Electrophoretic Studies of Calf Thymus Histone

By Nobuo Ui

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As was reported in the previous papers[1,2], calf thymus histone consists of two fractions (histones I and II). It was also shown that both were ultracentrifugally homogeneous. However, electrophoretic investigation on these fractions revealed that neither of them was homogeneous.

The present paper deals with the electrophoretic properties of histone I, the main component. A few experiments with histone II are also reported and the results are compared with those of histone I. Although electrophoretic heterogeneity of calf thymus histone has also been proved by other workers[3-7] the present experiments showed the presence of more components than had ever been reported before.

Experimental

Materials.—Two fractions of calf thymus histone used in this investigation, i.e., histones I and II, were prepared by the method described in a previous paper[1]. The preparation of histone I mainly used was obtained from the extract of isolated nuclei with 0.2 N sulfuric acid after removing histone II by extraction with 0.1 N sulfuric acid.

Methods.—Electrophorsis was carried out in a Tiselius-type apparatus[8] equipped with a Philpot-Svensson's shlieren optical system[9]. Prior to electrophoresis, each preparation was dissolved in a desired buffer and dialyzed against a large quantity of the buffer in a cold room which was maintained approximately at 0°C, although, in some experiments, the dialysis was omitted or performed at 25°C.

The pH value of some buffers, such as ammonium chloride ammonia buffers, markedly varied with temperature. Accordingly, pH measurements were made at a temperature nearly the same as that of electrophoresis. Acetate buffers used in most of the experiments did not show any change in pH with temperature.

In most cases, runs were made at a constant temperature of approximately 0°C (at 0.2°C or slightly higher), but some experiments were made at 25.0°C to see the effect of temperature.

A medium electrophoretic cell with a tall center section was used throughout this investigation, and electrophoresis was continued usually until the distance of migration of the fastest boundary reached 6 cm. or more, as it was found that separation of boundaries was poor when the migration distance was shorter.

Specific electric conductivity of the solution was measured in a bath maintained at 0.0°C (or 25.0°C when electrophoresis was carried out at 25.0°C). Therefore, the electrophoretic mobility

1) N. Ui, *Biochim. Biophys. Acta*, in press.
2) N. Ui, This Bulletin, **30**, 801 (1957).
3) J. Grégoire and M. Limozin, *Bull. Soc. Chim. Biol.*, **36**, 15 (1954).
4) J. A. V. Butler, P. F. Davison, D. W. F. James and K. V. Shooter, *Biochim. Biophys. Acta*, **13**, 224 (1954).
5) P. F. Davison, D. W. F. James, K. V. Shooter and J. A. V. Butler, ibid., **15**, 415 (1954).
6) H. J. Gruft, C. M. Mauritzen and E. Stedman, *Nature*, **174**, 580 (1954).
7) J. M. Luck, H. A. Cook, N. T. Eldredge, M. I. Haley, D. W. Kupke and P. S. Rasmussen, *Arch. Biochem. Biophys.*, **65**, 449 (1956).
8) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).
9) J. St., L. Philpot, *Nature*, **141**, 283 (1938); H. Svensson, *Kolloid-Z.*, **87**, 181 (1939).

(u) obtained was thought to be the value at 0.0°C (or 25.0°C).

The greatest difficulty encountered in this investigation was due to the fact that solutions of histone solubilized some kinds of water-insoluble material. Thus, when ground surfaces of flanged plates at each end of cell segments were greased as usual with vaseline or silicone grease (Dow Corning's high vacuum silicone grease), there occurred serious disturbance in initial boundaries formed between the protein solution and buffer at the beginning of the experiments. The difficulty was overcome by using silicone oil with high viscosity (Shin-Etsu Silicone Oil KF 96, 100,000 cs.). The author is indebted to Dr. K. Hayashi, Shin-etsu Chemical Co., for his kind supply of various preparations of silicone oil.

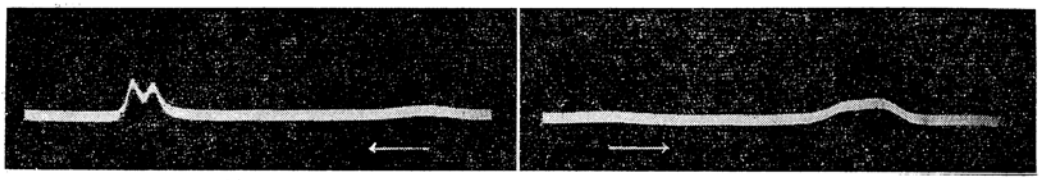
Results and Discussion

A. Electrophoretic Behavior of Histone I at pH 5.0.—The preparation of histone I was examined by electrophoresis in acetate buffers of pH 5.0 at various ionic strengths. The protein concentration was almost at one per cent. It was found that at least three components with similar electrophoretic mobilities migrated towards the cathodes, although separation was poor at higher ionic strength. Some electrophoretic patterns are shown in Fig. 1 (A—C).

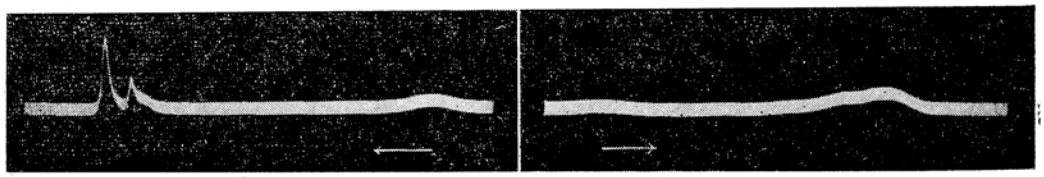
It was also noticed that the electrophoretic composition varied markedly with change in ionic strength. The apparent relative concentrations of the fastest migrating component computed from the ascending pattern was 48 per cent. at ionic strength of 0.2, while at ionic strength of 0.05 a much higher value, 73 per cent., was obtained. Slightly lower, but essentially the same relative concentration was obtained from the corresponding descending pattern, although the value was not accurate owing to the broadness and overlapping of the boundaries.

A similar change in apparent relative concentrations was also observed when the protein concentration was lowered from one to 0.5 or 0.3 per cent. at constant ionic strength of 0.1.

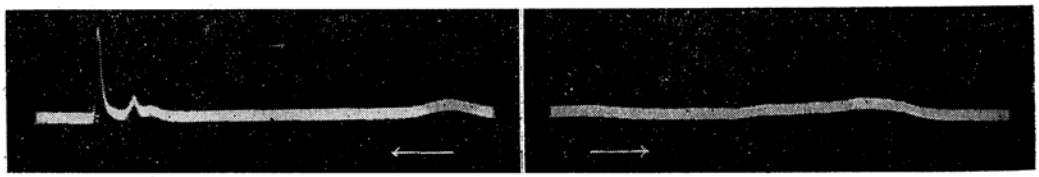
As the change in electrophoretic patterns with change in ionic strength or protein concentration was astonishingly great, it was felt, at first, that more than



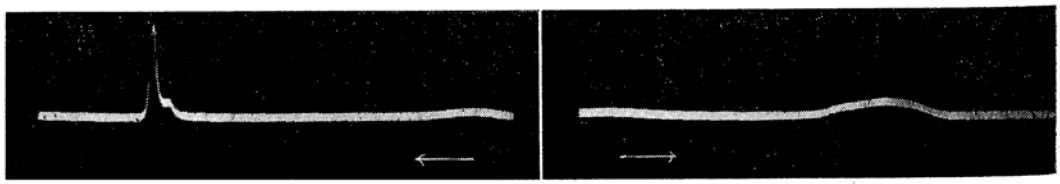
(A) : In acetate buffer of ionic strength 0.2.



(B) : In acetate buffer of ionic strength 0.1.



(C) : In acetate buffer of ionic strength 0.05.



(D) : In acetate—Na_2SO_4 (5/300 M) buffer ionic strength 0.1.

Fig. 1. Electrophoretic patterns of histone I at pH 5.0 (0°C). Ascending patterns on the left and descending patterns on the right.

one form of the protein existed in solutions and the state of equilibrium was sensitive to ionic strength or protein concentration. However, this view was soon found to be incorrect. When the deviation of apparent composition from the true composition was estimated with the aid of the moving-boundary theory developed by Dole[10], it was found that the apparently abnormal phenomenon could be accounted for on the basis of the so-called boundary anomalies[11], which were greatly exaggerated in the case of histone I solution; large errors in the apparent analysis would be made in the case of a mixture with nearly the same mobilities[12] as encountered in the present experiments, since the contribution of superimposed gradients of buffer ions and foreign proteins becomes great in such a system.

With the aid of the Dole's equation[10], change in the theoretical, apparent composition with change in ratio of protein concentration to ionic strength (c/μ)* was calculated for hypothetical mixtures of two components with the relative mobilities (r) of 0.28 and 0.26 which corresponded to the estimated mobility values of the two main components (faster components) of histone I. The net positive charge on each component was assumed to be 27 or 25 per molecule with a molecular weight of 37,000**. In addition, it was assumed, for the sake of simplicity, that the solution contained only sodium ($r=1.00$) and acetate ($r=-0.79$) ions besides the proteins. In so far as it is permissible to neglect the minor, third component in histone I observed in the case of lower ionic strength, such a solution would be a simplified system representing a histone I solution at pH 5.0. The refractive-index increment of each constituent in the solution was appropriately assumed.

The results of calculations for mixtures containing 30 and 40 per cent. of the fastest component are shown in Fig. 2. It is clear that the dependency of the apparent concentration upon the ratio of protein concentration to ionic strength is very great. In spite of many assumptions

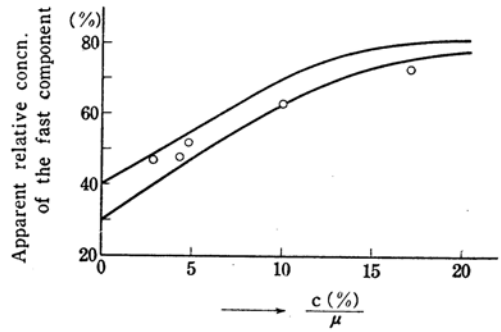


Fig. 2. Variation of apparent relative concentration of the fastest component of histone I with the ratio of protein concentration to ionic strength.

involved in theory and calculation, the theoretical curve for the solution containing 30 or 40 per cent. of the faster component fitted fairly well with the experimental points, which are represented as circles in this figure. Thus, the heterogeneity of histone I was confirmed and the true percentage of the fastest component was estimated to be 30—40 per cent. of the total proteins.

As might be expected from the results described above, it was shown that exposure of the solution to high ionic strength had no effect; when a 2 per cent. solution in the acetate buffer of ionic strength 0.2 (pH 5.0) was kept at 0°C for one day and subjected to electrophoresis without dialysis after being diluted with the same volume of distilled water, the electrophoretic pattern was found to be nearly the same as that obtained at ionic strength of 0.1.

A small variation in the electrophoretic composition of components was found with different preparations of histone I. It was thought that different procedures for extraction had caused this variation.

In Table I are shown the electrophoretic mobility data of histone I at pH 5.0. It should be noticed that the values, except those of the fastest component calculated from descending patterns, are only apparent ones, since the specific conductivity of

10) V. P. Dole, *J. Am. Chem. Soc.*, **67**, 1119 (1945).
11) L. G. Longsworth, *J. Phys. & Colloid Chem.*, **51**, 171 (1947).
12) C. Hoch-Ligeti and H. Hoch, *Biochem. J.*, **43**, 556 (1948).

* An apparent composition will agree with a true composition when the ratio of protein concentration to ionic strength becomes equal to zero.

** The net charge on either of two main components of histone I was calculated from its electrophoretic mobility by using Gorin's equation[13] for a long cylinder. Taking the results of sedimentation and diffusion studies[2] into consideration, a cylinder of 7.1 Å in diameter and 290 Å in length was assumed for both of the components. However, as it seems unlikely that the molecules are so elongated (see discussion in a previous paper[2]), the values of charges thus obtained would be only tentative. Calculated from amino acid composition using the data of Daly and Mirsky[14] on their arginine-rich histone, the valency was 57 at pH 5.0 and was almost twice as high as that adopted in the present study.

13) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," New York (1942).
14) M. M. Daly and A. E. Mirsky, *J. Gen. Physiol.*, **38**, 405 (1955).

Table I
Electrophoretic mobility data on histone I at 0°C in acetate buffers of pH 5.0

Ionic strength	Concn. (%)		$u \times 10^5$ cm²/sec. volt Comp. 1	Comp. 2	Comp .3
0.2	0.90	Des.	6.4	5.8	—
		As.	7.0	6.6	—
0.1	1.00	Des.	8.0	6.9	—
		As.	9.1	8.4	5.6
0.1	0.51	Des.	8.3	7.5	6.0
		As.	9.0	8.1	7.7
0.1	0.29	Des.	8.5	7.7	6.1
		As.	8.9	7.9	7.3
0.05	0.85	Des.	9.4	7.6	5.9
		As.	11.5	10.4	9.9

protein solutions was used for these calculations.

B. Effect of Sulfate Ion on the Electrophoretic Behavior of Histone I.—It has been found[15] that the aggregation of histone I was greatly accelerated when histone I sulfate was used or when the sulfate ion was added to the solution. Therefore, the effect of the sulfate ion upon electrophoretic behavior of histone I was studied, using acetate buffers of pH 5.0 containing sodium sulfate.

As shown in Table II, it was found that electrophoretic mobility markedly decreased when the sulfate ion was present. When sodium sulfate was added to one-half of the total ionic strength of 0.1, the mobility was about two-thirds of the value

Table II
Electrophoretic mobility data on histone I at 0°C in acetate buffers containing sodium sulfate

Ionic strength	Concn. of sodium sulfate		$u \times 10^5$ cm²/sec. volt Comp. 1	Comp. 2
0.2	1/30 M	Des.	4.3	3.9
		As.	4.9	4.6
0.1	5/300 M	Des.	5.6	4.7
		As.	6.5	6.2

in the absence of the sulfate ion. Perhaps, it would be due to the fact that a fairly large number of sulfate ions is bound to the proteins. Positively charged groups, such as guanidinium and ε-amino groups would be responsible for this interaction.

The effect of the sulfate ion on the electrophoretic composition was also observed. In accordance with the observations described in section **A**, the apparent concentration of the fastest component increased with the decrease in ionic strength, but, when compared at the same ionic strength, the value was higher than that obtained in the sulfate ion-free buffer (see Fig. 1D). The fact was again accounted for on the basis of boundary anomalies, since it was shown by the moving-boundary equation that the presence of bivalent sulfate ions accentuates the anomaly.

C. Effect of Aggregation Reaction on the Electrophoretic Behavior of Histone I.—As was shown in a previous paper[15], a component with a slightly higher mobility appeared when a solution of histone I formed an aggregate (see Fig. 9 in a previous paper[15]). Although the amount of the newly formed component could not be estimated owing to its poor separation from the fastest component in the original solution, this component was thought to correspond to the aggregation product as seen in sedimentation patterns. Although Davison, James, Shooter and Butler[5] reported that calf thymus histone contained two electrophoretically distinguishable components and that the third component appeared when the aggregation took place, it seemed that none of the boundaries described in **A** or **B** (see Fig. 1) was due to aggregation.

D. Electrophoretic Behavior of Histone I at 25°C.—To see the effect of temperature on the electrophoretic behavior of histone I, a series of experiments were carried out at 25°C using solutions of histone I which had been dialyzed at 0°C. The solvents used were acetate buffers of pH 5.0 at ionic strength of 0.2, 0.1 and 0.05, and acetate-sodium sulfate buffer of the same pH and ionic strength of 0.1.

Since the solutions used in these experiments had not been exposed to a high temperature before the initiation of electrophoresis except that they were kept at 25°C for a short period in the cell to establish a temperature equilibrium, no appreciable aggregation occurred. Only a small amount of aggregate was found in the case of solution at ionic strength of 0.2 (4 per cent.) or containing sodium sulfate (5 per cent.).

The electrophoretic patterns obtained are shown in Fig. 3. When they are compared with those obtained at 0°C (see Fig. 1), it is clear that they differ definitely at a higher ionic strength or in the presence of sulfate ions, while the difference was not large at a lower ionic strength. As these temperature dependencies could not entirely be interpreted by the spreading of boundaries due to diffusion, it was thought more likely that some changes in

15) N. Ui, This Bulletin, **30**, 806 (1957).

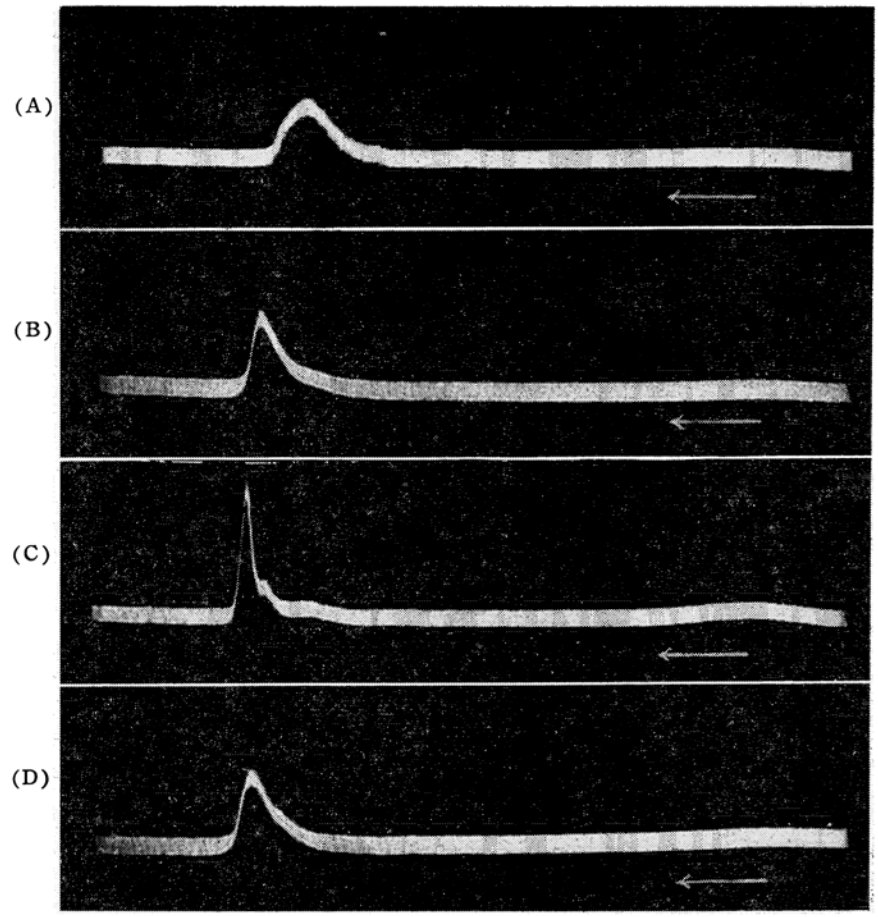


Fig. 3. Electrophoretic patterns of histone I at 25°C.
(A)—(C) : In acetate buffers of pH 5.0 at ionic strengths of 0.2, 0.1 and 0.05.
(D) : In acetate-Na_2SO_4(2/300 M) buffer of pH 5.0 at ionic strength of 0.1.
Only ascending patterns are shown.

the proteins occurred with change in temperature. However, it must also be considered that the aggregation might proceed, if not considerably, during the electrophoresis. Therefore, other interpretations might also be possible.

In other experiments, solutions of histone I aged and dialyzed at 25°C were subjected to electrophoresis. With these solutions which contained aggregate, appearance of a new component with higher mobility than that of any original component of histone I was always observed. The results agreed with the observation at 0°C described in **C**.

E. Electrophoretic Behavior of Histone I at pH's other than 5.0.—Effect of pH on the electrophoretic behavior of histone I was studied at 0°C using buffers* of various pH's at a constant ionic strength of 0.1. Although the solutions used had not been exposed to a high temperature, the aggregation product was present in the cases of alkaline solutions.

By this study, it was found that at least two components were present and they always moved toward the cathode in a range of pH's from 4.0 to 10.3. Some of the patterns are shown in Fig. 4.

Electrophoretic mobilities of the two main components (the fastest and the second fastest components) of this preparation calculated from descending patterns are plotted against pH in Fig. 5. The values decreased slowly with increase in pH below pH 9 and decreased rapidly at a higher pH region. Even at pH 10.3, however, each boundary migrated toward the cathode fairly rapidly. Although the

* The following buffers were used in this study : pH 4.0 and 5.0, acetate buffer ; pH 6.5, phosphate buffer ; pH 6.8, cacodylate or cacodylate-NaCl buffer ; pH 8.7, tris (hydroxymethyl)aminomethane buffer ; pH 9.3, veronal or veronal-NaCl buffer ; pH 10.0 and 10.3, NH_4Cl-NH_3 buffer.

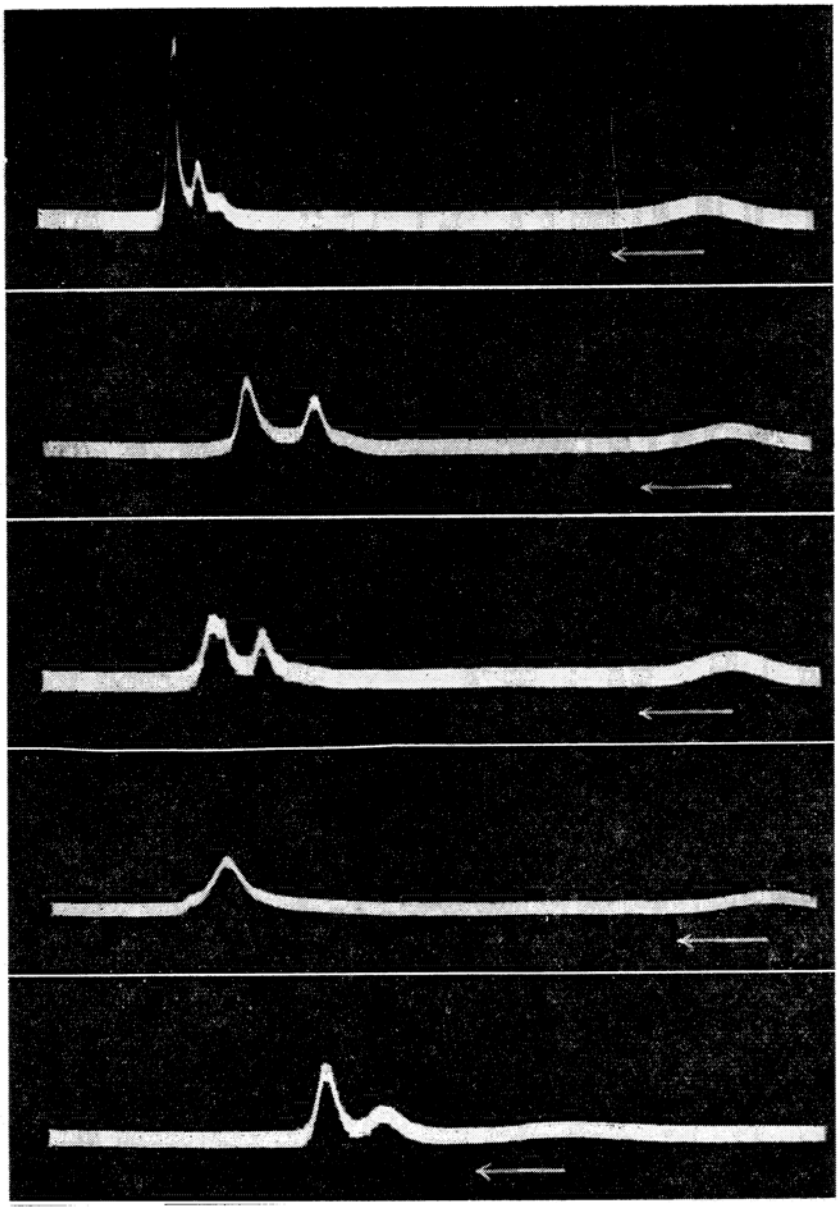


Fig. 4. Electrophoretic patterns of histone I at various pH's (0°C). The pH's are from top to bottom 4.0, 6.5, 6.8, 8.0 and 10.3, respectively. Ionic strength=0.1. Protein concn.=1%. Only ascending patterns are shown.

isoelectric point of each component could not be determined owing to the insolubility of this preparation in more alkaline solution, it was thought from the shape of the pH-mobility curve that the value was higher than pH 10.5.

At ionic strength of 0.2, similar patterns were obtained, but separation of boundaries was poorer in agreement with the results at pH 5.0.

F. Electrophoretic Behavior of Histone II.—The other fraction of calf thymus histone, histone II, was subjected to electrophoresis in acetate buffers of pH 5.0 at 0°C. As shown in Fig. 6 (A and B), one main and three minor components migrated toward the cathode. Unlike histone I, the apparent concentration of the fastest component did not vary appreciably with change in ionic strength; the values of relative concentration calculated from descending and ascending patterns were 63 and 62 per cent. at ionic strength of 0.2, and 62 and 65 per cent. at ionic strength

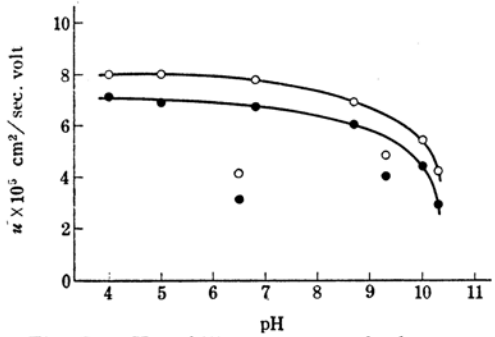


Fig. 5. pH-mobility curves of the two main components of histone I at ionic strength of 0.1 (0°C).

of 0.1. The independency of apparent concentration upon ionic strength would be due to the fact that a large mobility difference exists among the components of histone II.

The electrophoretic mobility data of histone II are shown in Table III. The mobility of the main component was even much higher than that of the fastest component in histone I. It was also found that the preparation of histone II was not contaminated with any component of histone I, since the electrophoretic mobility of none of the components of histone II agreed with the values obtained with histone I. The fact was also confirmed by an electrophoretic experiment on a mixture of histones I and II.

The isoelectric point of any of the components of histone II was found to be higher than pH 10.0, since none of the boundaries migrated toward the anode at this pH (see Fig. 6 and Table III).

Summary

Electrophoretic studies were made on histone I, the main fraction of calf thymus histone, at various ionic strengths, pH's and temperatures. It was found that at least three components are present in this fraction.

Histone II, the other fraction, was electrophoretically not homogeneous either. One main and three minor components were found to exist, and their mobilities differed from that of any component of histone I.

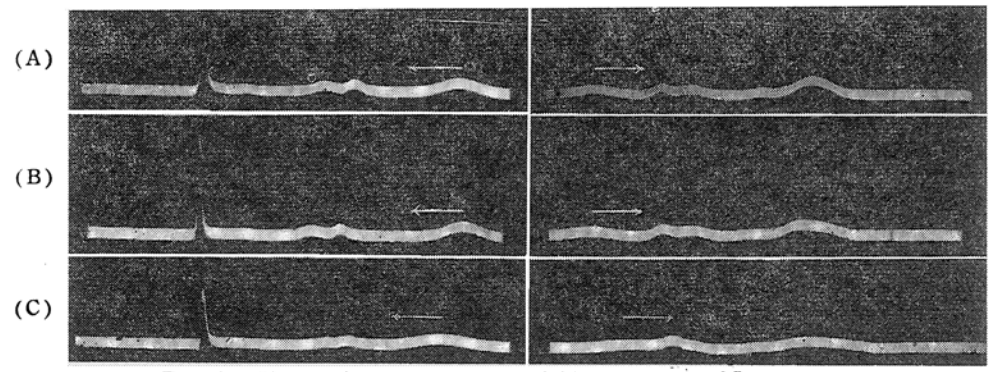


Fig. 6. Electrophoretic patterns of histone II at 0°C.
(A): In acetate buffer of pH 5.0 at ionic strength of 0.2.
(B): In acetate buffer of pH 5.0 at ionic strength of 0.1.
(C): In NH_4Cl-NH_3 buffer of pH 10.0 at ionic strength of 0.1.

TABLE III
ELECTROPHORETIC MOBILITY DATA ON HISTONE II AT 0°C*

Buffer	pH	Ionic strength		$u \times 10^5$ cm²/sec. volt			
				Comp. 1	Comp. 2	Comp. 3	Comp. 4
Acetate	5.0	0.2	Des.	**8.3**	6.5	3.9	2.7
			As.	**9.3**	7.9	4.9	3.9
Acetate	5.0	0.1	Des.	**10.1**	7.7	4.7	3.3
			As.	**12.2**	10.3	6.9	5.5
NH_4Cl-NH_3	10.0	0.1	Des.	**9.0**	6.5	3.7	2.0
			As.	**10.3**	9.1	4.9	3.9

* The values of the main component are shown in bold type.

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Institute of Science and Technology
The University of Tokyo
Meguro, Tokyo