*Sedimentation and Diffusion Studies of Calf Thymus Histone**

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In a previous paper[1], it was reported that calf thymus histone consists of two fractions, histones I and II, which were different in respect to chemical as well as physico-chemical properties. The present paper deals with the molecular-kinetic properties of these two fractions. In spite of their heterogeneity as revealed by electrophoresis, they behaved like a single protein in sedimentation and diffusion, and the molecular weights have been calculated.

As there have been few data about the molecular weight of histone, especially of mono-disperse preparation, it seems of value to present the results in some detail. A preliminary report of this investigation has already been published[2].

Experimental

Materials.—The preparations of histone used in this study were extracted from isolated calf thymus nuclei using sulfuric acid, purified by an ethanol-precipitation method at a temperature below 0°C, and finally lyophilized as histone chlorides. Histone I is the main fraction of histone precipitated with 20 per cent. ethanol, and histone II is the fraction precipitated by 45 per cent. Details of the procedure for the preparation and fractionation have been described[1].

As it was found that histone I had a tendency to aggregate, especially at higher temperature, care was taken to avoid the aggregation; i. e., each preparation was dissolved in a cold buffer solution and kept at about 0°C until measurements were performed (less than one day). In most of this study an acetate buffer of pH 5.0 at ionic strength of 0.2 was used as a solvent. In this the aggregation did not take place appreciably even if the solution was kept at 20°C for 24 hours.

Protein concentrations were usually determined by the micro-Kjeldahl method. The author is indebted to Miss T. Kitamura for this analysis.

Methods.—Ultracentrifugal characteristics of these materials have been studied using a Spinco model E ultracentrifuge. In the case of histone II, a synthetic boundary cell[3] was used in order to measure its low sedimentation coefficient. Runs were made always at 59,780 r. p. m. The temperature of each run was taken as the mean of the rotor temperature at the beginning and the end of the run without correction for a change in rotor temperature during acceleration and deceleration[4]. The observed sedimentation coefficient was corrected to the value in water at 20°C ($s_{20,w}$) by a conventional manner[5].

Diffusion measurements were made at 20.0°C in a Neurath-type diffusion cell[6] equipped with a Philpot-Svensson's schlieren optical system[7]. Diffusion coefficients were computed from the second moments of the diffusion curves[8] and corrected to the value in water at 20°C ($D_{20,w}$).

Partial specific volume of histone I was determined at 20.0°C, using a pycnometer of 5-cc. capacity.

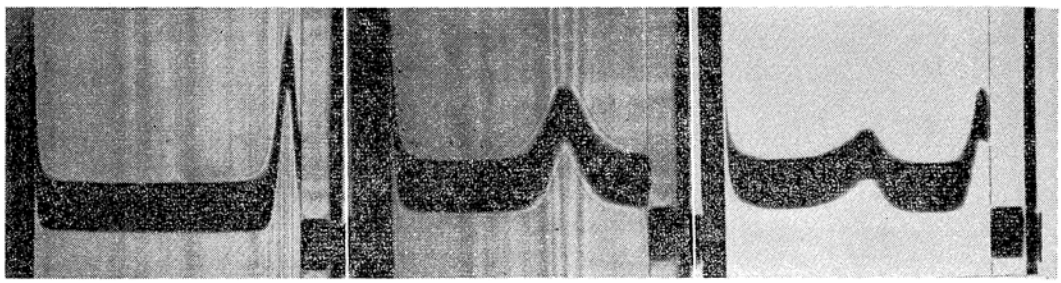
Results

A. Sedimentation Characteristics of Histone I.—The preparation of histone I, the main fraction of calf thymus histone, was examined in the ultracentrifuge in a pH range of between 4.0 and 9.9. Only a single homogeneous boundary was observed below pH 7, as is shown in Fig. 1 (1A and 1B). These photographs were taken using an acetate buffer of pH 5.0 and ionic strength of 0.2.

At pH values higher than 7, except in tris(hydroxymethyl)aminomethane buffer of pH 8.0, a faster component was observed (see Fig. 1C) even when the solution had not been exposed to a high temperature. The amount of the fast component varied with the pH and the composition of the buffer. It was also found that the faster boundary disappeared when the solution was quickly brought to pH 5.0 by dialysis in a cold room and examined by the ultracentrifuge. The presence of the fast component at a higher pH than 7 would not be due to the inhomogeneity of this preparation, but would

* A part of this work was read before the 8th Annual Meeting of the Chemical Society of Japan held in Tokyo, April 1, 1955.

1) N. Ui, *Biochim. Biophys. Acta*, in press.
2) N. Ui, *Biochim. Biophys. Acta*, **22**, 205 (1956).
3) H. K. Schachman, *Proc. Natl. Acad. Sci. U. S. A.*, **38**, 943 (1952).
4) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instruments*, **23**, 609 (1952).
5) T. Svedberg and K. O. Pedersen, "Ultracentrifuge," Oxford (1940).
6) H. Neurath, *Chem. Revs.*, **30**, 357 (1942).
7) J. St. L. Philpot, *Nature*, **141**, 283 (1938); H. Svensson. *Kolloid-Z.*, **87**, 181 (1939).
8) O. Lamm, *Nova Acta Regiae Soc. Sci. Upsaliensis*, Ser. IV, **10**, No. 6 (1937).



(A) (B) (C)

Fig. 1. Sedimentation patterns of calf thymus histone I.
(A): In acetate buffer of pH 5.0 at ionic strength of 0.2 after 23 min. at 59,780 r. p. m.
(B): The same as (A), but after 190 min.
(C): In veronal-NaCl buffer of pH 9.0 at ionic strength of 0.2, after 16 min. at 59,780 r. p. m.

be accounted for if a (reversible) formation of aggregate took place in an alkaline solution.

In Fig. 2 (curve I) is shown the concentration dependency of the sedimentation coefficient of histone I in the acetate buffer of pH 5.0 at ionic strength of 0.2. The sedimentation coefficient slightly increased with decrease in concentration. A value of 2.0_3 S (Svedberg unit) was obtained when extrapolated to infinite dilution by the least square method.

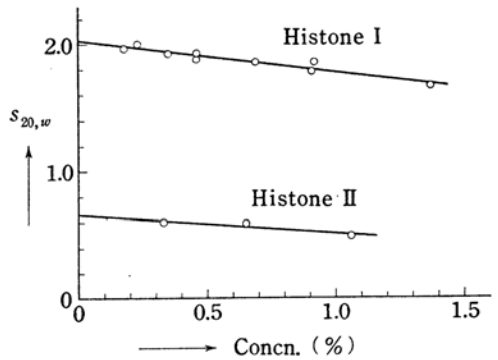


Fig. 2. Concentration dependency of sedimentation coefficients of histones I and II in an acetate buffer of pH 5.0 at ionic strength of 0.2.

The sedimentation coefficients obtained at other pH values are given in Table I. In all cases, the concentration was nearly 1.0 per cent. Amounts of the faster component, if present, are also recorded in the last column. It was noticed that a little higher sedimentation coefficient was obtained at a higher pH.

B. Diffusion of Histone I.—The measurements of free diffusion of histone I were made at 20.0°C in the acetate buffer of pH 5.0 at ionic strength of 0.2. Runs lasted less than 20 hours. Therefore, the effect of aggregation would be small.

Diffusion curves obtained were always symmetrical and showed nearly Gaussian curves. The homogeneity of this preparation was thus confirmed. The diffusion coefficients calculated from the second moments of the refractive-index gradient

TABLE I
SEDIMENTATION COEFFICIENT OF HISTONE I IN BUFFER SOLUTIONS OF IONIC STRENGTH 0.2 (Concn. = ca. 1%)

Buffer	pH	Sedimentation coefficient		Amount of fast comp.
		Slow comp. (S)	Fast comp. (S)	(%)
Acetate	4.0	1.5_9	—	0
〃	4.2_5	1.3_6	—	0
〃	5.0	1.7_8	—	0
Acetate-Na_2SO_4	〃	1.5_5	—	trace
Cacodylate-NaCl	6.0	1.7_6	—	0
NaCl (unbuffered)	6.3	1.8_3	—	trace
Cacodylate	6.8	1.8_9	—	0
Phosphate	7.7	2.2_8	19.1	8
Tris*	8.0	2.1_0	—	0
NH_4Cl-NH_3-NaCl	8.6	2.0_5	26.3	32
Veronal-NaCl	9.0	2.1_9	30.2	60
NH_4Cl-NH_3	9.9	2.4_2	22.0	70

* tris(hydroxymethyl)aminomethane buffer.

TABLE II
DIFFUSION DATA ON HISTONE I

Concn. (%)	$D_{20,w} \times 10^7$ (cm²/sec.)	Y_{max}	X_{max}
0.93	4.9_3	2.11	0.02
0.47	4.9_4	2.01	0.00
0.41	4.8_3	2.00	0.00
0.34	5.3_9	2.03	0.00
0.30	5.5_3	2.03	0.00
0.28	5.1_5	2.13	0.00
Mean	5.1_3	2.05	0.00

curves were almost independent of concentration and the averaged value was taken as the diffusion constant. The results of the experiments are shown in Table II together with the values of Y_{max} and X_{max}, which show the degree of homogeneity and of concentration dependency of diffusion coefficient, respectively*.

C. Partial Specific Volume of Histone I.—The partial specific volume ($\bar{V}$) of histone I was calculated using the equation,

$$\bar{V}=\frac{m_0-(m-h)}{\rho_0 h} \quad (1)$$

where m is the weight of the pycnometer filled with the solution in which h gram of protein is dissolved, and m_0 is the weight of the pycnometer filled with a solvent with density ρ_0.

The calculated values at the concentrations of 0.96_0, 0.64_0 and 0.42_7 per cent. were 0.73_7, 0.73_7 and 0.73_4, respectively. A value of 0.74 was taken as the mean value.

D. The Molecular Weight and Shape of Histone I.—From these results, the molecular weight (M) of histone I was calculated using the formula deduced by Svedberg and Pedersen[5], viz.,

$$M=\frac{RTs}{D(1-\bar{V}\rho)} \quad (2)$$

where R is the gas constant and T is the absolute temperature. The value (*unhydrated* molecular weight), 37,000, was obtained (see Table IV).

When sedimentation and diffusion constants are known, it is possible to calculate the frictional ratio, f/f_0, i.e., the ratio of the frictional coefficient of the protein to that for a compact spherical and unhydrated molecule of the same volume[5]. In the case of histone I, f/f_0 was calculated to be 1.88.

The fairly large value of f/f_0 raised a question concerning the molecular configuration of histone I. If it could be assumed that molecule is a rigid ellipsoid of revolution and the density of the hydrated water has the same value as that of free solvent, its axial ratio could be calculated using Perrin's equation[9]. Since the extent of hydration is not known, the plausible range of values usually accepted, i.e., 0—0.5 gram water per gram protein, was taken. Then the value of axial ratio, 17—11, was obtained for a prolate ellipsoid. However, in view of the assumption involved in this calculation, it is not convincing that histone I is really such an asymmetric molecule. Recently, Scheraga and Mandelkern[10] attempted to interpret observed physical properties of the protein in terms of a rigid "equivalent ellipsoid of revolution" characterized by its effective volume V_e and its axial ratio without any assumption concerning hydration. They showed that the function, β, defined as $Ns[\eta]^{1/3}\eta/M^{2/3}(1-\bar{V}\rho)$, is dependent only upon the shape of the hydrodynamic particle. For histone I, β was calculated using the above-mentioned values of sedimentation constant and molecular weight as well as its limiting viscosity number, $[\eta]$, measured in the same buffer, 0.111 (cc./g.). A value of 2.05×10^6 was obtained, which fell outside the range for a rigid ellipsoid ($\beta=2.12\times10^6$ for spheres and $\beta>2.12\times10^6$ for ellipsoids). As the validity of Scheraga and Mandelkern's treatment has not been established, no definite conclusion would be deduced from this consideration*. However, taking the extreme sensitivity of β to errors in experimental data into consideration, the fact that β was not far from 2.12×10^6 might show that histone I is not highly elongated, but is much swollen by imbibition of a solvent**. Recently, Noda[13] has proposed an experimental equation relating the volume fraction concentration and sedimentation coefficient in a suspension of spheres, and, taking advantage of this equation, he explained the large value of frictional ratio in histone I by the increase of apparent volume by solvation. Our finding that the limiting viscosity number of histone I was very sensitive to the change in ionic strength or pH of the solution[14] might support this view.

* For a homogeneous preparation with a single diffusion coefficient independent of concentration, $Y_{max}=2.00$ and $X_{max}=0^{8)}$. Slightly higher values of Y_{max} than 2.00 in some of the present experiments may be due to the presence of a small amount of aggregate.

9) F. Perrin, *J. phys. radium*, (7) **7**, 1 (1936).

10) H. A. Scheraga and L. Mandelkern, *J. Am. Chem. Soc.*, **75**, 179 (1953).

* Tanford and Buzzell[11] have shown that the value of β for bovine serum albumin calculated by using accurate molecular-kinetic data was definitely lower than the permissible range for rigid ellipsoids.

11) C. Tanford and J. G. Buzzell, *J. Am. Chem. Soc.*, **76**, 3356 (1954).

** Ogston[12] has also considered several methods for making an estimate of dimensions of solute particles from dynamic properties of their solution. According to one of his most reliable methods, IIb, the axial ratio of the spheroidal hydrodynamic model, J, the effective hydrodynamic volume per gram of unhydrated mass, V', and the molecular weight were calculated for histone I from s_0, $d(1/s)/dc$, and $[\eta]$; the values, $J=5$, $V'=1.9$, $M=35{,}000$, were obtained. It was again suggested that histone I is only moderatly elongated, and it carries a fairly large amount of solvent with it.

12) A. G. Ogston, *Trans. Faraday Soc.*, **49**, 1481 (1953).

13) H. Noda, This Bulletin, **30**, 495 (1957).

14) N. Ui, unpublished.

E. Results on Histone II.—Similar measurements were made on histone II, the other fraction of calf thymus histone.

Ultracentrifugal patterns of histone II (see Fig. 3) showed only one boundary

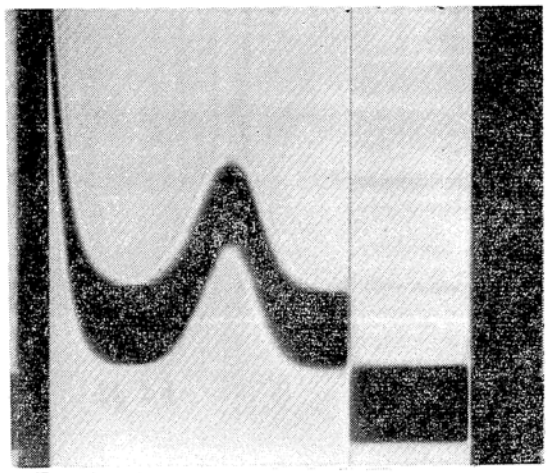


Fig. 3. Sedimentation pattern of calf thymus histone II in acetate buffer of pH 5.0 at ionic strength of 0.2.
After 110 min. at 59,780 r. p. m. in a synthetic boundary cell.

indicating the homogeneity of this preparation. The sedimentation coefficient of this protein was definitely lower than that of histone I in the acetate buffer of pH 5.0 at ionic strength of 0.2. Unlike histone I, histone II did not aggregate even if the solution was kept at 30°C for several days. The sedimentation coefficient vs. concentration curve is given in Fig. 2 (curve II). The sedimentation constant at infinite dilution was obtained by extrapolation as 0.6_6 S.

Diffusion study also confirmed the homogeneity of this preparation. The diffusion coefficients together with the values of Y_{max} and X_{max} calculated from the diffusion curves in the acetate buffer are tabulated in Table III. The mean value was taken as the diffusion constant.

TABLE III
DIFFUSION DATA ON HISTONE II

Concn. (%)	$D_{20,w}\times10^7$ (cm²/sec.)	Y_{max}	X_{max}
0.85	7.1_6	2.00	0.00
0.36	7.5_9	2.02	0.00
0.29	7.1_5	2.02	0.01
Mean	7.3_0	2.01	0.00

Assuming the same value of partial specific volume as histone I, the molecular weight of histone II was calculated using equation (2). The value, 8,400, was nearly a quarter of that of histone I. A value of 2.16 was obtained for the frictional ratio, which was even higher than that for histone I.

Several data obtained for histone I and II are listed and compared in Table IV.

TABLE IV
MOLECULAR-KINETIC DATA ON HISTONES I AND II

	Histone I	Histone II
Sedimentation constant, $s_{20,w}$ (S)	2.0_3	0.6_6
Diffusion constant, $D_{20,w}\times10^7$ (cm²/sec.)	5.1_3	7.3_0
Partial specific volume	0.74	(0.74)*
Molecular weight	37,000	8,400
Frictional ratio, f/f_0	1.88	2.16
Axial ratio, assuming a rigid ellipsoid without hydration	17	25

* Assumed value.

Discussion

As shown in Table IV, a difference in molecular-kinetic properties is clear between the two fractions of histone, histone I and histone II. The view that histone II is due to the disaggregation of histone I seems unlikely, since histone I could not be converted into a smaller molecule under several conditions examined and the difference of these two fractions was definitely shown by chemical analyses[1]. Therefore, it might be reasonable to assume that calf thymus histone consists of two fractions with different molecular weights; the main fraction (80—90 per cent. of total histone) has a molecular weight of 37,000, and a lower molecular-weight fraction (molecular weight=8,400) is also contained as a minor fraction.

In this connection, it is of interest to compare the results of the present investigation with those reported by other workers. Numerous values of sedimentation constant have been recorded on the preparations of calf thymus histone prepared by various methods, although most other workers used ultracentrifugally inhomogeneous preparations. The presence of a faster component with much higher sedimentation coefficient than 2 S was often reported[15—20], but it is likely that

15) L. Ahlström, *Arki. Kemi, Mineral. Geol.*, **24A**, No. 31 (1947).
16) D. Hamer, *Brit. J. Cancer*, **5**, 130 (1951).

such a component is due to the aggregation that occurred during the isolation or in the solution in which sedimentation was performed. The view was supported by the fact that a similar boundary was also observed in our experiments on histone I at pH values higher than 7 or on using a solution (pH<7) which had been kept at room temperature for a while. The component with a sedimentation coefficient of approximately 2 S was also observed by most investigators[16-20]; the somewhat lower value recorded would be due either to the fact that the values were not extrapolated to infinite dilution or to insufficient separation of a slower component. Although it was reported by some workers that the amount of 2 S component was less than that of the component with a higher sedimentation coefficient[19,20], our conclusion that 2 S component (histone I) is the main fraction of histone would be reasonable, if we consider the great tendency of this component to aggregate. The results of Butler and coworkers aggreed to a fair extent with our results. However, their "slow component" purified as a homogeneous fraction showed higher value of sedimentation constant, 1.06 S[18], than histone II. As the properties and the amino acid composition of these two preparations are similar, further study would be necessary to explain the discrepancy.

The diffusion constant of histone has been measured by Cruft et al.[21], and Butler et al.[17,18] The former authors found that the diffusion coefficient of their preparation (β-histone) depended markedly upon pH and accounted for this phenomenon by aggregation. The highest value reported by them, 4.2×10^{-7} cm^2/sec., which was obtained at pH 1.7, nearly coincided with our values for histone I at pH 5.0. The value obtained by Butler et al. markedly varied with preparations, and it was noticed that even an ultracentrifugally inhomogeneous preparation showed nearly a Gaussian curve[17]. The diffusion constant of a homogeneous fraction with a low molecular weight[18] was 5.3×10^{-7} cm^2/sec., definitely lower than our value for histone II. Luck et al.[20] noted that the diffusion curves of their two fractions were not Gaussian.

Molecular weight of calf thymus histone has been calculated by Butler et al. by combining the values of sedimentation and diffusion constants[17,18]. For the homogeneous fraction with a low sedimentation constant (the slow component) a value of 18,000 was obtained, which was approximately twice as great as that of our histone II. The molecular weights of unfractionated preparations were also calculated by them, but, owing to the scattered values obtained, no definite conclusion are to be expected. Other recorded values of molecular weight calculated from sedimentation constant (or sedimentation coefficient) by assuming that the molecule is spherical would not be worth mentioning, as it was found by the present study as well as by Butler et al. that the frictional ratio of histones always showed a high value of about 2. Of interest is the view of Luck et al.[20] that the monomeric molecular weight of any fraction of histones is as low as about 10,000. They came to this conclusion by measurements of osmotic pressure and sedimentation in the presence of guanidinium chloride or urea and by end-group analysis. However, the results of our N-terminal analysis[22] have failed to support their view, although more work is necessary to see if the dissociation of our preparation does occur.

As the preparation used in this study had been obtained under milder conditions, and measurements were made under a suitable condition using ultracentrifugally homogeneous samples, it is more likely that the values of molecular weight obtained in this investigation would be reliable. It might be noted that, although rather high values of frictional ratio were obtained with both fractions, it does not necessarily mean that they are asymmetric molecules. The results in favor of this view have been obtained as described in the previous section, and further research is now in progress.

Summary

Two fractions of calf thymus histone, histone I and histone II, were examined by sedimentation and diffusion.

Histone I was ultracentrifugally homogeneous in the buffer solutions below pH

17) J. A. V. Butler, P. F. Davison, D. W. F. James and K. V. Shooter, *Biochim. Biophys. Acta*, **13**, 224 (1954).
18) P. F. Davison, D. W. F. James, K. V. Shooter and J. A. V. Butler, ibid., **15**, 415 (1954).
19) B. Bakay, J. J. Kolb and B. Toennies. *Arch. Biochem. Biophys.*, **58**, 144 (1955).
20) J. M. Luck, H. A. Cook, N. T. Eldredge, M. I. Haley, D. W. Kupke and P. S. Rasmussen, ibid., **65**, 449 (1956).
21) H. J. Cruft, C. M. Mauritzen and E. Stedman, *Nature*, **174**, 580 (1954).
22) M. Yamasaki, N. Ui, S. Ishii, H. Fujioka, K. Iwai and T. Ando, The 29th General Meeting of the Japanese Biochemical Society, Fukuoka, Nov. 1, 1956.

7, but showed the presence of an aggregate above pH 7. The sedimentation constant (at infinite dilution) of histone I was calculated to be 2.0_3 S in an acetate buffer of pH 5.0 at ionic strength of 0.2. The homogeneity of this sample was also confirmed by diffusion experiments in the same buffer, and $5.1_3 \times 10^{-7}$ cm^2/sec. was obtained as the diffusion constant. Combining the values of sedimentation and diffusion constants as well as the value of partial specific volume, 0.74, the molecular weight was calculated to be 37,000.

Histone II was also found to be homogeneous by sedimentation and diffusion measurements in the acetate buffer of pH 5.0. Its sedimentation constant, diffusion constant, and molecular weight were calculated as 0.6_6 S, $7.3_0 \times 10^{-7}$ cm^2/sec., and 8,400, respectively.

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