Further Studies on the Preparation of Calf Thymus Histone

By Nobuo Ui

(Received May 31, 1957)

In a previous paper[1], it was reported that calf thymus histone, which had been extracted with sulfuric acid from the isolated nuclei and precipited by addition of ethanol at a low temperature, showed the presence of two fractions (histones I and II). In spite of their ultracentrifugal homogeneity, their electrophoretic patterns were complex and it was concluded that calf thymus histone consists of several components. However, as acids often caused irreversible modifications of many proteins, it was thought necessary to prove that no changes had occurred during the course of the extraction of histone with an acid.

In this investigation, histone was prepared by three methods without using acid, and their properties were compared with those of two preparations obtained by the acid and ethanol method[1].

Experimental and Results

(1) Preparation of Histone by Ultracentrifugation.—Calf thymus nucleohistone (TNH) is generally believed to dissociate into histone and desoxyribonucleic acid (DNA) in a concentrated saline solution[2]. In fact, when TNH was subjected to a prolonged high-speed centrifugation in concentrated saline, histone was left separate in the upper layer of the supernatant[3].

In this study, TNH* solutions both in 1 M and 2 M sodium chloride were used and different preparations of histone were obtained from the supernatants. Typical examples are as follows.

i) TNH was dissolved in 1 M sodium chloride in a concentration of about 0.4 per cent. and centrifuged at 42,040 r. p. m. in a Spinco model E ultracentrifuge using a preparative rotor B. After centrifugation for 6 hours at about 10°C, centrifuge tubes were removed as gently as possible. A sharp boundary was observed at the middle of the tube. The upper layer was withdrawn with a syringe without disturbing the boundary. It was dialyzed against distilled water and lyophilized after removing a small amount of insoluble material formed during the dialysis with a centrifuge. About 40 mg. of histone was obtained from 400 mg. of TNH.

ii) TNH solution in 2 M sodium chloride at a concentration of about 0.2 per cent. was centrifuged in the same apparatus, but a preparative rotor A was used this time and centrifuged at 50,740 r. p. m. for 5 hours. The temperature was nearly 15°C. Although no boundary was observed in this case, the upper half of the supernatant was found from the ultraviolet absorption curve to be free from nucleic acid. It was with-drawn with a syringe, dialyzed against cold distilled water and lyophilized. Twenty three mg. of histone was obtained from 160 mg. of TNH.

The preparations of histone thus obtained were essentially free from DNA and usually showed only one boundary in an ultracentrifuge. In an acetate buffer of pH 5.0 at ionic strength of 0.2, the sedimentation coefficient of the preparation obtained from the supernatant of 1 M

1) N. Ui, *Biochim. Biophys. Acta*, in press.

2) E. Chargaff, in E. Chargaff and J N. Davidson, "The Nucleic Acids," New York, Vol. 1, p. 307 (1955).

3) A. E Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).

* TNH was prepared by the following method. Fresh calf thymus glands, freed from fat and extraneous tissues, were frozen over solid carbon dioxide, minced, and homogenized in an electric mixer with 0 14 M NaCl (pH 7) at a temperature below 5°C. The homogenate was then centrifuged for about ten minutes at 4,000 r. p. m. The sediment was resuspended in 0.14 M NaCl, placed in the mixer for a few minutes and centrifuged as before. The same washing was repeated three times more.

Cold 1 M NaCl was added to the sediment and the mixture was stirred overnight in a cold room. The solution was then clarified by centrifugation for 2 hours at 20,000 r. p m. The clean but slightly opalescent supernatant was poured into six volumes of cold distilled water and a fibrous precipitate of TNH was obtained. The precipitate was washed twice with 0.14 M NaCl, and as much water as possible was pressed out of it. It was then briefly washed in water and lyophilized.

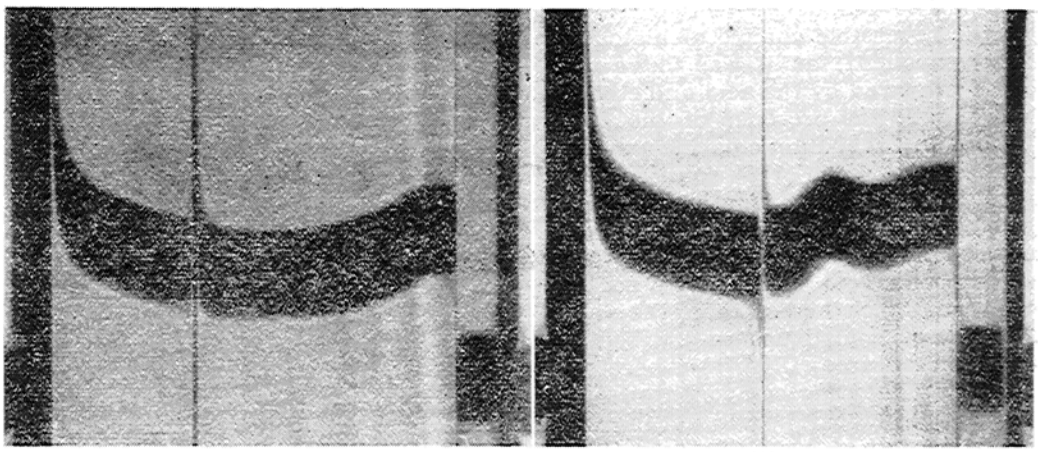
TABLE I

SEDIMENTATION COEFFICIENTS OF NUCLEOHISTONE AND SOME PREPARATIONS OF HISTONE

Sample	Solvent	Concn. (%)	$s_{20,w}$ (S) Fast	Middle	Slow
TNH	1 M NaCl	0.6	10.6	—	<1
〃	〃	0.5	11.2	—	<1
〃	2 M NaCl	0.8	4.9	3.4	<1
〃	〃	0.7	5.3	3.4	<1
Histone obtained by ultracentrifugation in 1 M NaCl	Acetate buffer pH 5.0, μ=0.2	0.7	—	—	0.7
Histone obtained by ultracentrifugation in 2 M NaCl	〃	1.0	—	1.4	?
Histone obtained by means of adsorption on Hyflo Super-Cel	〃	0.6	—	2.2	—
Histone obtained by using La salt	〃	0.7	—	1.8	?
Histone I*	〃	—**	—	2.0_3	—
Histone I*	2 M NaCl	1.0	—	3.5, 1.7	—
Histone I* once exposed to 2 M NaCl	Acetate buffer pH 5.0, μ=0.2	1.0	—	1.7	—
Histone II*	〃	—**	—	—	0.6

* These samples were prepared by the method described in a previous paper[1].

** The values were extrapolated to infinite dilution[1,4],



(A) (B)

Fig. 1. Sedimentation patterns of TNH.

(A) In 1 M NaCl after 66 min. at 59,780 r. p. m. (c=0.6%).

(B) In 1 M NaCl after 109 min. at 59,780 r. p. m. (c=0.8%).

sodium chloride solution was found to be 0.7 S and agreed with the value of histone II in previous papers[1,4] while the preparation from 2 M sodium chloride solution gave a value of 1.4 S, which was slightly lower than that of histone I (see Table I). In the case of latter preparations, the presence of a slower component was suspected from the shape of sedimentation pattern. The difference between these two kinds of preparation was also found in their solubility behavior; although the preparation from 2 M sodium chloride almost precipitated by the addition of ammonia or the same volume of 10% trichloroacetic acid, the solutions of the preparation obtained from 1 M sodium chloride remained clear on these additions. Judged from these results, the preparation from 2 M sodium chloride was thought to correspond

4) N. Ui, This Bulletin, **30**, 801 (1957).

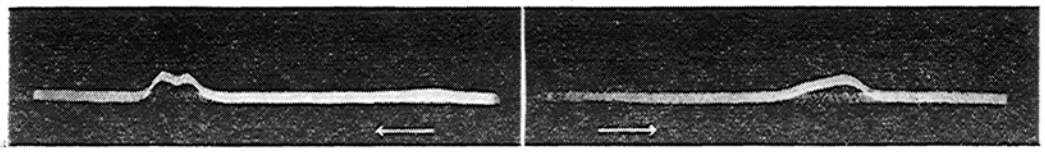


Fig. 2. Electrophoretic pattern of a histone preparation obtained by means of adsorption on Hyflo Super-Cel. Solvent: an acetate buffer of pH 5.0 at ionic strength of 0.2. Ascending pattern on left and descending pattern on right.

to a mixture of histones I and II, while the preparation from 1 M sodium chloride corresponded to histone II.

The fact that the proteins left in the upper layer of the fluid differed with the change in saline concentration, was also and more directly shown by sedimentation study of TNH. The sedimentation patterns of TNH in 1 M and 2 M sodium chloride (pH 6.3) are shown in Fig. 1. It is clear that, besides a sharp, faster boundary, one slower boundary exists in 1 M sodium chloride, and two in 2 M sodium chloride. The sedimentation coefficient of the slowest components in each case was below 1 S. A 3.4-S component was found only in the case of 2 M sodium chloride (see Table I). This component would be due to the reversible change of histone I, the 2-S component, as it was found that histone I prepared by the acid and ethanol method showed the presence of the 3.4-S component in 2 M sodium chloride and that it disappeared when the solution was brought to an ionic strength of 0.2 by dialysis.

The sedimentation coefficient of the faster component of TNH in 1 M sodium chloride was much higher than the value of the fastest component in 2 M sodium chloride or of free DNA[5]. Therefore, the faster sedimenting component of TNH in 1 M sodium chloride would not be free nucleic acid but a partially dissociated nucleohistone.

(2) Preparation of Histone by Means of Adsorption.—As histone is adsorbed on Hyflo Super-Cel from TNH solution in concentrated saline[6], an attempt was made to isolate histone by elution of the adsorbent.

TNH was extracted from the cell nucei with 1 M sodium chloride. To the clarified extract, solid sodium chloride was added to saturation. The solution became milky and a small amount of precipitate was formed. After being kept in the cold, it was filtered with suction using Hyflo Super-Cel on a Buchner funnel. The filter cake containing histone was thus obtained*.

After being washed several times with saturated saline, the filter cake was mixed with a large volume of distilled water. Then the suspension was centrifuged and the supernatant was dialyzed against distilled water. After clarification by a centrifuge, it was lyophilized. About 400 mg. of histone was obtained from 200 g. of the glands.

The preparation of histone thus obtained contained less than 0.1 per cent. of phosphorus, so the contamination of DNA in this preparation was thought to be negligible. In an acetate buffer of pH 5.0 at ionic strength of 0.2, it showed only one sedimentation boundary and the sedimentation coefficient at a concentration of 0.6 per cent. was found to be 2.2 S. The electrophoretic pattern of this preparation in the same buffer is shown in Fig. 2. It closely resembled the pattern of histone I reported previously[1,7]. It could be precipitated by addition of ammonia or trichloroacetic acid (5 per cent.). Therefore, according to our designation, it corresponds to histone I.

Thus, only one fraction of histones, histone I, was obtained by this method and the other fraction, histone II, was not. It might be due to the failure of this histone II fraction to be retained on the filter cake under the condition mentioned above.

(3) Preparation of Histone Using Lanthanum Salt.—Lanthanum salt is known as a powerful precipitant of DNA[2]. Therefore, an attempt was made to isolate histone from TNH using lanthanum acetate.

To 100 mg. of purified TNH described before (see method (1)), 400 ml. of 2 M sodium chloride containing 3% lanthanum acetate was added and the mixture was kept in a cold room with occasional stirring. TNH swelled slowly and histone was released from it. After two days this suspension was centrifuged. The same extraction was repeated once more. The two extracts were

5) Y. Kawade, *Biochem. Biophys. Acta*, in press.
6) N. Schwander and R. Singer, *Helv. Chim. Acta*, **33**, 1521 (1950).

* This filter cake was kindly supplied by Dr. Y. Kawade
7) N. Ui, This Bulletin, **30**, 815 (1957).

combined and, after clarification, dialyzed against distilled water and lyophilyzed. Yield, 30 mg.

When the preparation of histone obtained was subjected to ultracentrifugation in an acetate buffer of pH 5.0 at ionic strength of 0.2, only one boundary appeared. The sedimentation coefficient varied a little with the preparations i. e., 1.5—1.8 S. As the sedimentation pattern did not give a symmetrical peak, it was thought that this preparation also contained a slower component. When ammonia or the same volume of 10 per cent. trichloroacetic acid was added to its solution, a precipitate was formed, but part of the proteins remained in the solution. Therefore, this preparation was considered to be a mixture of histone I and histone II.

When TNH was extracted with the solutions of lower ionic strengths, a smaller amount of histone was extracted, presumably owing to the insufficient dissociation of TNH. In the case of 1 M sodium chloride—3% lanthanum acetate a fairly large amount of histone remained in the residue and could be extracted with 0.5 N sulfuric acid. An extract with 0.5 M sodium chloride—1.5% lanthanum did not give a precipitate in 5 per cent. trichloroacetic acid, and contained only a fraction corresponding to histone II.

Copper sulfate could be substituted for lanthanum acetate and a similar preparation was obtained.

Discussion

The three methods for the preparation of histone described above are milder than those usually adopted; neither acid, alkali nor organic solvent was used in these methods. Therefore, these preparations would be least likely to be modified during the purification. Although degradation of histone by the action of proteolytic enzyme at neutral pH was suggested by Butler et al.[8], the experiments presented here have failed to reveal it.

It was shown by the examination of these preparations that histone contained two fractions with different molecular weights. The lower molecular-weight fraction was isolated by the high-speed centrifugation experiments on TNH in 1 M sodium chloride, while the higher molecular-weight fraction was obtained by the other methods including the centrifugation in 2 M sodium chloride. Sedimentation as well as solubility studies indicated that the former corresponded to histone II described in a previous paper[1] and the latter to histone I. It was also found that the higher molecular-weight fraction was electrophoretically not homogeous.

From these results it was confirmed that the preparations of histone obtained by the standard method adopted by us[1] were not altered with acid or ethanol. The view that calf thymus histone consists of several components was also ascertained. In view of these findings, the previously reported method using acid and ethanol will be recommended, since it is easy to operate and gives a good yield.

Recently, evidences[5,9,10] were accumulated indicating that the progressive dissociation of TNH into components is induced by increasing concentrations of sodium chloride. It was also shown that dissociation was not complete in 1 M sodium chloride[5,9-11]. Failue of histone I, the higher molecular-weight fraction of histone, to be obtained by the centrifugation experiments in 1 M sodium chloride would be due to the binding of this fraction with DNA under this condition; lower molecular-weight fraction, histone II, was the only fraction present as the free protein in 1 M sodium chloride. In fact, it was shown[5] by the measurements of viscosity and sedimentation that histone I interacted with DNA in 1 M sodium chloride, while histone II did not.

Summary

Calf thymus histone was prepared as gently as possible without using acids, alkalis or organic solvents. It was clearly shown that two components with sedimentation coefficients of 2 S and 0.7 S are present.

Sedimentation, electrophoresis and solubility behaviors of these preparations were similar to those of the preparations obtained by the previously reported method using acid and ethanol, and it was confirmed that histone consists of two fractions, histones I and II. It was also ascertained that acid as well as ethanol causes no alteration of the proteins.

8) J. A. V. Butler, P. F. Davison, D. W. F. Jamse and K. V. Shooter, *Biochim. Biophys. Acta*, **13**, 224 (1954).

9) C. F. Crampton, R. Lipshitz and E. Chargaff, *J. Biol. Chem.*, **206**, 499 (1954).

10) L. B. Smillie, A. M. Marko and G. C. Butler, *Can. J. Biochem Physiol.*, **33**, 263 (1955).

11) M. Fleming anb D. O. Jordan, *Discussions Faraday Soc.*, No. **13**, 580 (1953).

The author wishes to express his sincere thanks to Professor Itaru Watanabe for his valuable suggestion and encouragement. His thanks are also due to Professors S. Sasaki and O. Taniguchi of Tokyo Institute of Technology for their kind permission to use their ultracentrifuge. The expense of this research was partly defrayed from a Grant in Aid for Scientific Research from the Ministry of Education.

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