are larger than the Arg:P input ratios. The examination of melting profiles of the supernatant fractions revealed that essentially free DNA was present in these fractions. These results suggest that, in the case of unsonicated DNA, incompletely complexed DNA molecules

and essentially free DNA molecules are present in the solutions after dialysis. Thus, our results give a clear-cut evidence for a cooperative binding of clupeine to DNA under the conditions of gradient dialysis. Similar observations were made by Olins *et al.* (1967, 1968). Leng and Felsenfeld (1966) also showed cooperative binding of polylysine and polyarginine to DNA in a high salt solution. We have extended the study to see if there is preferential binding of clupeine to DNA regions containing a special base sequence. The solution of DNA–clupeine formed by gradient dialysis at an Arg:P input ratio of 0.25 was fractionated by centrifugation at 25,000*g*. The precipitate was dissolved in 4 M $NaClO_4$ and the melting profile was compared with that of the unfractionated DNA in the same solvent. This solvent was used because the complex is dissociated and DNA present in this system behaves like free DNA. Moreover, T_m of DNA is unusually lowered and the dependence of T_m on the nucleotide composition in this solvent is larger than in other solvents (Hamaguchi and Geiduschek, 1962). Essentially identical melting profiles (T_m = 62°) were observed for the precipitated DNA and the unfractionated DNA. These results suggest that clupeine does not show selective binding to A-T-rich or G-C-rich regions of DNA.

It is noted that in the presence of free DNA molecules, 1:1 complexes of DNA with clupeine are dispersed in solutions and precipitated only by high-speed centrifugation. This is in contrast to the fact that, in the absence of free DNA, the 1:1 complexes precipitates without centrifugal force even at 0.1 concentration of that used in the above experiments. Thus free DNA appears to have an effect of solubilizing the complexed DNA when both species are present in the same solution. Complexes formed by the mixing method did not precipitate by high-speed centrifugation when Arg:P ratios were less than unity. In this case, the distribution of clupeine onto DNA molecules appears to be random at low Arg:P ratios (S. Inoue and T. Ando, to be published).

TABLE III: Sedimentation of DNA–Clupeine Complexes Formed by Gradient Dialysis.

DNA	Clupeine	Arg:P Input	Sedimented DNA: Total DNA	Arg:P in the Sediment
Sonicated				
		0.2	0.21	1.1
	YI	0.4	0.41	1.0
		0.6	0.61	0.94
		0.2	0.21	0.90
	YII	0.4	0.36	0.97
		0.6	0.59	0.97
		0.2	0.17	0.80
	Z	0.4	0.38	0.92
		0.6	0.58	0.85
Unsonicated				
		0.2	0.24	
	YI	0.4	0.51	
		0.6	0.80	
		0.8	0.88	
		0.2	0.22	
		0.4	0.51	
	YII	0.6	0.78	
		0.8	0.89	
		0.2	0.23	
		0.4	0.52	
	Z	0.6	0.73	
		0.8	0.81	

Discussion

In the nuclei of fish spermatozoa, the nucleoprotamine exists in an insoluble state. It is soluble in high salt solutions (*e.g.*, 2 M NaCl) in a dissociated form, but hardly soluble in low salt solutions (below 0.1 M), in contrast to the nucleohistone (Oth and Desreux, 1957). The use of EDTA and shearing by mechanical homogenization or sonication can solubilize only a small portion of the chromosomal nucleoprotamine (Raukas *et al.*, 1966). Thus it seems almost impossible to solubilize the nucleoprotamine without modifying its native structure, although this approach has been successful in the case of the nucleohistone (Bonner *et al.*, 1968).

Because of these difficulties, we have studied the properties of DNA–clupeine complexes reconstituted in solution from isolated DNA and clupeine, in order to gain a better understanding of the nature of the chromatin. In these studies, sonicated DNA having an average molecular weight of 3.7×10^5 (approximately 1000 nucleotide units) was used to prepare "soluble" complexes. Complexes showing only small turbidity were formed between the sonicated DNA and clupeine (or its model peptides) by the mixing method.

The sonicated DNA–clupeine complexes showed sharp biphasic melting transitions at Arg:P ratios below unity. The appearance of the biphasic transition was found to depend on the chain length of the basic peptide rather than the nature of the basic side chain of the peptide, on the basis of comparative melting studies of the DNA–$(Arg)_4$, DNA–$(Arg)_{20}$, DNA–$(Lys)_{20}$, and DNA–$(Orn)_{19}$. The order of stability of these complexes changed according to the nature of the basic groups in the peptides. The T_m values of the second transition of the DNA–clupeine complexes were close to those of the DNA–$(Arg)_{20}$ complexes, although the formers were slightly lower than the latters.

This difference between DNA–clupeine and DNA–$(Arg)_{20}$ should be ascribed to an effect of neutral amino acid residues present in the molecules of clupeine. Another difference between the DNA–clupeine complexes and the DNA–$(Arg)_{20}$ complexes was found in the width of the second transition: the transition of the DNA–clupeine complexes was sharper than that of the DNA–$(Arg)_{20}$ complexes. The transition for DNA–$(Lys)_{20}$ and DNA–$(Orn)_{19}$ was broader than that of DNA–$(Arg)_{20}$. In going to the DNA–$(Lys)_{100}$ complexes, both the first and the second transitions underwent further broadening. These differences observed between the DNA–clupeine and DNA–polyamino acid complexes suggest the difference in the mechanism of complex formation between these two systems. It is probable that in the binding of clupeine having

neutral amino acid residues between clusters of arginine residues, a kinetic barrier is smaller than in the binding of polyarginine and polylysine, and thus the complexes with thermodynamically favored structures are formed under the conditions of low ionic strength. It should be noted that Olins *et al.* (1968) also reported their nonannealed (direct mixing) and annealed (gradient dialysis) DNA–protamine complexes differ much less in melting properties than annealed and nonannealed complexes of DNA with basic polypeptides.

DNA–clupeine complexes formed by gradient dialysis can be fractionated into the fully bound and free DNA by high-speed centrifugation. The formation of such rigid aggregates appears to be due to a nonrandom distribution of clupeine onto DNA molecules through a selective binding at the initial stage of the binding process followed by the preferential binding of clupeine to sites adjacent to those already occupied by the peptide. Under such conditions of cooperative binding, specificity in the binding of clupeine toward base sequences of DNA cannot be detected by analyzing the nucleotide composition of DNA in the complexes. Our results support the situation.

The present studies failed to observe any significant difference in the properties of the three different components of clupeine. This is not unexpected from the close similarity in the covalent structure of these components. Unfractionated clupeine or an equimolar mixture of the three components did not show noticeable difference from any one component in the interaction with DNA. As it has been demonstrated that in the testis of a fish, three components of clupeine exist in nearly equimolar amounts (Ando and Sawada, 1962), it may be a problem to be solved whether such characteristic mode of occurrence of clupeine components is of any functional significance or it is an evolutionary consequence as proposed by Black and Dixon (1967).

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