PREPARATION, FRACTIONATION AND PROPERTIES OF CALF THYMUS HISTONE

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It is well known that histone is a main constituent protein of cell nuclei in most animal tissues[1]. In the cell, this characteristic, basic protein is present together with deoxyribonucleic acid, presumably in the form of a complex known as nucleohistone. In view of the possible role of deoxyribonucleoprotein in the multiplication of cells and in protein synthesis, it will no doubt be of value to study the nature of its protein component.

In recent years, evidence has accumulated indicating that calf thymus histone is not a homogeneous protein. AHLSTRÖM[2] reported that his preparation was not homogeneous with respect to molecular weight; part of the preparation was dialyzable through a cellophane membrane and the rest when subjected to ultracentrifugation showed two components. Variations in amino acid composition of histone preparations isolated from the same tissue by different procedures have been pointed out by several investigators[3-7], and it was suggested that more than one kind of histone, each being different in amino acid composition, is present in thymus glands. Heterogeneity of histone has also been indicated by electrophoresis[6,8-13], sedimentation[9-11,13,14] and ion exchange chromatography[15]. Many efforts have been made to fractionate calf thymus histone.

While these observations clearly show the heterogeneity of histone, there are some discrepancies between the data obtained by several investigators. Some of these may be attributable to differences in the isolation procedures and the methods employed for the characterization. However, there is a possibility that the properties of histone preparations change during the course of isolation. A tendency of histone to aggregate has been reported by CRUFT, MAURITZEN AND STEDMAN[12] and by the present author[16]. BUTLER, DAVISON, JAMES AND SHOOTER[9] have reported that the preparation extracted at neutral pH was considerably degraded probably by the action of intra-nuclear cathepsin.

The purpose of this communication is to describe the most satisfactory and simple procedure now adopted by us for the purification and fractionation of calf thymus histone. Some properties of the fractions are also described.

EXPERIMENTAL AND RESULTS

Preparation and fractionation of histone

In this study, histone was fractionally extracted from isolated calf thymus nuclei, with sulfuric acid of different concentrations, and precipitated by addition of ethanol

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to the extracts. Taking advantage of solubility differences in ethanol–water mixtures, a fractionation of histone sulfate into two fractions was easiy achieved. Each fraction was then converted from the sulfate to the chloride before storage, because it was found that the presence of sulfate ion accelerated the aggregation of histone. The method used for purification is described below. All operations were carried out near o° C, except the precipitation by ethanol which was effected below o° C.

(i) *Purification of cell nuclei.* Fresh calf thymus glands were obtained from a slaughter-house. Cell nuclei were separated from cytoplasmic constituents as thoroughly as possible by the citric acid method of MIRSKY AND POLLISTER[17]. The nuclei appeared clean when examined microscopically.

(ii) *Extraction of histone.* The preparation of nuclei obtained was then extracted with acid. Sulfuric acid was the most suitable when ethanol was used as a precipitant of histone. Hydrochloric acid was not so suitable, because a large excess of ethanol was needed to precipitate histone chloride. It was found, in the preliminary experiments using nucleohistone, that a larger part of histone could be extracted with 0.2 *N* sulfuric acid, while 0.1 *N* acid was sufficient to extract its lower molecular-weight fraction. Nearly complete extraction was achieved with 0.5 *N* sulfuric acid. Therefore, extraction was carried out successively using sulfuric acid of increasing concentrations as is shown in the following example.

A suspension of purified thymus nuclei, which had been obtained from 1.15 kg of thymus glands, was centrifuged and the supernatant was discarded. In order to extract histone with 0.1 *N* sulfuric acid from the wet preparation of nuclei, a slightly higher concentration of acid than 0.1 *N* was used in the first extraction; *i.e.*, 800 ml of 0.13 *N* sulfuric acid was added to the wet nuclei and allowed to stand for 20 min with occasional stirring. The suspension was then centrifuged at 4,000 r.p.m. for 5 min. The supernatant (1.17 l) was slightly opalescent and it was found to contain 0.93 g of nitrogen. The residue was then extracted with 300 ml of 0.1 *N* sulfuric acid for 20 min, and centrifuged as before. This time 450 ml of extract was obtained, which contained 0.56 g of nitrogen. Both extracts (0.1 *N*) were combined and clarified again by centrifugation at 8,000 r.p.m. for 15 min. This was designated as Extract A.

The residue of the extraction with 0.1 *N* sulfuric acid was then extracted with 500 ml of 0.2 *N* sulfuric acid. After 20 min, the suspension was centrifuged. Extraction was repeated thrice more using a total of 900 ml of 0.2 *N* sulfuric acid. The concentration of nitrogen in each supernatant decreased gradually as extraction was repeated. Each extract was clarified by centrifugation at 8,000 r.p.m. for 15 min and combined (Extract B). About 2.66 g of nitrogen was found in 1.53 l of supernatant.

Further extractions were carried out three times using a total of 1.2 l of 0.5 *N* sulfuric acid. In the first two extractions the suspension was allowed to stand for an hour, while the last extraction was continued overnight. 0.90 g of nitrogen was obtained (Extract C).

These extracts (Extracts A, B and C) contained a total of 5.05 g of nitrogen, which corresponded to nearly 26 g of histone. Further extraction was not attempted, although there remained a small amount of nitrogen extractable by the acid.

Extract A differed markedly from Extracts B and C. When ammonia was added, precipitation occurred in Extracts B and C, while in Extract A the solution remained almost clear. Judging from the ultracentrifugal patterns, too, Extracts A and B were clearly different, while a difference between B and C was not clear by this test.

(iii) *Precipitation and fractionation.* To a cooled Extract A (1.6 l), chilled ethanol was added slowly. During the addition the temperature was kept as low as possible but without falling below the freezing point. When the solution was brought to an ethanol concentration of 20 % by volume (400 ml of absolute ethanol was added), it became turbid and a precipitate began to form. The mixture was allowed to stand at —5° C for three hours in order to complete formation of the precipitate, which was then separated by a refrigerated centrifugation (histone I, see below). Ethanol was added slowly to the supernatant until the ethanol concentration was 45 % by volume, when the solution became turbid and precipitation occurred gradually. After standing for a few

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hours at —10° C, the precipitate was separated as before (histone II). The supernatant did not contain any more histone fractions.

Each fraction was dissolved in cold distilled water, clarified by centrifugation at 8,000 r.p.m. for 30 min, and then reprecipitated twice more. The final precipitate was dissolved in distilled water and sulfate ions were removed by addition of 0.2 *M* barium chloride. Precipitated barium sulfate was separated by centrifugation next morning, the supernatant was thoroughly dialyzed against cold distilled water and lyophilized (yield: 2.6 g of histone I and 1.2 g of histone II).

Extract B (1.53 l) was brought to an ethanol concentration of 20% at −5° C. After standing overnight, the precipitate formed was collected by centrifugation. The supernatant was discarded, because only a small amount of precipitate formed by further addition of ethanol.

The precipitate formed by 20% ethanol was dissolved in distilled water and reprecipitated twice more after clarification. It was converted to the chloride, dialyzed and lyophilized as before (histone I). This preparation consisted of nearly half of the histone in the total extracts (12.0 g).

Extract C (1.2 l) was treated the same as Extract B. A fraction which precipitated only at ethanol concentration of 20% was obtained (histone I) (yield: 2.8 g).

It was evident from this result that calf thymus histone contained at least two fractions of different solubility in ethanol–water mixture. Extract A contained two fractions, while Extracts B and C contained only one. As fractions precipitated by 20% ethanol were found to be similar to each other in ultracentrifugal behaviour, they were tentatively designated as "histone I" whether they were obtained from Extract A, B or C. The fraction precipitated from Extract A by 45% ethanol was designated as "histone II"*.

In this case the amount of histone I was more than 90% of the total products obtained, but, taking into consideration a fairly large loss of histone II during the reprecipitation, the content of histone I was thought to be 80–90% of the total histone**.

Homogeneity and some properties of histone fractions

The homogeneity and properties of these two histone fractions (histones I*** and II) were studied by various methods. Differences between the two fractions were clearly shown.

(i) *Sedimentation.* The sedimentation experiments were carried out in an acetate buffer of pH 5.0, ionic strength 0.2, using a Spinco model E ultracentrifuge at 59,780 r.p.m. In the case of histone II a synthetic boundary cell[18] was used to determine its low sedimentation coefficient.

Ultracentrifugal patterns of histones I and II (see Fig. 1 of the previous paper[16]) showed that both fractions were homogeneous. The preparations of histone I obtained from Extract A, B and C, could not be distinguished from each other. Sedimentation constants ($s_{20,w}$) of histones I and II at infinite dilution are shown in Table I.

It has often been reported[2,9-11,13,14,19] that histone contains a fraction with a much higher sedimentation coefficient than 2 *S*. In fact, a small amount of 5-10 *S*

* Histone II could be separated from histone I merely by fractional precipitation with ethanol from the extract obtained from the nuclei with acid of higher concentration than 0.2 *N*, although, in this study, histone II was initially separated from a large portion of histone I by extraction with 0.1 *N* sulfuric acid in order to facilitate subsequent fractionation.

** In one experiment, histone was extracted from isolated nuclei with 0.5 *N* sulfuric acid as thoroughly as possible and the same volume of 10% trichloroacetic acid was added to the extract. The mean value of the ratio of nitrogen precipitated by the addition of trichloroacetic acid, to the total nitrogen in the original extract was 0.85. Since histone I is precipitated by 5% trichloroacetic acid while histone II is not, the relative amount of histone I can be calculated. After correction for nitrogen content in both fractions, the content of histone I in the total histone was estimated to be 84%.

*** In the following investigations of histone I, the preparation obtained from Extract B was used unless otherwise stated.

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TABLE I

MOLECULAR KINETIC DATA OF HISTONES I AND II

	Histone I	*Histone II*
Sedimentation constant, $S_{20,w}$ (S)	2.0_3	0.6_6
Diffusion constant, $D_{20,w} \times 10^7$ cm²/sec	5.1_8	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

* Assumed.

component was sometimes observed in our experiments with histone I sulfate. It was also found that the faster boundary appeared and increased slowly with time when an ultracentrifugally homogeneous solution of histone I in the acetate buffer was kept at 30° C, and that sulfate ions accelerated the aggregation[16]. Therefore, it was concluded that such a component was not an independent component but an aggregation product. In an alkaline solution, histone I aggregated even if the solution was kept at low temperature.

Histone II, on the contrary, had no tendency to aggregate.

(ii) *Diffusion.* Diffusion measurements were carried out in an acetate buffer of pH 5.0, ionic strength 0.2, using a NEURATH-type diffusion cell[20] equipped with a PHILPOT-SVENSSON optical system[21].

Diffusion patterns obtained showed nearly Gaussian curves indicating that both fractions were fairly homogeneous. Diffusion constants at infinite dilution ($D_{20,w}$) calculated by the second moment method[22] were almost independent of concentration; the average values are shown in Table I.

(iii) *Molecular weights.* Combining the values of sedimentation and diffusion constants[23], molecular weights of histones I and II were calculated. The value of the partial specific volume, necessary for this calculation, was determined as 0.74 for histone I, and the same value was assumed in the case of histone II. It was clear that the molecular weight of histone I is much higher than that of histone II (see Table I)*.

(iv) *Electrophoresis.* Electrophoresis was carried out at 0° C in a TISELIUS-type apparatus[24] equipped with a PHILPOT-SVENSSON optical system[21]. A cell with tall center section[25] was used in order to obtain a better separation of components. Prior to electrophoresis, the solution of histone was dialyzed against a large quantity of buffer solution for two days in a cold room.

The preparation of histone I, which had been obtained from Extract B, was electrophoretically not homogeneous under all conditions examined. Electrophoretic patterns of this sample at 0° C in acetate buffer solutions of pH 5.0 at ionic strengths of 0.2, 0.1 and 0.05 are shown in Fig. 1. Separation of boundaries was better in the case of lower ionic strength; more than three components moved to the cathode at an ionic strength of 0.05, while only two were found at 0.2. Electrophoretic mobility values were not accurate, since the boundaries in the descending limb were very broad. In the acetate buffer at ionic strength 0.2, the mobility of the fastest component

* Although the molecular weight was as low as 8,400, histone II was not dialyzable through a cellophane membrane; it was found that only 1 % of the total protein nitrogen was lost during three days' dialysis.

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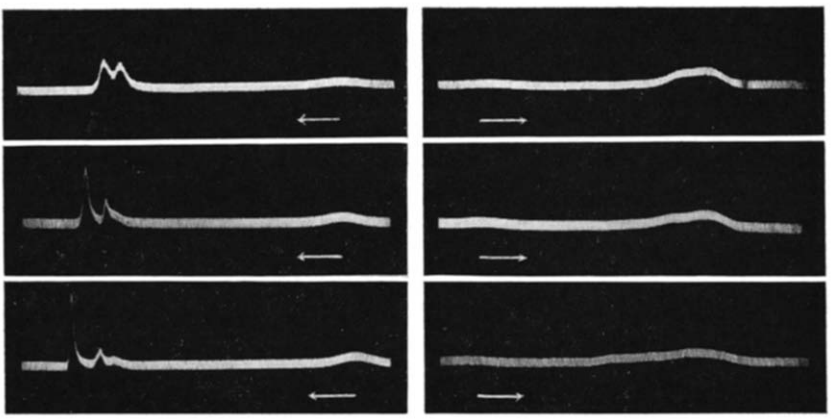

Fig. 1. Electrophoretic patterns of histone I (from Extract B) at 0° C in acetate buffers of pH 5.0. Protein concentrations are about 1 %. Ascending patterns on left and descending patterns on right. (A) $\mu = 0.2$, (B) $\mu = 0.1$, (C) $\mu = 0.05$.

was $6.4 \cdot 10^{-5}$ cm²/sec.volt, and the value of the second boundary was estimated to be $5.9 \cdot 10^{-5}$ cm²/sec.volt.

The variation of the electrophoretic patterns of histone I with ionic strength was astonishingly great. At an ionic strength of 0.2, the fastest component amounted to 48% of the total area of the ascending pattern not including the δ-boundary, while at ionic strengths of 0.1 and 0.05, the values were 63 and 73%, respectively. This phenomenon was considered to be due to the boundary anomalies[26] encountered in moving boundary electrophoresis, which were especially exaggerated in this case. From both theoretical[27] and experimental considerations, the true percentage of the fastest component was estimated to be 30–40%.

Electrophoretic heterogeneity of this preparation was also shown at other pH values, between 4.0–10.3.

The preparation of histone I that had been obtained from Extract A or C was also electrophoretically heterogeneous, but its electrophoretic composition differed somewhat from that of the preparation from Extract B.

In the case of histone II, one main and three minor components were found by electrophoresis at pH 5.0 (see Fig. 2). In contrast to histone I, the electrophoretic patterns did not change markedly with ionic strength. The main component, which migrated fastest of all, constituted nearly 60% of the whole preparation. Its mobility, $8.3 \cdot 10^{-5}$ cm²/sec.volt, in the acetate buffer of pH 5.0 at ionic strength 0.2, was higher than that of any other component of histone. This preparation was not contaminated with histone I.

Therefore it was concluded that histone I as well as histone II were mixtures of components in spite of their ultracentrifugal homogeneity. All the components

Fig. 2. Electrophoretic pattern of histone II at 0° C in an acetate buffer of pH 5.0, ionic strength = 0.2.

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had the same basic characteristics; their isoelectric points were definitely higher than pH 10, since none of them migrated toward the anode at pH 10.0.

(v) *Ultraviolet absorption spectra.* Measurements of ultraviolet absorption were made with a Beckman model DU spectrophotometer using 1-cm quartz cells.

The absorption maxima of histones I and II, both in water (pH 6.3) and in 0.1 *N* NaOH, agreed with each other. However, the extinction coefficient at the absorption maximum of histone I was approximately five times higher than that of histone II in either solvent.

Tyrosine and tryptophan contents were estimated from the optical density readings[28] of solutions in 0.1 *N* NaOH by the method of GOODWIN AND MORTON[29]. The values of the tyrosine content were 3.4% for histone I and 0.7% for histone II. Tryptophan seemed to be absent in both fractions, as the values obtained (0.07 and 0.01% respectively) were very low. Nearly the same values for the tryptophan content of both fractions, 0.05% (histone I) and 0.02% (histone II), were obtaiend on chemical analysis by the method of SPIES *et al.*[30].

(vi) *Elementary analyses.* The nitrogen content was determined by the micro-Kjeldahl method, using preparations that had been dried over P_2O_5 *in vacuo* at 105° C. For this analysis the author is indebted to Mr. Y. KAWANISHI of Prof. T. ANDO's laboratory in this Institute.

Sulfur was determined gravimetrically as $BaSO_4$. The author is indebted to the Research Laboratory of Daiichi Pharmaceutical Co. for this analysis.

Phosphorus was determined by the method of KING[31].

It is clear from Table II that the nitrogen content of histone I is higher than that of histone II, and that sulfur is almost absent in histone II. The phosphorus content was below 0.01% in both fractions, indicating the absence of nucleic acid in these preparations.

TABLE II

ANALYTICAL DATA ON HISTONE FRACTIONS

	Histone I	*Histone II*
N, %	19.3 ± 0.2	18.0 ± 0.2
S, %	0.42	0.06
P, %	< 0.01	< 0.01

(vii) *Basic amino acid composition.* Analyses of basic amino acids were kindly carried out by Dr. S. ISHII in Prof. T. ANDO's laboratory in this Institute. These analyses were carried out by the ion exchange chromatographic method of MOORE AND STEIN[32] as modified by ISHII[33] (Amberlite IRC-50 was used).

The results are shown in Table III. The difference between the two fractions is clearly shown. Histone I was richer in arginine than histone II, while the preparation of histone II contained a larger amount of lysine*. With respect to basic amino acid composition, the former was similar to the arginine-rich histone of DALY AND MIRSKY[7], and the latter to their lysine-rich histone.

* The preparations of histone used in this analysis were not the same samples as those used in the other investigations. As it was suspected from the electrophoretic and ultracentrifugal patterns that the preparation of histone II used in this case was slightly contaminated with histone I, the result obtained with this preparation should be treated with some reserve. The preparation of histone I did not differ appreciably from that used in other studies.

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TABLE III

BASIC AMINO ACID COMPOSITION OF HISTONE FRACTIONS

Amino acid	Mole amino acid per 100 moles of protein nitrogen	
	Histone I	Histone II
Arginine	5.9	1.6
Histidine	1.5	0.24
Lysine	7.3	18.9

(viii) *Other properties.* It was found that various properties of histones I and II are alike in some respects.

When the solution of histone I or histone II was mixed with a solution of sodium deoxyribonucleate and the concentration of sodium chloride in the solution was brought to nearly 0.14 *M*, a fibrous precipitate formed immediately.

Both fractions were soluble in a hot or cold solution of 0.34 *M* $HgSO_4$ in 1.88 *M* H_2SO_4. MIRSKY AND POLLISTER[17] found, that the non-precipitability by this reagent was a specific property of histones.

On the other hand, the two fractions differed as regards several properties other than those described before.

Although insolubility in an alkaline solution was regarded by KOSSEL[34] as a characteristic of histones, histone II could not be precipitated by addition of alkali, while most of the histone I precipitated between pH 10.5–12, although precipitation began at lower pH values if the solution was kept for a long time*.

Histone I could more easily be salted out than histone II; histone I could be precipitated at 60% saturation with sodium chloride, while histone II solution remained clear in saturated sodium chloride solution. In order to precipitate histone II, ammonium sulfate had to be added to nearly complete saturation**.

Separation of these two fractions could also be achieved with trichloroacetic acid; a precipitate was formed in histone I solutions when the same volume of 10% trichloroacetic acid solution was added, but histone II solutions remained clear and more acid had to be added to precipitate this fraction.

It has been found[35] that unaggregated histone I, unlike histone II or aggregated histone I, is capable of interacting with deoxyribonucleic acid in 1 *M* NaCl solution just as in natural nucleohistone.

DISCUSSION

From the foregoing results, it is clear that calf thymus histone can be separated into two fractions (histones I and II) with different properties and compositions. Although each fraction had been precipitated from the acid extract at a definite ethanol concentration and was homogeneous with respect to molecular weight, its electrophoretic pattern was always complex. Therefore, it was concluded that calf thymus histone consists of a number of different basic proteins, which can be classified as subfractions of histone I or II.

Any difference in these preparations could not have been caused by the purification procedure. Acid had no effect on the preparations[36]. The precipitation by ethanol

* Part of histone I (nearly 10%) did not precipitate on the addition of alkali, but this fraction was ultracentrifugally not different from the rest and showed an $s_{20,w}$ of about 2 *S*.

** A fractionation of histone similar to the one described in this paper was also achieved by means of salting-out using ammonium sulfate or other reagents.

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also would not lead to any modification of the proteins, since no significant difference in either electrophoretic or ultracentrifugal behaviour was detected before and after the addition of ethanol. Therefore, the acid and ethanol method described here was thought to be a mild and convenient method for the purification of calf thymus histone provided that care is taken to keep the temperature at which the operations are performed as low as possible. BUTLER *et al.*[9] have also reported that the best preparations of histone were obtained from acid extracts.

The yields of the products were found to be rather high in this method. Although the content of histone in thymus gland is not exactly known, it was estimated to be about 3% of the wet gland by assuming that the amount of histone present in the nuclei is nearly the same as that of deoxyribonucleic acid, the concentration of which is 2.8%[37]. As mentioned before, the extracts from 1.15 kg of glands contained about 26 g of histone (*ca.* 80%) and the total amount of purified product amounted to 18.6 g (*ca.* 50%).

Various values for the sedimentation velocity of calf thymus histone have been recorded by different investigators. All the authors found one or two components with a sedimentation coefficient below about 2 *S*; in addition, the presence of a faster component was often reported. Our experiments on the fractionated samples clearly showed the presence of two components of 2.0_3 and 0.6_8 *S*, respectively, which nearly agreed with the results of BUTLER *et al.* (2 *S* and 1 *S*)[10]. Although a value of *ca.* 1.5 *S* was often reported[13,14], it might be due either to the fact that the value was not extrapolated to infinite dilution or to incomplete separation of 2 *S* and 0.7 *S* components. In fact, unfractionated preparations of ours sometimes showed only one broad boundary and an apparent value of nearly 1.5 *S* was obtained when the duration of the sedimentation experiments was not so long. A component much heavier than 2 *S* would be an artifact of histone as was pointed out by HAMER[19]. In view of the results of our experiments, it seems to be due to the aggregation of the 2 *S* component (histone I) which occurred during the isolation of histone or in the solution in which sedimentation was performed.

From the present experiments on the electrophoresis of histone fractions, it was concluded that in calf thymus histone there are at least four components present in fairly large amounts (three components of histone I and one component of histone II). CRUFT *et al.*[12] found three components (designated as α-, β- and γ-histones), while GRÉGOIRE *et al.*[6,8], who examined their histone fractions (P and S) by electrophoresis, found three components in fraction S and one almost homogeneous component in fraction P. BUTLER *et al.*[10] observed only two components but they showed that a third component appeared when aggregation took place. In their opinion, the presence of the third component in the experiments of the previous authors could be attributed to aggregated histone. However, it should be noted that the electrophoretic patterns described in this paper were obtained without formation of an aggregate, and that different patterns were obtained when aggregation took place.

It has been reported that one fraction of histone contained much arginine and tyrosine, and that another was rich in lysine[3,5–7]. From the results of chemical analyses, it was concluded that our histone I corresponded to the former fraction and histone II to the latter. A homogeneous preparation with high lysine content (the slow component) was successfully obtained by BUTLER *et al.*[5,10], but the molecular weight was nearly twice as high as that of histone II, our corresponding fraction. In

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addition, the main component of histone II migrated most rapidly of all histones at pH 5.0 and its behaviour differed from that of the slow component of BUTLER *et al.* According to CRUFT *et al.*[12] their subsidiary histone with low arginine content was the component with the highest electrophoretic mobility (α-histone). The other fraction rich in arginine was also fractionated and examined by BUTLER *et al.*, but, unlike histone I, its behaviour in the ultracentrifuge was complex[5].

A fractionation based on ultracentrifugal separation has been reported by some other workers[13,14], two components being obtained thereby. As was described before, however, it seems likely that the heavier fraction is not an independent component but an aggregation product of a component of the lighter fraction.

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SUMMARY

1. A simple and useful method for the purification and fractionation of calf thymus histone is presented. Taking advantage of solubility differences in ethanol–water mixtures, two fractions, histones I and II, were obtained.

2. Physicochemical and chemical properties of both fractions were studied and compared with each other. Histone I, the main fraction, markedly differed from histone II in several properties, *i.e.*, molecular weight, electrophoretic behaviour, chemical composition, etc.

3. Although each fraction showed a good degree of homogeneity in an ultracentrifuge, it was electrophoretically not homogeneous. Histone I contained at least three components and histone II showed mainly one component with three minor components. It was, therefore, concluded that there are more than four basic proteins present in calf thymus histone.

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ETHANOL PRECIPITATION ANALYSIS OF THYMUS HISTONE

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Though thymus histone, and occasionally also the histone prepared from other tissues, has been subjected to electrophoresis and ultracentrifugation and various fractions have been obtained from it by preparative procedures, no certainty has been obtained about the number of components bound to deoxyribonucleic acid in the nucleus. In our opinion one of the reasons for this uncertainty is the fact that not all workers in this field have been careful enough to avoid the possibilities of denaturation and aggregation in the course of the isolation of the total histone and during the storage of the product obtained, before the number of components was determined. With the ultimate aim of isolating the total histone from various tissues under the mildest well-controlled conditions, necessary for a comparison of the histone from various sources, we have thoroughly examined two well-known methods of isolation of thymus histone. These methods, described by BUTLER and associates[1], and CRAMPTON, LIPSHITZ AND CHARGAFF[2], respectively, belong to the group of methods that start with the preparation of nucleohistone from the total gland. We preferred to test these methods, and not those that begin with the isolation of the nuclei, since we are not convinced that the isolation of sufficient amounts of nuclei is possible without loss of chromosomal protein.

We have chosen an analytical ethanol precipitation technique at —5° C for the

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